American Heart Association | American Stroke Association

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BCVS 2015

Basic Cardiovascular Sciences

2015 Scientific Sessions: Pathways to Cardiovascular Therapeutics

Final Program

July 13–16 | Hilton New Orleans Riverside | New Orleans, Louisiana

Sponsored and organized by the Council on Basic Cardiovascular Sciences.

my.americanheart.org/bcvssessions

Program at a Glance

	Sunday July 12	Monday July 13	Tuesday July 14	Wednesday July 15	Thursday July 16
7:00 AM			7–8:00 AM **Continental Breakfast/ Registration/Exhibits**	7–8:00 AM **Continental Breakfast/ Registration/Exhibits**	7–8:00 AM **Continental Breakfast/ Registration**
8:00 AM			8:00–9:40 AM **Session 4** Regulation of Mitochondrial Function and Metabolism	8:00–9:40 AM **Session 8** Stem Cells: Renaissance of Regenerative Mechanisms	8:00–9:40 AM **Session 13** Exploing the Functional Importance of Non-myocytes in Heart Failure
9:30 AM			9:40–10:00 AM **Refreshment Break/ Exhibits**	9:40–10:00 AM **Refreshment Break/ Exhibits**	
10:00 AM			10:00–11:40 AM **Session 5** Cellular Quality Control Mechanisms	10:00–11:40 AM **Session 9** Aging and Heart Failure	9:40–11:20 AM **Session 14** Genetics of Cardiac Development and Disease
10:30 AM					
11:00 AM					
NOON	NOON–6:00 PM **ACRE (Academy of Cardiovascular Research Excellence) Meeting**		NOON–1:30 PM **Early Career Workshop/Lunch** *Ticket required for box lunch*	NOON–1:30 PM **Early Career Workshop/Lunch** *Ticket required for box lunch*	**Adjourn**
1:00 PM					
1:30 PM		1:45–2:05 PM **Welcome Remarks**	1:30–3:10 PM **Session 6** The Micochondrial Calcium Microdomain in Cardiac Function and Disease	1:30–2:15 PM **Session 10** Outstanding Early Career Investigator Award Finalists	
2:00 PM		2:05–3:45 PM **Session 1** Novel Signaling Networks in the Heart		2:15–3.35 PM **Session 11** Heart Failure with Preserved Ejection Fraction	
3:00 PM			3:10–3:30 PM **Refreshment Break/ Exhibits**		
3:30 PM			3:30–5:10 PM **Session 7** Sources of New Myocytes in Diseased Hearts	3:35–3:55 PM **Refreshment Break/ Exhibits**	
4:00 PM		3:45–4:30 PM **Session 2** Keynote Lecture		3:55–5:35 PM **Session 12** Moving Novel Targets from Bench to Bedside: Academic-Industry Collaboration	
4:30 PM		4:30–4:50 PM **Refreshment Break**			
5:00 PM	5:00–7:00 PM **Registration Opens**	4:50–6:30 PM **Session 3** Exercise and Cardiovascular Disease	5:10–6:40 PM **Poster Session 2**		
6:00 PM				5:35–7:05 PM **Poster Session 3**	
6:30 PM		6:30–8:00 PM **Poster Session 1**			
7:00 PM				7:05–11:00 PM **Council Dinner** *Ticket required*	
7:30 PM					

Legend

- Plenary Session
- Poster Session
- Meals/Breaks
- Other

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Contact Information

Questions

If you have questions after reading this program, contact the American Heart Association National Center, Dallas, Texas:

Telephone 888.242.2453 (inside the United States)
214.570.5935 (outside the United States)
Email *scientificconferences@heart.org*
Website *my.americanheart.org*

Professional Membership Customer Service

Lippincott Williams & Wilkins
Telephone 800.787.8984 (inside the United States)
301.223.2307 (outside the United States)
Email *ahaonline@LWW.com*

Letter From the Chairs

Dear Colleague,

On behalf of the American Heart Association and the Scientific Council on Basic Cardiovascular Sciences, welcome to the Basic Cardiovascular Sciences 2015 Scientific Sessions: Pathways to Cardiovascular Therapeutics.

The Basic Cardiovascular Sciences conference has become the meeting of choice for both investigators and trainees. It is considered by many to be the premier basic and translational cardiovascular research meeting in the world, and attracts our field's best and brightest from across the globe.

The primary goal of this meeting is to provide a forum for timely discussion of the latest findings from leaders in the field of cardiovascular sciences. As a result, we hope the conference will foster new ideas and collaborations to accelerate translation. Attendees will hear state-of-the-art presentations on a broad array of topics, including signaling, genetics/genomics, lncRNA/microRNAs, cardiac fibrosis and remodeling, cardiac development, tissue engineering, iPS cells, and cardiac cell therapy. Invited speakers represent institutions from the United States, Canada, Europe, Asia, Latin/South America, and Australia as we further broaden our international scope and partnerships.

In addition to the Outstanding Early Career Investigator Award competition, we will have 3 lively poster sessions, and two lunch workshops targeting early career development. The Tuesday lunch workshop will be a networking/mentoring session where young investigators will have the opportunity to interact with meeting Faculty and members of the BCVS Leadership Committee, and the Wednesday workshop will be Speed Networking with senior faculty panelists.

As your hosts, we hope you will find the conference an educational experience and a great opportunity to network with scientists from around the world who are dedicated to building healthier lives, free of cardiovascular diseases and stroke. Let us know if there is anything we can do to enrich your stay in New Orleans and thank you for sharing your insight and expertise. We look forward to meeting you.

Sincerely,

Åsa Gustafsson, PhD, FAHA
Program Co-Chair, BCVS 2015

David Lefer, PhD, FAHA
Program Co-Chair, BCVS 2015

Anthony Rosenzweig, MD, FAHA
Program Co-Chair, BCVS 2015

The American Heart Association is a national voluntary health organization whose mission is:
"Building healthier lives, free of cardiovascular diseases and stroke."

The American Heart Association Council on Basic Cardiovascular Sciences gratefully acknowledges the educational grants provided for the support of this conference by the following:

Cardiovascular Center of Excellence at
Louisiana State University Health Science Center

The Carlyle Fraser Heart Center of Emory Hospital Midtown

The Center for Translational Medicine,
Temple University School of Medicine

Heart Institute of Cincinnati
Children's Hospital Medical Center

Massachusetts General Hospital

Pfizer, Inc.

The Stanford Cardiovascular Research Institute

University of California, San Diego
Sulpizo Cardiovascular Center

The American Heart Association is grateful to the members of the Program Committee for their dedication and leadership in planning the program.

Basic Cardiovascular Sciences 2015 Program Committee

Asa Gustafsson, PhD, FAHA, Co-Chair, University of California San Diego, La Jolla, California
David Lefer, PhD, FAHA, Co-Chair, Louisiana State University Health Sciences Center, New Orleans, Louisiana
Anthony Rosenzweig, MD, FAHA, Co-Chair, Beth Israel Deaconess Medical Center, Boston, Massachusetts
Burns Blaxall, PhD, FAHA, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio
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Howard Rockman, MD, Duke University, Durham, North Carolina
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Invited Presenters

Zoltan Arany, MD, PhD, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania
Robert Balaban, PhD, NHLBI-NIH, Bethesda, Maryland
David M. Bers, PhD, FAHA, University of California, Davis, Davis, California
Thomas Brand, PhD, Heart Science Centre, Imperial College of London, Harefield, United Kingdom
John Calvert, PhD, FAHA, Emory University School of Medicine, Atlanta, Georgia
Paul Cohen, MD, PhD, Rockefeller University, New York, New York
Wilson Colucci, MD, FACC, Boston University Medical Center, Boston, Massachusetts
Gianluigi Condorelli, MD, PhD, Humanitas Research Hospital, University of Milan, Milan, Italy
Stuart Cook, MD, PhD, Duke-National University of Singapore, Singapore, Singapore
Jennifer Davis, PhD, Children's Hospital Medical Center, Cincinnati, Ohio
Federica del Monte, MD, PhD, Beth Israel Deaconess Medical Center, Boston, Massachusetts
Gerald Dorn, MD, FACC, FAHA, Washington University, St. Louis, Missouri
Konstantinos Drosatos, PhD, MS, Temple University School of Medicine, Philadelphia, Pennsylvania
John Elrod, PhD, Temple University School of Medicine, Philadelphia, Pennsylvania
Loren Field, PhD, Riley Hospital for Children, Indianapolis, Indiana
Thomas Force, MD, FAHA, Vanderbilt University School of Medicine, Nashville, Tennessee
Stefan Frantz, MD, Universitätsklinik und Poliklinik für Innere Medi, Halle (Saale), Germany
Henk Granzier, PhD, University of Arizona, Tucson, Arizona
Eric Green, MD, PhD, MyoKardia, Inc., San Francisco, California
Joshua Hare, MD, FAHA, University Of Miami, Miami, Florida
Joseph Hill, MD, PhD, FAHA, UT Southwestern Medical Center, Dallas, Texas
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Ahsan Husain, PhD, FAHA, Emory University School of Medicine, Atlanta, Georgia
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Steven Jones, PhD, FAHA, University of Louisville, Louisville, Kentucky
Navin Kapur, MD, Tufts Medical Center MCRI, Boston, Massachusetts
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Mark Keating, MD, Novartis Institutes for BioMedical Research, Cambridge, Massachusetts
Richard Kitsis, MD, FAHA, Albert Einstein College Medicine, Bronx, New York
Walter Koch, PhD, FAHA, Temple University School of Medicine, Philadelphia, Pennsylvania
Maria Kontaridis, PhD, FAHA, Beth Israel Deaconess Medical Center, Boston, Massachusetts
Jonathan Lederer, MD, PhD, FAHA, University of Maryland School of Medicine, Baltimore, Maryland
Annarosa Leri, MD, FAHA, Brigham and Women's Hospital, Boston, Massachusetts
Gregory Lewis, MD, Massachusetts General Hospital, Boston, Massachusetts
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Heidi McBride, PhD, Montreal Neurological Institute, Montreal, Canada
Timothy McKinsey, PhD, University of Colorado, Aurora, Colorado
Julie McMullen, PhD, FAHA, Baker IDI Heart and Diabetes Institute, Melbourne, Australia
Elizabeth McNally, MD, PhD, Northwestern University Feinberg School of Medicine, Chicago, Illinois
Joseph Metzger, PhD, University of Minnesota, Minneapolis, Minnesota
Shigeki Miyamoto, DVM, PhD, University of California, San Diego, La Jolla, California
Anthony J. Muslin, MD, Sanofi, Cambridge, Massachusetts
Satoshi Nishimura, MD, PhD, University of Tokyo, Jichi Medical University, Tokyo, Japan
Eric Olson, PhD, FAHA, UT Southwestern Medical Center at Dallas, Dallas, Texas
Brian O'Rourke, PhD, Johns Hopkins University, Baltimore, Maryland
Jeffrey Robbins, PhD, FAHA, Cincinnati Children's Hospital, Cincinnati, Ohio
Marcello Rota, PhD, FAHA, Brigham and Women's Hospital-Harvard Medical School, Boston, Massachusetts
Hesham Sadek, MD, UT Southwestern Medical Center, Dallas, Texas
Junichi Sadoshima, PhD, MD, FAHA, Rutgers New Jersey Medical School, Newark, New Jersey
Alison Schecter, MD, Johnson and Johnson Innovation, Cambridge, Massachusetts
Luca Scorrano, MD, PhD, University of Padova, Padua, Italy

(continued on next page)

Invited Presenters (continued)

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Mark Sussman, PhD, FAHA, San Diego State University, Heart Institute and Integrated Regenerative Research Institute, San Diego, California
Rong Tian, MD, PhD, FAHA, University of Washington, Seattle, Washington
Jop van Berlo, MD, PhD, University of Minnesota, Minneapolis, Minnesota
Eva van Rooij, PhD, Hubrecht Institute, Utrecht, Netherlands
Thomas Vondriska, PhD, FAHA, UCLA, Los Angeles, California
Rory Weiner, MD, Massachusetts General Hospital, Boston, Massachusetts
Joseph Wu, MD, PhD, FAHA, Stanford University School of Medicine, Stanford, California
Junjie Xiao, PhD, Shanghai University, Shanghai, China

Invited Moderators

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Donald M. Bers, PhD, FAHA, University of California Davis, Davis, California
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Jeffrey Madwed, PhD, Merck Research Laboratories, Rahway, New Jersey
Jeffery Molkentin, PhD, Children's Hospital Medical Center, Cincinnati, Ohio
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Abstract Reviewers

The conference organizers gratefully acknowledge the following individuals for their assistance with the abstract grading process:

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Hossein Ardehali, MD, PhD, FAHA
Burns Blaxall, PhD, FAHA
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John Calvert, PhD, FAHA
Ju Chen, PhD
Wilson Colucci, MD, FACC
Gianluigi Condorelli, MD, PhD
Stuart Cook, MD, PhD
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Yibin Wang, PhD, FAHA
Joseph Wu, MD, PhD, FAHA
Junjie Xiao, PhD
Jianyi Zhang, MD, PhD

Room Locator

Activity	Hotel Location, Floor
Sunday, July 12	
Speaker Resource Room	Grand Salon 7
Registration Opens	Grand Ballroom B
ACRE Meeting	Belle Chasse
Monday, July 13	
Speaker Resource Room	Grand Salon 7
Registration	Grand Ballroom B
General Session	Grand Ballroom A
Refreshment Break/Exhibits	Grand Ballroom B
Poster Session	Grand Ballroom C/D
Tuesday, July 14	
Speaker Resource Room	Grand Salon 7
Registration	Grand Ballroom B
Continental Breakfast/Exhibits	Grand Ballroom B
General Session	Grand Ballroom A
Refreshment Break/Exhibits	Grand Ballroom B
Early Career Workshop/Lunch	Grand Salon C
Poster Session	Grand Ballroom C/D
Wednesday, July 15	
Speaker Resource Room	Grand Salon 7
Registration	Grand Ballroom B
Continental Breakfast/Exhibits	Grand Ballroom B
General Session	Grand Ballroom A
Refreshment Break/ Exhibits	Grand Ballroom B
General Session	Grand Ballroom A
Early Career Workshop/Lunch	Grand Salon C
Poster Session	Grand Ballroom C/D
Council Dinner	Napoleon Ballroom
Thursday, July 16	
Speaker Resource Room	Grand Salon 7
Registration	Grand Ballroom B
Continental Breakfast	Grand Ballroom B
General Session/Adjourn	Grand Ballroom A

General Information

Program Description

The 11th annual BCVS 2015 Scientific Sessions — Pathways to Cardiovascular Therapeutics has become the premier conference for molecular cardiovascular biology and disease. Sponsored by the American Heart Association Basic Cardiovascular Sciences Council, the world's leading organization of cardiovascular scientists, the conference attracts leading researchers in fields such as microRNAs, cardiac gene and cell therapy, cardiac development and most recently tissue engineering and iPS cells.

The planned agenda includes 14 fast-paced sessions over three-and-a-half days in a forum that promotes the relaxed exchange and discussion of cutting-edge research in molecular and translational cardiovascular biology and disease. The program includes a diversity of speakers representing the best cardiovascular scientists from around the world, and encourages interaction between young scientists and more established scientists to foster dialogue and facilitate the exchange of ideas.

Conference Registration

Registration will be in Grand Ballroom B. Registration will be open during the following hours:

Sunday, July 12.....5:00–7:00 pm

Monday, July 13.....7:00 am–7:00 pm

Tuesday, July 14.....7:00 am–7:00 pm

Wednesday, July 15.....7:00 am–7:00 pm

Thursday, July 16.....7:00 am–NOON

Exhibits

Beginning Monday, at 10:00 AM, visit our Exhibitors, located in Grand Ballroom B. This year we welcome:

- Alfa Aesar, a Johnson Matthey Company
- ACRE-APS
- American Heart Association
- Miltenyi Biotec
- OmegaBrite
- Visual Sonics

You can also renew your AHA membership and bring your non-member colleagues to learn the latest information about the benefits of membership.

Learning Objectives

After completing this program, participants will be able to:

- Evaluate the most recent evidence supporting the use of cardioprotective strategies in the setting of acute myocardial infarction.
- Discuss advances in understanding the underlying mechanisms of cardiomyopathies and the identification of new therapeutic targets.
- Assess new models for the use of cell- and gene-based therapies in repairing damaged cardiac tissue.
- Examine progress in the development of novel therapeutic targets for heart failure.
- Identify new targets for drug discovery and opportunities to participate in the translational research required to identify new interventions and bring them into clinical trials.

General Information (continued)

Continuing Medical Education Accreditation — Physicians

The American Heart Association is accredited by the Accreditation Council for Continuing Medical Education to provide continuing medical education for physicians.

The American Heart Association designates this live activity for a maximum of 23.00 *AMA PRA Category 1 Credits*™. Physicians should claim only the credit commensurate with the extent of their participation in the activity.

All persons who develop and/or control educational content in CME activities sponsored by the American Heart Association will disclose to the audience all financial relationships with any commercial supporters of this activity as well as with other commercial interests whose lines of business are related to the CME-certified content of this activity. In addition, presenters will disclose unlabeled/unapproved uses of drugs or devices discussed in their presentations. Such disclosures will be made in writing in course presentation materials.

Continuing Medical Education Accreditation — Physician Assistants

AAPA accepts certificates of participation for educational activities certified for *AMA PRA Category 1 Credit*™ from organizations accredited by ACCME or a recognized state medical society. Physician assistants may receive a maximum of 23.00 hours of Category 1 credit for completing this program.

Continuing Medical Education Accreditation — Nurse Practitioners

American Academy of Nurse Practitioners (AANP) accepts AMA PRA Category 1 Credit™ from organizations accredited by the ACCME.

**ACCME credit must be claimed within 6 months of attendance. CME/CE credit will no longer be available to claim for this activity after November 16, 2015.

CME/CE Credit

We offer two ways to complete your conference evaluation and claim your CME/CE credits for the conference:

1. Stop by the Communication Center by the registration desk in the Grand Ballroom B.

2. Visit learn.heart.org from any computer with Internet connection.

For both options:

- Sign in with your username and password at learn.heart.org. If you do not have an account, please create one.
- Find the course Basic Cardiovascular Sciences 2015 Sessions under the “Activity Catalog” tab and select to enroll.
- Complete the evaluation and claim your credit.
- Authorization code to claim your credit is **bcvs2015**.

You are strongly encouraged to claim your CME/CE credit within 30 days of the conference, and you must claim your credit by Nov. 16, 2015. For assistance, please contact our National Support Center at (888) 242-2453 or learn@heart.org.

The International Attendance Verification forms will also be available at registration.

Web Resources

HealthJobsPlus
The American Heart Association and Lippincott Williams & Wilkins (a Wolters Kluwer business) offers HealthJobsPlus, a first-rate source for those seeking and posting jobs by connecting qualified healthcare professionals with top-notch employers. Go to http://healthjobsplus.com to learn more.

Professional Education Center - learn.heart.org
Please visit learn.heart.org to register/claim your CME certification using the conference’s unique code. The AHA is the premier provider in quality science, evidence-based, continuing education for healthcare professionals. Our course offerings are available in a multitude of formats, including live presentations and online activities. The Professional Education Center website also provides access to webcasts, satellite broadcasts and podcasts.

my.americanheart.org
My AmericanHeart for Professionals is the American Heart Association/American Stroke Association’s Internet resource for healthcare professionals devoted to the fight against cardiovascular disease and stroke. Depending on the level of membership selected, AHA/ASA professional members may have access to all 12 AHA scientific journals, biweekly clinical updates, core clinical textbooks, a continually updated drug database and much more. Also available from this site are links to the BCVS Scientific Sessions website, Science News and the AHA’s Professional Online Network.

Information for Presenters

Speaker Resource Room

The Speaker Resource Room is located in Grand Salon 7. Speakers are asked to deliver their presentations on CD-ROM, DVD-ROM or a USB storage device to the Speaker Resource Room at least three hours before the beginning of the session in which they will speak. *It is imperative that you review your presentation in the Speaker Resource Room if it contains video files or was created on a Mac.* Speakers will be directed to a preloading station where a technician will be on hand to load the presentations. Speakers may also use this room to review and practice their presentations on both PCs and Mac computers.

The Speaker Resource Room will be open during these hours:

Sunday, July 12	5:00–7:00 PM	Wednesday, July 15	7:00 AM–5:00 PM
Monday, July 13	7:00 AM–5:00 PM	Thursday, July 16	7:00 AM–NOON
Tuesday, July 14	7:00 AM–5:00 PM		

Abstract Presentations

Abstract presentations for the Basic Cardiovascular Sciences 2015 Scientific Sessions are embargoed for release at the time of presentation or time of an AHA news event. Information may not be released before the scheduled presentation time.

Abstracts will be published in the October online edition of the AHA journal *Circulation Research*.

Abstracts will be presented as follows:

Poster Session 1-P01	Monday, July 13	6:30–8:00 PM	Abstracts 1–134
Poster Session 2-P02	Tuesday, July 14	5:10–6:40 PM	Abstracts 150-283, 340, 343
Poster Session 3-P03	Wednesday, July 15	5:35–7:05 PM	Abstracts 300–432

Poster Presenters, please note the schedule below:

Poster Session 1

Set-up time:	Monday, July 13	NOON–5:00 PM
Attended time:	Monday, July 13	6:30–8:00 PM
Tear-down time:	Tuesday, July 14	before 9:00 AM

Poster Session 2

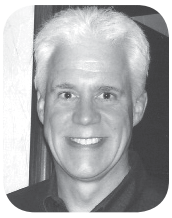
Set-up time:	Tuesday, July 14	NOON–5:00 PM
Attended time:	Tuesday, July 14	5:10–6:40 PM
Tear-down time:	Wednesday, July 15	before 9:00 AM

Poster Session 3

Set-up time:	Wednesday, July 15	NOON–5:00 PM
Attended time:	Wednesday, July 15	5:35–7:05 PM
Tear-down time:	Thursday, July 16	before 9:00 AM

Conference Highlights

Keynote Lecture



Eric N. Olson, PhD, will present **New Insights into Muscle Development, Disease and Regeneration** on Monday, July 13, at 3:45 PM.

Dr. Olson is a professor and chair of the Department of Molecular Biology at the University of Texas Southwestern Medical Center in Dallas, where he also holds the Robert A. Welch Distinguished Chair in Science, the Annie and Willie Nelson Professorship in Stem Cell Research and the Pogue Distinguished Chair in Research on Cardiac Birth Defects.

Dr. Olson grew up in North Carolina and attended Wake Forest University, receiving a BA in chemistry and biology, a PhD in biochemistry and an honorary doctorate. After postdoctoral training at the Washington University School of Medicine, he began his scientific career at MD Anderson Cancer Center in Houston, Texas. In 1995, he founded the Department of Molecular Biology at the University of Texas Southwestern Medical Center in Dallas.

Eric Olson has dedicated his career to deciphering the mechanisms that control development and disease of the heart, cardiovascular system and skeletal muscle tissue. He and his colleagues discovered many of the key transcription factors and mechanisms responsible for cardiac gene regulation and formation of the heart and, in so doing, unveiled the molecular underpinnings of congenital and acquired diseases of the heart. Most recently, Olson has focused on epigenetic mechanisms and microRNAs as regulators of muscle development and disease.

Olson is among the most highly cited researchers, with his publications cited more than 70,000 times in literature. He has trained numerous students and postdoctoral fellows, many of whom are emerging as the next generation of leaders in cardiovascular biology.

Dr. Olson co-founded multiple biotechnology companies to translate basic discoveries into new therapeutics for muscle disease. He was co-founder of Myogen, Inc., a biotechnology company focusing on therapies for heart muscle disease. In 2007, he co-founded miRagen Therapeutics, which is developing new therapeutics for cardiovascular disease, based on microRNAs. In 2010, he and his colleagues founded Lone Star Heart, which is working to develop new approaches for heart regeneration and repair.

In his spare time, Eric Olson plays guitar and harmonica with The Transactivators, a rock band inspired by the Texas icon, Willie Nelson, who created the professorship that Olson holds.

Conference Highlights

Outstanding Early Career Investigator Award Finalists' Presentations

The three finalists will present their abstracts on Wednesday, July 15, at 1:30 PM. The winner will be announced Wednesday evening during the Basic Cardiovascular Sciences Council Dinner. Refer to page 14 for more information on award finalists

Career Development Workshops

Join us on Tuesday and Wednesday for these sessions targeted for Early Career attendees. These workshops are open to all attendees; however, a ticket is required for lunch. Check with staff at the Registration Desk for availability.

Tuesday, July 14, NOON–1:30 PM

Publish or Perish: Knowing when it's Time to Publish
Jeffrey Robbins, PhD, FAHA

Beyond the Bench – Marketing Yourself for Non-Academic Positions
Timothy A. McKinsey, PhD

Mentorship vs. Sponsorship and the Importance of Having Both
Eric N. Olson, PhD, FAHA

Wednesday, July 15, NOON–1:30 PM

New in 2015 — Speed Networking with Senior Faculty Panelists

Success – Learning the Art of Networking
Mark A. Sussman, PhD

Speed Networking – Faculty Panelists

Reza Ardehali, MD
Burns Blaxall, PhD, FAHA
John Calvert, PhD, FAHA
Gerald Dorn, MD, FACC, FAHA
Thomas Force, MD, FAHA
Sarah Franklin, PhD
Steven Houser, PhD, FAHA
Walter Koch, PhD, FAHA
Maria Kontaridis, PhD, FAHA
Donald Menick, PhD, FAHA
Nicole Purcell, PhD
Sakthivel Sadayappan, PhD, MBA, FAHA
Ivonne Schulman, MD
Susan Steinberg, MD, FAHA
Wolfram Zimmermann, MD, FAHA

Please Note: The workshop is open to all attendees; however, a ticket is required for lunch.

Council on Basic Cardiovascular Sciences Dinner

On Wednesday, July 15, at 7:05 PM, please plan to join us for food, drinks and entertainment at the BCVS Council Dinner, being held in the Napoleon Ballroom. Tickets, if available, may be purchased at the AHA Registration Desk ($60 per person for registrants and their guests).

Conference Highlights – Awards

The American Heart Association Council on Basic Cardiovascular Sciences provides educational programs, awards/scholarships, travel grants and mentoring opportunities that support the ongoing training and development of individuals in the early stages of their careers.

The council is pleased to announce the finalists and winners of the following awards:

Outstanding Early Career Investigator Award Finalists

The Outstanding Early Career Investigator Award finalists will present their abstracts during a special oral session on Wednesday, July 15, at 1:30 PM. The winner will be announced Wednesday evening during the Basic Cardiovascular Sciences Council Dinner.

Name/Institution	Abstract Number
Johannes Backs, University of Heidelberg	433
Prabhakara Nagareddy, University of Kentucky	116
Kunhua Song, University of Colorado School of Medicine	126

Cardiovascular Outreach Award Recipients

Name	Abstract/Poster Number
Rene Begay	194
Ilton Cubero-Salazar	302
Malina Ivey	250
Jasmin Kristianto	45
Tania Nevers	406
Brisa Pena	9
Pearl Quijada	16

New Investigator Travel Award Recipients

Name	Abstract/Poster Number
Cristina Beltrami	329
Matthew Brody	412
Jaqueline da Silva	363
Alicia D'souza	430
Elvira Forte	12
Zaniar Ghazizadeh	312
Laurel Grisanti	414
Michal Handzlik	239
Prince Jeyabal	321
Onur Kanisicak	380
Jason Karch	173
Sadiya Khan	179
Kazuki Kodo	249
Paolo Kunderfranco	282
Timothy Luongo	93
Catherine Makarewich	339
Sonomi Maruyama	229
Kevin Morine	64
Ane Miren Salvador	211
Moshi Song	91
Hasahya Tony	323
Zhihua Wang	283
An-Chi Wei	389
Huiliang Zhang	391
Sen Zhu	361

Policy Information

Disclaimer
The Basic Cardiovascular Sciences Scientific Sessions is a scientific and educational conference for exchanging and discussing research results and scientific developments in the field of cardiovascular disease. Accordingly, the American Heart Association cannot and does not offer any assurance or warranty of the accuracy, truthfulness or originality of the information presented at the conference.

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Please note: The American Heart Association shall not be liable for cancellation of the Basic Cardiovascular Sciences 2015 Scientific Sessions caused by labor strikes, civil disorders, fires, weather conditions, or other acts of God for any damages or losses resulting from such cancellations.

Program Agenda

MONDAY, JULY 13

7:00 AM
Registration
Grand Ballroom B

10:00 AM
Exhibits Open
Grand Ballroom B

1:45–2:05 PM
Welcome Remarks
Grand Ballroom A

1:45 **Joshua Hare, MD, FAHA**
BCVS Council Leadership Chair,
University of Miami, Miami, Florida

1:50 **Mark Creager, MD**
Dartmouth-Hitchcock Medical Center,
Geisel School of Medicine. Lebanon, NH
and President, American Heart
Association

2:05–3:45 PM
Grand Ballroom A
Session 1
Novel Signaling Networks in the Heart

Moderators:
Ju Chen, PhD, UCSD School of Medicine,
La Jolla, California
Susan Steinberg, MD, FAHA, Columbia University
Medical Center, New York, New York

2:05 **Non-GPCR Roles for GRKs in the Heart**
Walter Koch, PhD, FAHA, Temple University School of Medicine, Philadelphia, Pennsylvania

2:25 **RhoA and Cardiac Signaling**
Maria Kontaridis, PhD, FAHA, Beth Israel Deaconess Medical Center, Boston, Massachusetts

2:45 **S-Nitrosylation Can Control Biased GPCR Signaling**
Jonathan Stamler, MD, FAHA, Case Western Reserve University, Cleveland, Ohio

3:05 **Cardiomyocyte Klf5: A Novel Regulator of Cardiac and Systemic Energetics**
Konstantinos Drosatos, PhD, MS, Temple University School of Medicine, Philadelphia, Pennsylvania

3:25 **Mechanistic Insights from Genetic Discoveries in Dilated Heart Failure Syndromes**
Elizabeth McNally, MD, PhD, Northwestern University Feinberg School of Medicine, Chicago, Illinois

3:45–4:30 PM
Grand Ballroom A
Session 2
Keynote Lecture

Moderator:
Joshua Hare, MD, FAHA, BCVS Council Leadership Chair, University of Miami, Miami, Florida

3:45 **New Insights into Muscle Development, Disease and Regeneration**
Eric Olson, PhD, FAHA, UT Southwestern Medical Center, Dallas, Texas

4:30–4:50 PM
Refreshment Break/Exhibits
Grand Ballroom B

4:50–6:30 PM
Grand Ballroom A
Session 3
Exercise and Heart Disease

Moderators:
Joseph Libonati, PhD, FAHA, University of Pennsylvania, Philadelphia, Pennsylvania
Glenn Rowe, PhD, University of Alabama at Birmingham, Birmingham, Alabama

4:50 **Cardiovascular Effects of Exercise: Clinical Insights from Imaging**
Rory Weiner, MD, Massachusetts General Hospital, Boston, Massachusetts

5:10 **PI3Kinase Signaling in the Benefits of Exercise**
Julie McMullen, PhD, FAHA, Baker IDI Heart and Diabetes Institute, Melbourne, Australia

5:30 **Non-coding RNA basis of Physiological Hypertrophy**
Junjie Xiao, PhD, Shanghai University, Shanghai, China

5:50 **Nitric Oxide Pathways and the Protective Effects of Exercise**
John Calvert, PhD, FAHA, Emory University School of Medicine, Atlanta, Georgia

6:10 **Learning from PGC-1 in Skeletal Muscle**
Zoltan Arany, MD, PhD, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania

6:30– 8:00 PM
Grand Ballroom C/D
Poster Session 1

TUESDAY, JULY 14

7:00–8:00 AM
Continental Breakfast/Registration/Exhibits
Grand Ballroom B

8:00–9:40 AM
Grand Ballroom A
Session 4
Regulation of Mitochondrial Function and Metabolism

Moderators:
Douglas Lewandowski, PhD, FAHA, University of Illinois at Chicago, Chicago, Illinois
Qiangrong Liang, MD, PhD, New York Institute of Technology College of Osteopathic Medicine, Old Westbury, New York

8:00 **Epigenetic Modification and Metabolism**
Rong Tian, MD, PhD, FAHA, University of Washington, Seattle, Washington

8:20 **Keeping Mitochondria in Shape: A Matter of Life and Death**
Luca Scorrano, MD, PhD, Venetian Institute of Molecular Medicine, Padua, Italy

8:40 **Mitochondrial Derived Vesicles**
Heidi McBride, PhD, Montreal Neurological Institute, Montreal, Quebec

9:00 **Metabolic and Thrombotic Processes Revealed by in vivo Multi-photon Imaging**
Satoshi Nishimura, MD, PhD, University of Tokyo, Jichi Medical University, Tokyo, Japan

9:20 **Molecular Regulation of Adaptive Thermogenesis in Beige Fat**
Paul Cohen, MD, PhD, The Rockefeller University, New York, New York

9:40–10:00 AM
Refreshment Break/Exhibits
Grand Ballroom B

10:00–11:40 AM
Grand Ballroom A
Session 5
Cellular Quality Control Mechanisms

Moderators:
Fedrica del Monte, MD, PhD, Beth Israel Deaconess Medical Center, Boston, Massachusetts
Xuejun Wang, MD, PhD, FAHA, University of South Dakota, Vermillion, South Dakota

10:00 **Serpins, Cell Death, and Diabetes**
Richard Kitsis, MD, FAHA, Albert Einstein College of Medicine, Bronx, New York

10:20 **Alternative Autophagy**
Junichi Sadoshima, PhD, MD, FAHA, Rutgers New Jersey Medical School, Newark, New Jersey

10:40 **Postnatal Metabolic Remodeling by Parkin-mediated Mitophagy**
Gerald Dorn, MD. FACC, FAHA, Washington University, St. Louis, Missouri

11:00 **Proteotoxicity and Heart Failure**
Jeffrey Robbins, PhD, FAHA, Cincinnati Children's Hospital, Cincinnati, Ohio

11:20 **Hexokinase II and Mitochondrial Quality Control**
Shigeki Miyamoto, DVM, PhD, University of California San Diego, La Jolla, California

NOON–1:30 PM
Grand Salon C
Early Career Development Luncheon I
(Ticket required for box lunch)

Moderators:
Konstantinos Drosatos, MSc, PhD, Temple University School of Medicine, Philadelphia, Pennsylvania
Il-Man Kim, PhD, Georgia Regents University, Augusta, Georgia

12:00 **Publish or Perish – Knowing When it's Time to Publish**
Jeffrey Robbins, PhD, FAHA, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

12:30 **Beyond the Bench – Marketing Yourself for Non-academic Positions**
Timothy McKinsey, PhD, University of Colorado, Aurora, Colorado

1:00 **Mentorship vs. Sponsorship and the Importance of Having Both**
Eric Olson, PhD, FAHA, UT Southwestern Medical Center, Dallas, Texas

Program Agenda (continued)

1:30–3:10 PM
Grand Ballroom A
Session 6: The Mitochondrial Calcium Microdomain in Cardiac Function and Disease

Moderators:
Donald M. Bers, PhD, FAHA, University of California Davis, Davis, California
Xin Ma, MD, Thomas Jefferson University, Philadelphia, Pennsylvania

1:30 **Calcium Movement in Cardiac Mitochondria**
Jonathan Lederer, MD, PhD, FAHA, University of Maryland School of Medicine, Baltimore, Maryland

1:50 **The Mitochondria Reticulum: Role in Muscle Energy Distribution and Ca Signaling**
Robert S. Balaban, PhD, NHLBI – NIH, Bethesda, Maryland

2:10 **Mitochondrial Malfunction in Heart Failure and Sudden Death**
Brian O'Rourke, PhD, Johns Hopkins University, Baltimore, Maryland

2:30 **Mitochondrial Uniporter and Transient Permeability Transition Opening**
Donald M. Bers, PhD, FAHA, UC Davis, Davis, California

2:50 **Mitochondrial Calcium Exchange Mechanisms: Physiological Function and Contribution to Disease**
John Elrod, PhD, Center for Translational Medicine, Philadelphia, Pennsylvania

3:10–3:30 PM
Refreshment Break/Exhibits
Grand Ballroom B

3:30–5:10 PM
Grand Ballroom A
Session 7
Sources of New Myocytes in Diseased Hearts

Moderator:
Ronglih Liao, PhD, FAHA, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts
Mark Sussman, PhD, FAHA, San Diego State University, Heart Institute and Integrated Regenerative Research Institute, San Diego, California

3:30 **Mechanisms of New Myocyte Formation after Cell Therapy**
Eduardo Marban, MD, PhD, FAHA, Cedars-Sinai Medical Center, Los Angeles, California

3:50 **Mesenchymal Stem Cell Therapy: Mechanisms Underlying Myogenesis**
Joshua Hare, MD, FAHA, University of Miami, Miami, Florida

4:10 **Bone Stem Cell Derived New Myocytes**
Steven Houser, PhD, FAHA, Temple University School of Medicine, Philadelphia, Pennsylvania

4:30 **Induced Pluripotent Stem Cell Derived New Myocytes**
Joseph Wu, MD, PhD, FAHA, Stanford University School of Medicine, Stanford, California

4:50 **Reprogramming of Cardiac Fibroblasts**
Masaki Ieda, MD, PhD, Keio University School of Medicine, Tokyo, Japan

5:10–6:40 PM
Grand Ballroom C/D
Poster Session 2

WEDNESDAY, JULY 15

7:00–8:00 AM
Continental Breakfast/Registration/Exhibits
Grand Ballroom B

8:00–9:40 AM
Grand Ballroom A
Session 8
Stem Cells: Renaissance of Regenerative Mechanisms

Moderators:
Jeffrey Molkentin, PhD, Children's Hospital Medical Center, Cincinnati, OH
Ivonne Schulman, MD, Veterans Affairs Medical Center, Miami, Florida

8:00 **Regulation of Cardiomyocyte Cell Cycle**
Hesham Sadek, MD, UT Southwestern Medical Center, Dallas, Texas

8:20 **Proliferation of Cardiac Myocytes During Preadolescence**
Ahsan Husain, PhD, FAHA, Emory University School of Medicine, Atlanta, Georgia

8:40 **c-kit Positive Cardiac Progenitor Cells**
Jop van Berlo, MD, PhD, Lillehei Heart Institute, Minneapolis, Minnesota

9:00 **Protein O-GlcNAcylation in the Heart**
Steven Jones, PhD, FAHA, University of Louisville, Louisville, Kentucky

9:20 **Mechanisms Driving the Cardiomyocyte Cell Cycle**
Loren Field, PhD, Riley Hospital for Children, Indianapolis, Indiana

9:40–10:00 AM
Refreshment Break/Exhibits
Grand Ballroom B

10:00–11:40 AM
Grand Ballroom A
Session 9
Aging and Heart Failure

Moderators:
Evangelia Kranias, PhD, FAHA, University of Cincinnati College of Medicine, Cincinnati, Ohio
Honglian Li, MD, PhD, FAHA, Wuhan University, Wuhan, China

10:00 **A Tangled Web: Is Heart Failure Alzheimer's of the Heart?**
Federica del Monte, MD, PhD, Beth Israel Deaconess Medical Center, Boston, Massachusetts

10:20 **The Role of Dystrophin in Age-related Cardiac Dysfunction**
Joseph Metzger, PhD, University of Minnesota, Minneapolis, Minnesota

10:40 **Popeye Domain Containing (Popdc) Genes are Associated with Cardiac Arrhythmia and Muscular Dystrophy**
Thomas Brand, PhD, Heart Science Centre, Imperial College of London, Harefield, United Kingdom

11:00 **Myocardial Aging in a Large Animal Model**
Marcello Rota, PhD, Brigham and Women's Hospital-Harvard Medical School, Boston, Massachusetts

11:20 **Cardiac Progenitor Cells and Aging**
Annarosa Leri, MD, FAHA, Brigham and Women's Hospital, Boston, Massachusetts

NOON–1:30 PM
Grand Salon C
Early Career Development Luncheon 2
(Ticket required for box lunch)

Moderators:
Pilar Alcaide, PhD, Tufts Medical Center, Boston, Massachusetts
Saumya Das, MD, Beth Israel Deaconess Medical Center, Boston, Massachusetts

12:00 **Success – Learning the Art of Networking**
Mark A. Sussman, PhD, FAHA, San Diego State University, Heart Institute and Integrated Regenerative Research Institute, San Diego, California

12:30 **Speed Networking**
Faculty Panelists
Reza Ardehali, MD, UCLA Medical Center, Los Angeles, California
Burns Blaxall, PhD, FAHA, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio
John Calvert, PhD, FAHA, Emory University School of Medicine, Atlanta, Georgia
Gerald Dorn, MD, FAHA, Washington University, St. Louis, Missouri
Thomas Force, MD, FAHA, Vanderbilt University School of Medicine, Nashville, Tennessee
Sarah Franklin, PhD, University of Utah, Salt Lake City, Utah
Steven Houser, PhD, FAHA, Temple University School of Medicine, Philadelphia, Pennsylvania
Walter Koch, PhD, FAHA, Temple University School of Medicine, Philadelphia, Pennsylvania
Maria Kontaridis, PhD, FAHA, Beth Israel Deaconess Medical Center, Boston, Massachusetts
Donald Menick, PhD, Medical University South Carolina, Charleston, South Carolina
Nicole Purcell, PhD, University of California San Diego, San Diego, California
Sakthivel Sadayappan, PhD, FAHA, Loyola University Chicago, Maywood, Illinois
Ivonne Schulman, MD, Veteran's Affairs Medical Center, Miami, Florida
Susan Steinberg, MD, FAHA, Columbia University Medical Center, New York, New York

1:30–2:15 PM
Grand Ballroom A
Session 10
Outstanding Early Career Investigator Award Finalists

Moderators:
Lorrie Kirshenbaum, PhD, FAHA, St. Boniface Hospital Research Center, Winnipeg, Manitoba
Junichi Sadoshima, PhD, MD, FAHA, Rutgers New Jersey Medical School, Newark, New Jersey

1:30 **Disrupting the Interaction Between CaM Kinase II and Histone Deacetylase 4 – an Epigenetic Therapy for Heart Failure?** **433**
Johannes Backs, Tao He, Lorenz H. Lehmann, Andrea Schmidt, Jan Beckendorf, Univ of Heidelberg, Heidelberg, Germany; Joe Lewis, European Molecular Biology Lab, Heidelberg, Germany; Vasileios Askoxylakis, Radiation Oncology, Heidelberg, Germany; Hugo A. Katus, Univ of Heidelberg, Heidelberg, Germany

1:45 **Myelopoiesis Following Myocardial Ischemia (MI) Involves Activation of the Nlrp3 Inflammasome by Neutrophil-derived S100a8/a9** **116**
Prabhakara R. Nagareddy, Rahul Annabathula, Saojing Ye, Yuri Klyachkin, Ahmed Abdel-Latif, Susan Smyth, Univ of Kentucky, Lexington, KY

2:00 **High Efficiency Reprogramming of Fibroblasts Into Cardiomyocytes Requires Suppression of Pro-fibrotic Signaling** **126**
Kunhua Song, Yuanbiao Zhao, Pilar Londono, Emily Sharpe, Joshua R. Clair, Catherine Proenza, Rebecca O'Rourke, Kenneth L. Jones, Mark Y. Jeong, Lori A. Walker, Peter M. Buttrick, Timothy A. McKinsey, Yingqiong Cao, Univ of Colorado Sch of Med, Aurora, CO

2:15–3:35 PM
Grand Ballroom A
Session 11
Heart Failure with Preserved Ejection Fraction

Moderators:
Xinli Li, MD, PhD, First Affiliated Hospital/Nanjing Medical University, Nanjing, China
Jil Tardiff, MD, PhD, FAHA, University of Arizona, Tucson, Arizona

2:15 **HFpEF: Insights from the Clinic and Metabolomic Profiling**
Gregory Lewis, MD, Massachusetts General Hospital, Boston, Massachusetts

2:35 **Multiple Etiologies and Approaches to HFpEF**
David Kass, MD, FAHA, Johns Hopkins University, Baltimore, Maryland

2:55 **Titin's Role(s) in Diastolic Dysfunction and Heart Failure**
Henk Granzier, PhD, University of Arizona, Tucson, Arizona

3:15 **Diet-induced Diastolic Dysfunction**
Wilson Colucci, MD, FACC, Boston University Medical Center, Boston, Massachusetts

3:35–3:55 PM
Refreshment Break/Exhibits
Grand Ballroom B

3:55–5:35 PM
Grand Ballroom A
Session 12
Moving Novel Targets from Bench to Bedside: Academic-Industry Collaboration

Moderators:
Jeffrey Madwed, PhD, Merck Research Laboratories, Rahway, New Jersey
Albert Kim, MD, PhD, Pfizer CVMED Research Unit, Cambridge, Massachusetts

3:55 **How Do We Evaluate Novel Therapeutic Strategies for Heart Failure?**
Alison Schecter, MD, Johnson and Johnson Innovation, Cambridge, Massachusetts

4:15 **Targeting Fibrosis in Heart Disease**
Anthony J. Muslin, MD, Sanofi, Cambridge, Massachusetts

4:35 **Discover, Launch, Build: From Ideas to Transformative Companies**
Eric Green, MD, PhD, MyoKardia, Inc., San Francisco, California

4:55 **What Can We Learn From the Recent Success of the PARADIGM Trial About How We Should Approach Heart Failure Therapies?**
Mark Keating, MD, Novartis Institutes for BioMedical Research, Cambridge, Massachusetts

5:15 **Group Discussion/Q&A**

5:35–7:05 PM
Grand Ballroom C/D
Poster Session 3

7:05–11:00 PM
Napoleon Ballroom
BCVS Council Dinner
(Ticket required)

THURSDAY, JULY 16

7:00–8:00 AM
Continental Breakfast/Registration
Grand Ballroom B

8:00–9:40 AM
Grand Ballroom A
Session 13
Exploring the Functional Importance of Non-myocytes in Heart Failure

Moderator:
Burns Blaxall, PhD, FAHA, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio I

8:00 **What Makes a Myofibroblast?**
Jennifer Davis, PhD, Children's Hospital Medical Center, Cincinnati, Ohio

8:20 **Cardiomyocyte GSK-3 Deletion Leads to Mitotic Catastrophe and Fatal Dilated Cardiomyopathy**
Thomas Force, MD, FAHA, Vanderbilt University School of Medicine, Nashville, Tennessee

8:40 **Targeting Epigenetics to Block Cardiac Fibrosis**
Timothy McKinsey, PhD, University of Colorado, Aurora, Colorado

9:00 **A Functional Role for Inflammatory T-cells on Healing after Myocardial Infarction**
Stefan Frantz, MD, MUniversitätsklinik und Poliklinik für Innere Medi, Halle (Saale), Germany

9:20 **TGF-beta and Endoglin Signaling in Non-myocyte Cells of the Heart**
Navin Kapur, MD, Tufts Medical Center MCRI, Boston, Massachusetts

9:40–11:20 AM
Grand Ballroom A
Session 14
Genetics of Cardiac Development and Disease

Moderators:
Sakthivel Sadayappan, PhD, FAHA, Loyola University Chicago, Maywood, Illinois
Yibin Wang, PhD, FAHA, David Geffen School of Medicine at UCLA, Los Angeles, California

9:40 **Chromatin Remodeling in Cardiac Hypertrophy and Failure**
Gianluigi Condorelli, MD, PhD, Humanitas Research Hospital, Rozzano, Milan, Italy

10:00 **Epigenomic Dissection of Cardiac Chromatin and Disease Implications**
Tom Vondriska, PhD, University of California Los Angeles, Los Angeles, California

10:20 **Reversible Protein Acetylation**
Joseph Hill, MD, PhD, FAHA, UT Southwestern Medical Center, Dallas, Texas

10:40 **Spatial Expression Analysis of the Ischemic Heart Identifies Novel Factors Involved in Cardiac Remodeling and Disease**
Eva van Rooij, PhD, Hubrecht Institute, Utrecht, Netherlands

11:00 **Big Imaging-Genetic Data to Understand Big Hearts**
Stuart Cook, MD, PhD, Duke-National University of Singapore, Singapore, Rep of Singapore

11:20 AM
Adjourn

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Disrupting the Interaction Between CaM Kinase II and Histone Deacetylase 4 - an Epigenetic Therapy for Heart Failure?

Johannes Backs, Tao He, Lorenz H Lehmann, Andrea Schmidt, Jan Beckendorf, Univ of Heidelberg, Heidelberg, Germany; Joe Lewis, European Molecular Biology Lab, Heidelberg, Germany; Vasileios Askoxylakis, Radiation Oncology, Heidelberg, Germany; Hugo A Katus, Univ of Heidelberg, Heidelberg, Germany

CaM Kinase II (CaMKII) critically drives adverse cardiac remodeling. During the process of remodeling, CaMKII binds and phosphorylates Histone Deacetylase 4 (HDAC4), resulting in activation of the transcription factor MEF2. However, it remained unclear whether binding between CaMKII and HDAC4 causes heart failure and whether this interaction represents a novel therapeutic target. We used mouse genetics, HDAC4-based peptides and chemical biology to address these questions. First, we generated CaMKII-resistant HDAC4 mutant mice (CrH) by replacing Arg-598 (corresponds to Arg-601 in humans) of HDAC4 with Phe, because we found Arg-598 to be essential for the CaMKII-HDAC4 interaction. CrH were protected from cardiac dysfunction, hypertrophy and fibrosis in response to both pathological pressure overload or ischemia/reperfusion injury. CrH showed reduced CaMKII binding and less MEF2 activation. These data provided a proof-of-principle that the disruption of the CaMKII-HDAC4 interaction may have therapeutic potential. Thus, in a second step we engineered an HDAC4-derived peptide with homology to the CaMKII binding domain of HDAC4. This peptide competed with HDAC4 for binding with CaMKII, resulting in decreased MEF2 activation and attenuated agonist-induced cardiomyocyte hypertrophy. These data encouraged us to carry the translational pipeline one step further and we screened for small molecules that disrupt the CaMKII-HDAC4 interaction in an in vitro ALPHAScreen Assay (medium-throughput format using 78000 compounds). After a stringent validation process, 38 compounds showed > 40% inhibition. Out of these, 13 compounds effectively inhibited MEF2 activity in a cell-based assay without obvious signs for cellular toxicity, providing now potential cell permeable drug-like candidates. Chemical optimization and in vivo validation strategies are currently ongoing. In summary, we show that the CaMKII-HDAC4 interaction contributes to the development of heart failure and we identified drug-like molecules that specifically disrupt this protein-protein interaction. These findings lay the ground for a novel epigenetic therapeutic approach to combat heart failure.

J. Backs: None. **T. He:** None. **L.H. Lehmann:** None. **A. Schmidt:** None. **J. Beckendorf:** None. **J. Lewis:** None. **V. Askoxylakis:** None. **H.A. Katus:** None.

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Myelopoiesis Following Myocardial Ischemia (MI) Involves Activation of the Nlrp3 Inflammasome by Neutrophil-derived S100a8/a9

Prabhakara R Nagareddy, Rahul Annabathula, Saojing Ye, Yuri Klyachkin, Ahmed Abdel-Latif, Susan Smyth, Univ of Kentucky, Lexington, KY

Ischemic myocardial damage triggers leukocytosis particularly the production of monocytes and neutrophils from the bone marrow and spleen (myelopoiesis). These cells infiltrate the evolving myocardial wound, degrade extracellular matrix and aid in the clearance of dead cardiac myocytes and their debris. Although this inflammatory process is a prerequisite for tissue healing, it is non-specific and often blunt. If unchecked, excessive production of monocytes and neutrophils may result in abnormal ventricular remodeling and heart failure. The myocardial cellular and molecular events that orchestrate with the BM/spleen to regulate myelopoiesis remain unclear. We report here that the number of circulating monocytes and neutrophils peak within 24 hours following coronary artery ligation (LAD) in mice. This is due to expansion and proliferation of hematopoietic stem and multi-potential progenitor cells (HSPC) in the BM as well as extra medullary hematopoiesis in the spleen. MI induced -myelopoiesis was associated with a dramatic increase in the expression of S100a8/a9 (a damage associated molecular pattern), its receptor (Tlr4), the Nlrp3 inflammasome and pro-IL1β in the heart. Cell separation studies revealed that the infiltrating neutrophils and cardiac fibroblasts are the predominant source of S100a8/a9 and the Nlrp3 inflammasome respectively in the heart. Further, deletion of s100a8/a9 not only reduced MI -induced myelopoiesis but also significantly improved the mortality and cardiac function in mice following LAD. These data supports our hypothesis that neutrophil-derived S100a8/a9 interact with Tlr4 on cardiac fibroblasts to induce the Nlrp3 inflammasome and produce IL1β, which in turn stimulates IL-1R on HSPCs to promote myelopoiesis. Pharmacological strategies aimed at inhibition of S100a8a/9 or the Nlrp3 inflammasome-mediated production of IL1β may be a promising approach to limit inflammation following acute coronary syndrome.

P.R. Nagareddy: None. **R. Annabathula:** None. **S. Ye:** None. **Y. Klyachkin:** None. **A. Abdel-Latif:** None. **S. Smyth:** None.

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High Efficiency Reprogramming of Fibroblasts Into Cardiomyocytes Requires Suppression of Pro-fibrotic Signaling

Kunhua Song, Yuanbiao Zhao, Pilar Londono, Emily Sharpe, Joshua R Clair, Catherine Proenza, Rebecca O'Rourke, Kenneth L Jones, Mark Y Jeong, Lori A Walker, Peter M Buttrick, Timothy A McKinsey, Yingqiong Cao, Univ of Colorado Sch of Med, Aurora, CO

ABSTRACTS

The mammalian heart is composed of ~30% cardiomyocytes which have limited capacity to regenerate and ~70% non-cardiomyocytes including endothelial cells and cardiac fibroblasts. Direct reprogramming of fibroblasts into cardiomyocytes by forced expression of cardiomyogenic transcription factors, GMT (GATA4, Mef2C, Tbx5) or GHMT (GATA4, Hand2, Mef2C, Tbx5), has recently been demonstrated, suggesting a novel therapeutic strategy for cardiac repair. Despite extensive efforts, the efficiency of direct reprogramming of embryonic or adult fibroblasts into cardiomyocytes has yet to exceed 20%, or 0.1% respectively, leading many in the field to question the clinical translatability of this method.
Here, we demonstrate that pro-fibrotic signaling events governed by transforming growth factor-β (TGF-β) and Rho kinase (ROCK) are concomitantly activated in GHMT-expressing fibroblasts, leading to potent suppression of cardiac reprogramming (**Figure 1**). Remarkably, pharmacological inhibition of TGF-β, or ROCK leads to conversion of ≥ 60% of fibroblasts into highly functional cardiomyocytes, displaying global cardiac gene expression, spontaneous contractility, action potentials and calcium transients. Furthermore, inhibition of TGF-β, or ROCK dramatically enhances the kinetics of cardiac reprogramming, with spontaneously contracting cardiomyocytes emerging in less than two weeks, as opposed to 4 weeks with GHMT alone. These findings provide new insights into the molecular mechanisms underlying cardiac conversion of fibroblasts, and should enhance efforts to generate cardiomyocytes for clinical applications.

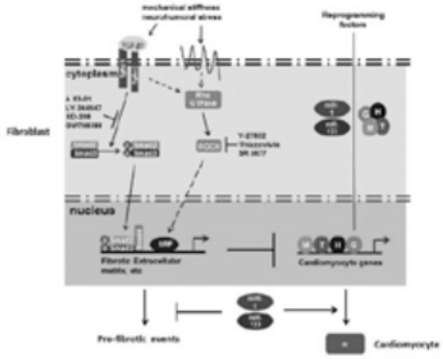

Figure 1. Pro-fibrotic signaling event serve as a barrier to cardiac reprogramming. Activated pro-fibrotic signaling inhibits cardiac reprogramming. Small molecules that inhibit pro-fibrotic events by targeting either TGF-β/SMAD or Rho-ROCK signaling are able to enhance reprogramming of fibroblasts into beating cardiomyocytes. miR-1 and miR-133 enhance cardiac reprogramming by attenuating pro-fibrotic events and other unidentified mechanisms.

K. Song: None. **Y. Zhao:** None. **P. Londono:** None. **E. Sharpe:** None. **J.R.S. Clair:** None. **C. Proenza:** None. **R. O'Rourke:** None. **K.L. Jones:** None. **M.Y. Jeong:** None. **L.A. Walker:** None. **P.M. Buttrick:** None. **T.A. McKinsey:** None. **Y. Cao:** None.

Abstracts

1
Chemical Biology of Cardiac Regeleration: an Instructive Hydrogel-based Platform for Heart Repair
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Regeleration is the myocardium's natural adaptive remodeling response to implanted biopolymer hydrogels, a new heart failure treatment modality with promising success in early clinical trials. Classified as a medical device capable of long-term myocardial engraftment, implanted hydrogels of varying molecular composition provide mechanical bulking and scaffolding support that can stabilize or reverse adverse ventricular remodeling. Additionally, natural or synthetically designed hydrogels encoding specific bioactivities or signaling functions can directly regulate myocardial biology to mediate heart repair. To gain mechanistic insight into the molecular and cellular biology and biochemistry of the biopolymer-myocardial interface, we studied two clinically relevant hydrogels - seaweed-derived alginate (Alg) and myomatrix (MMx), extracellular matrix molecules prepared from decellularized pig heart - in a mouse model. Alg and MMx differentially activated signal transduction cascades, recruited different cell types and produced distinctive gene expression signatures and patterns of cardiomyocyte hypertrophy, including muscle enhancer factor-2 (MEF2) and fetal gene program (re)activation. Chemically modifying Alg's backbone structure correspondingly altered myocardium's biological response, demonstrating the synthetic tunability of this repair process. These observations demonstrate that implanted biopolymer hydrogels drive unexpectedly robust and versatile regelerative responses in myocardium, transducing physical and biochemical signals to the cardiac genome that contribute to hydrogel function, providing a potential therapeutic target for enhancing hydrogel-mediated heart repair without stem cells.
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2
Mesenchymal Stem Cell Therapy Prevented Doxorubicin Induced Cardiac Dysfunction in Diabetics Rats
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Background: It is now established that having diabetes not only increases the chances of cancer also it complicates cancer treatment therapy. Doxorubicin (DOX) is a well known anticancer drug, however the clinical use of DOX was limited due to its cardiotoxic effects. One of the major concerns with DOX therapy has been its toxicity in patients who are less robust and more prone to toxic side effects, particularly patients with comorbid diseases such as diabetes mellitus. Several studies have demonstrated that mesenchymal stem cell (MSC) therapy has the potential to restore cardiac function following DOX induced cardiac injury. However, there is no study available on the effects of MSC therapy on DOX induced cardiac dysfunction in diabetics.
Methods and Results: Diabetes was induced in male Wistar rats by streptozotcin injection (STZ, 65mg/kg body weight, i.p.). After 4 weeks of STZ injection, blood glucose levels in STZ group

(301.58±23.97mg/dl) were significantly greater than control group (83.51±7.91mg/dl). These diabetic rats were treated with adriamycin (2.5mg/kg body weight, i.p) 3 times/week for two weeks (AD group); or with adriamycin+bone marrow MSCs (BM-MSC; 2x106 cells, via tail vein) or with adriamycin+adipose tissue derived MSCs (AD-MSC; 2x106 cells, via tail vein). Echocardiographic measurements showed a significant decline in cardiac function (%EF) following adriamycin treatment. Both BM-MSC and AT-MSC treatment improved %EF at 4 weeks. After 4 weeks of MSC injection, hearts from all the groups were excised and subjected to retrograde Langendorff perfusion and baseline levels of left ventricular developed pressure (LVDP), maximum rate of pressure rise dp/dt max and rate pressure product (RPP) were recorded. AD treatment caused a significant decrease in LVDP, dp/dt max and RPP levels. Both BM-MSCs and AD-MSCs injection significantly improved all these parameters. Conclusion: Both BM-MSC and AT-MSC were equally effective in preventing deterioration of cardiac function following doxorubicin therapy in diabetic rats. Furthermore, these findings should act as a stimulus for further research on the benefits of MSC therapy for diabetic subjects suffering from cancer.

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3

Human CD34+ Cells Deficient in miR-377 Attenuates Cardiac Dysfunction and Repair After Ischemia-reperfusion Injury

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microRNAs (miRNA/miR) dysregulation has been implicated in cardiac remodeling after injury or stress, however its effects on Human $CD34^+$ cells ($hCD34^+$) biology and function, particularly in the context of cell-based therapy for cardiomyopathy is not fully understood. miRNA array data analysis indicates that miR-377 is a potential interest. pre-miR-377 transfection in EPCs inhibits their migration and vascular tube formation ability in HUVECs. Furthermore, $hCD34^+$ cells treated with miR-377 mimic showed decrease in expression of STK35 (a novel serine/threonine kinase). Moreover, STK35 is predicted as a potential target gene of miR-377 by computational analysis. Interestingly, in a relevant mouse model of ischemia reperfusion, intramyocardial transplantation of miR-377-silenced $hCD34^+$ cells promotes neovascularization leading to improvement in myocardial function and repair. Echocardiography showed LV function was significantly improved in mice receiving miR-377-silenced $hCD34^+$ cells compared to control-miR-transfected $hCD34^+$ cells. Taken together, these data suggest that inhibiting miR-377 in $hCD34^+$ cells promotes their angiogenic ability after transplantation into ischemic myocardial tissue, potentially through activation of STK35 signaling.

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4

Porcine Parthenotes as a Model to Evaluate Developmental Potential of Human Inducible Pluripotent Stem Cells

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The use of human induced pluripotent stem cells (hiPSCs) has tremendous potential for regenerative medicine by providing an unlimited source of personalized cells. A number of protocols have been established for efficient differentiation of hiPSCs to the desired lineage in vitro, such as cardiomyocytes and blood. However, the field lacks an in vivo system to evaluate the differentiation potential and quality of hiPSCs. Developmental potential of stem cells derived from experimental animals can be readily assessed by generating blastocyst chimeras and examination of the contribution to the embryos, or by the potential of teratoma formation. However, this is not possible in the case of humans. As a potential solution for this issue, we examined whether porcine parthenotes could be used as an experimental model to test the developmental potential of the hiPSCs. Parthenotes are generated by electrical activation of the oocytes collected at the abattoir and will develop up to gestational day 53 if transferred to a pseudo-pregnant sow. The embryonic culture conditions have also been established and the zygotes can develop normally to the expanded blastocyst stage (day 7 post fertilization/activation), in vitro. We took advantage of this in vitro system and examined the ability of hiPSCs to proliferate and integrate into the parthenogenetic embryos. Parthenogenetic embryos were injected with ten undifferentiated hiPSCs at day 4 (8 cell ~ morula stage) and cultured up to 72 hours. During this period, parthenotes underwent blastocoel cavity formation and hatching. Cell tracing experiments demonstrated that hiPSCs proliferated and integrated into the parthenotes. They retained pluripotency marker expression during this period. hiPSCs and their derivatives were found both in trophoectoderm and embryo proper. We further observed that the hiPSCs underwent cellular proliferation and promoted developmental progression of the parthenote in vitro. In summary, the porcine parthenote model system is an efficient high throughput system to examine the developmental capacity of human stem cell populations.

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5

Novel Tools for Identification and Purification of Cardiomyocytes, Fibroblasts and Endothelial Cells From Neonatal Mouse and Rat Hearts

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Although isolation of vital cardiac cells from neonatal hearts is one of the most used experimental model in cardiac research, the manual dissociation of neonatal hearts is a laborious and difficult-to-standardize procedure. To shorten sample processing time and increase reproducibility, we developed a fully automated dissociation process followed by magnetic purification of cardiomyocytes (CMs), cardiac fibroblasts (CFs) and endothelial cells (ECs). Neonatal hearts were dissociated within 1 h resulting in high yields of single cells with excellent viability. Depending on age and species following cell ratios were recovered after dissociation: 50-70% CMs, 30-40% CFs, 10-20% ECs.

In order to purify CMs from dissociated neonatal hearts, we defined antibody cocktails enabling non-myocyte removal. Our depletion strategy allowed for the enrichment of CMs from whole hearts or preparations of heart chambers with purities of up to 98%. To enable simultaneous detection of CMs and subtypes, we developed recombinant antibodies against general CM markers like alpha Actinin, Myosin Heavy Chain or cardiac Troponin T as well as CM subtype-specific antibodies against MLC2a and MLC2v distinguishing between atrial and ventricular CMs respectively. Besides, we developed magnetic enrichment strategies for the purification of CFs and ECs. Current purification strategies that are solely based on antibodies against EC surface markers do not result in sufficiently pure cardiac ECs. Therefore, we established a 2-step enrichment protocol allowing for the complete removal of contaminating fibroblasts and leukocytes. Our data indicate that this protocol significantly improves the purity of primary cardiac ECs. Functionality of ECs before and after enrichment was proven by Dil-Ac-LDL endocytosis.
Similarly, our magnetic enrichment protocol for cardiac fibroblasts revealed homogenous expression of fibroblast markers like Vimentin and Prolyl-Hydroxylase, but virtually no contaminations with CMs or ECs.
In summary, we established an automated protocol for dissociation of neonatal hearts enabling subsequent purification, characterization and cultivation of CMs, CFs and ECs which can readily be used for cell culture assays or to generate heart muscle models.

D. Eckardt: 1. Employment; Significant; Miltenyi Biotec GmbH. **M. Kernbach:** 1. Employment; Significant; Miltenyi Biotec GmbH. **V. Czichowski:** 1. Employment; Significant; Miltenyi Biotec GmbH. **C. Hintzen:** 1. Employment; Significant; Miltenyi Biotec GmbH. **A. Bosio:** 1. Employment; Significant; Miltenyi Biotec GmbH.

6

Novel Tools for Generation, Purification, and Analysis of Pluripotent Stem Cell Derived Cardiomyocytes

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Pure and well-characterized cardiac cells derived from human pluripotent stem cells (hPSCs) are of high interest for cardiovascular disease modeling, drug safety studies and development of cell replacement strategies. Although several protocols for cardiac differentiation of hPSCs have been developed, major limitations are clone-to-clone variations in differentiation efficacy as well as heterogeneity of generated cardiomyocyte populations. Therefore, we have developed novel tools for cardiomyocyte differentiation, magnetic cell sorting-based purification and cell analysis using new antibody-conjugates enabling for flow cytometry- and immunofluorescence-based identification of cardiomyocytes and subtypes. hPSCs were maintained under xeno-free conditions in our recently developed StemMACS iPS-Brew XF medium to keep pluripotency for more than >20 passages and enable for efficient cardiac differentiation. We chose a monolayer differentiation protocol based on the timely regulated activation and inhibition of Wnt signaling by small molecules.
In order to identify antibodies suitable for cardiomyocyte enrichment or depletion of non-myocytes, we performed a surface marker screen with more than 400 antibodies between days 10-20 of differentiation. Besides identification of new surface markers, our screen confirmed expression of recently published markers like CD172a and CD106. In order to evaluate kinetics of their expression and correlation with intracellular markers of cardiomyocytes and subtypes, we performed a flow cytometry-based analysis of marker expression. Our data indicate a dynamic expression pattern for both CD172a and CD106, either completely or partially overlapping with intracellular cardiomyocyte marker expression. Based on these data we developed magnetic cell separation procedures for the isolation of cardiomyocytes. Magnetically enriched cardiomyocytes initiated contractions after replating and could be stably maintained in culture.
Taken together, we have developed novel tools supporting the workflow for efficient generation of PSC-derived cardiomyocytes, magnetic purification and flow cytometry or immunofluorecence-based characterization of cardiomyocytes.

D. Eckardt: 1. Employment; Significant; Miltenyi Biotec GmbH. **K. Noack:** 1. Employment; Significant; Miltenyi Biotec GmbH. **A. Bosio:** 1. Employment; Significant; Miltenyi Biotec GmbH.

8

Reprogramming of Adult Mammalian Cardiomyocytes Towards Proliferation and Regeneration Through Comparative Analyses of Epicardial and Myocardial Cells

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Heart development and regeneration require an elegant balance of cell proliferation and differentiation. In both adult zebrafish and neonatal mouse cardiac regeneration, new cardiomyocytes are shown to originate from pre-existing cardiomyocytes through de-differentiation of mature cells to the immature progenitor cell state, with developmental gene program reactivation and cell cycle reentry. Interestingly, after adult mammalian cardiac injury, both cardiac muscle cells and the epicardial cells that envelope the heart reinitiate developmental gene programs. However, adult epicardial cells are able to proliferate following injury but adult cardiomyocytes are not capable. We investigated the genetic circuitry of mouse epicardial cells and myocardial cells in different developmental stages and in response to injury. Such comparative analyses revealed a group of ~50 candidate genes that may be responsible for the permanent cell cycle arrest of cardiomyocytes. We generated adenoviruses that express these candidate genes individually, and demonstrated that the mixed viral pool possessed a robust activity to promote proliferation of adult mouse cardiomyocytes. Our comparative approach and functional screens may lead to identification of the dormant genetic circuitry in adult mammalian heart that can be reactivated to drive robust cardiomyocyte proliferation and regeneration.

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Temperature-responsive Cell Delivery Biopolymers for Cardiac Tissue Engineering

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Background: Advances in cell therapy and material science have made tissue engineering a promising strategy for heart regeneration. We developed an injectable biomimetic reverse thermal gel (RTG) that is liquid at room temperature but gel-like at body temperature, with the ultimate goal of being able to serve as a vehicle for cell-based delivery (liquid) to targeted tissue areas (gel-phase at 37°C). In this study we tested the suitability of this biomimetic RTG on cell viability.
Methods and results: We tested different biomimetic RTG systems with and without the chemical

incorporation of lysine. In vitro 3D culture experiments were performed with neonatal rat ventricular myocytes (NRVM) by mixing 3x104 cells with 50 µl of polymeric solution and allowing gel formation at 37°C. The cultured cells were incubated for 21 days. For controls we used NRVMs plated on 2D traditional gelatin coated dishes. We found that the 3D polymeric matrix induces rapid coordinated contraction with improved functionality when compared with standard 2D-cultured NRVM. By immunostaining for the morphology of the sarcomere (alpha-actinin) and DAPI, we also observed that the 3D polymeric matrix stimulates cells to spread and form 3D syncytia.
Conclusion: These proof-of-concept results demonstrate long-term cell viability in this unique biomimetic system and therefore provide feasibility of a polymeric cell delivery system that permits reversible liquid-to-gel transition at body temperature. These results offer potential for a tissue engineering approach to cardiac regeneration.

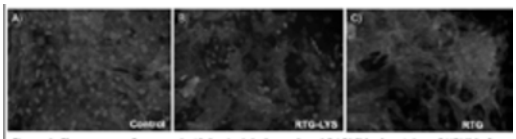

Figure 1. Fluorescence Sarcomeric Alpha Actinin (green) and DAPI (blue) staining of NRVM after 21 days of culture in different conditions: A) 2D tissue culture plate coated with gelatin (control), B) 3D RTG-Lysine, and C) 3D RTG. Compared to control groups, we found that the 3D polymeric matrix allows NRVM to spread resulting in 3D functional syncytia.

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MicroRNA 17 in Angiogenesis: Lessons Learned From Immobilized Vascular Endothelial Growth Factor

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Several pre-clinical and clinical studies are exploring the therapeutic effect of cell-based therapies in ischemic diseases, including Peripheral Artery Disease (PAD). Unfortunately, most of the cells (more than 80%) die few days after delivery. We postulate that better understanding of VEGF Biology might be important for the design of more effective pro-survival strategies. Release of VEGF from ECM activates a carcinogenic program since it triggers vasculogenesis, tumor growth and metastasis. Contrarily, immobilized VEGF might have all the properties of soluble VEGF without inducing a carcinogenic program. Thus, identification of downstream players such as miRNAs mediating this process might be important. Herein, we evaluated therapeutic effect of miR-17 downregulation, which mediates the effect of immobilized VEGF both in vitro and in vivo.
We recently showed that conjugated VEGF modulates cell activity by decreasing the expression miR-17 both in vitro and in vivo. In the present study, cell survival and angiogenesis were evaluated firstly in vitro using endothelial cells (ECs) transfected with antagomiR-17 to mimic the down-regulation of miR-17 by conjugated VEGF. AntagomiR-17 increased EC survival at least 1.5 times (n=6) compared to pro-angiogenic miRNAs reported in the literature (e.g. miR-424 and miR-132) and sprout formation on Matrigel at least 2 times (n=5) compared to all groups. The effect of antagomiR-17 was more pronounced under hypoxia conditions. In vivo, antagomiR-17 accelerated hemodynamic recovery of the whole limb (n=12) in unilateral limb ischemia obtained by occlusion of the left femoral artery. Blood flow recovery evaluated by Laser Doppler analysis was significantly higher 21 days after surgery in antagomiR-17 group compared to all other groups. Immunohistochemical analyses showed an increase in the capillary density of skeletal muscle in antagomiR-17 condition. In order to determine the gene target and potential pathway involved in the biological effect of antagomiR-17, next generation mRNA sequencing was performed.
In conclusion, here we show the potential and underlying molecular mechanism of antagomiR-17 treatment in endothelial cell survival and angiogenesis both in vitro and in vivo.

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Acquisition of Mitochondrial DNA Mutations Impairs Mitochondrial Function in Cardiac Progenitor Cells

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Activation of cardiac progenitor cells (CPCs) are critical for effective repair in response to pathologic injury. Stem cell activation and commitment involve increased energy demand and mitochondrial biogenesis. We have previously shown that incubation of c-kit+ CPCs in differentiation medium led to expansion of the mitochondrial network and lineage commitment. CPC function is reduced with age but the underlying mechanism is still unclear. Mitochondria contain their own DNA (mtDNA) which accumulates mutations over time that can impair mitochondrial function. In this study, we investigated the effects of acquiring mtDNA mutations on CPC proliferation, survival, and differentiation. We utilized a mouse model in which a mutation in the mtDNA polymerase gamma (POLGm/m) leads to accumulation of mtDNA mutations, mitochondrial dysfunction, and accelerated aging. Isolated CPCs from hearts of 2-month old POLGm/m mice had reduced proliferation and were more susceptible to oxidative stress and chemotherapeutic agents compared to WT CPCs. Incubation in differentiation medium resulted in fewer lineage committed POLGm/m CPCs compared to WT. In addition, the POLGm/m CPCs failed to activate mitochondrial biogenesis and did not increase levels of proteins involved in mitochondrial oxidative phosphorylation. We measured mitochondrial respiration with the Seahorse XF Analyzer and found that POLGm/m CPCs had undetectable oxygen consumption but still generated similar amounts of ATP as WT CPCs. Interestingly, POLGm/m CPCs produced increased amounts of l-lactate and were more sensitive to 2-deoxyglucose treatment, suggesting that these cells rely on glycolysis for energy production. Both WT and POLGm/m CPCs downregulated expression of glycolytic enzymes during differentiation. However, POLGm/m CPCs failed to undergo the metabolic transition from glycolysis to OXPHOS, which led to activation of cell death during differentiation. These data demonstrate that mitochondria play a critical role in CPC function, and accumulation of mtDNA mutations impairs CPC function and reduces their repair potential.

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Physiological Role of Endogenous Adult Cardiac Colony-forming Unit Fibroblasts

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Colony-forming unit fibroblasts (CFU-Fs), analogous to those giving rise to bone marrow (BM) mesenchymal stem cells (MSCs), have been identified in different tissues. CFU-Fs are multipotent progenitor cells, with a supportive paracrine and immunomodulatory function. They share the expression of surface antigens, are capable of self-renewal, and to differentiate into osteogenic, adipogenic and chondrogenic lineages in vitro. Our laboratory has isolated, for the first time, CFU-Fs from the adult murine heart. These cells are included within the Sca1+ PDGFRα+ CD31- fraction of interstitial cells, mainly associated with microvessels and the perivascular adventitial niche of larger vessels. Here we show that resident cCFU-Fs, are quiescent in homeostatic conditions, relatively more hypoxic and have a lower metabolic profile compared to other interstitial cells. Treatment with PDGF-AB ligand stimulates cCFU-Fs to exit the quiescent state, potentially making them more responsive to mitogenic and differentiative factors. In a mouse with a H2B-eGFP fusion gene knocked-in the Pdgfrα locus, cCFU-Fs lay in the GFPhigh population. After myocardial infarction, a GFPmed population appears, which has partially lost the expression of Sca1 and PDGFRα proteins, and consists largely of activated myofibroblasts. Likely derived from cCFU-Fs or related stromal fraction, GFPmed cells are significantly reduced by inhibiting PDGFRα signalling and increased by systemic PDGF-AB treatment. Despite augmenting the GFPmed myofibroblasts-like population, short-term PDGF-AB treatment is beneficial, leading to reduced scar, and increased vascular density and ejection fraction. A crucial factor in cardiac repair is the fine balance between pro-inflammatory (type1) and pro-reparative/pro-angiogenic (type2) immune responses, which can be regulated by MSCs. interestingly PDGF-AB treatment seems to be associated with an earlier resolution of the type1 response at 5 days after MI, compared to controls. We hypothesize that the beneficial effects of short term PDGF-AB treatment are due to early activation of cCFU-Fs and stromal cells from quiescence, leading to enhanced pro-angiogenic, anti-apoptotic and immunomodulatory impacts.

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Hippo Signaling Deletion During Heart Failure Reverses Functional Decline

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The heart has long been thought of as a static organ incapable of repair. Recent findings have challenged this view of the heart, and have demonstrated that mature cardiomyocytes are capable of re-entering the cell-cycle. However, there is still paucity in understanding endogenous mechanisms preventing cardiomyocyte self-renewal. Our approach is to apply developmental mechanisms of cardiomyocyte cell-cycle control to the damaged heart by altering the Hippo signaling pathway.

During development Hippo signaling regulates intrinsic organ size. The core mammalian Hippo pathway includes the Ste20-like serine/threonine kinases Mst1 and Mst2, homologous to the Drosophila Hippo kinase. A subsequent kinase cascade leads to the phosphorylation of the transcription factor Yap. Phosphorylated Yap is sequestered in the cytoplasm, thus preventing transcriptional activity. We previously demonstrated Hippo signaling controls cardiomyocyte proliferation during development to restrain heart size. Additionally, using both the Apex resection (AR) and LAD-ligation (MI) models of cardiac damage, in a Hippo signaling deletion mouse, we demonstrated cardiac regeneration. Indicated by preserved cardiac function and reduced fibrotic scar formation. Additionally, these hearts display cardiomyocyte proliferation as marked by EDU incorporation, pHH3, AurkB, and Ki67 staining. We are taking a new approach to determine the effect of Hippo signaling deletion on the failing heart, by inducing Hippo deletion after fibrotic scar formation has already occurred. To Thus far our results indicate functional recovery of the failing heart only after inducible deletion of Hippo signaling. Consistent with our previous data, preliminary results indicate adult cardiomyocytes after Hippo deletion re-enter the cell cycle. By altering Hippo signaling during heart failure and subsequently inducing cardiomyocyte proliferation we have established recovery of cardiac function. These results will greatly advance strategies to induce cardiac repair.

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Impact of Gata4, Mef2c and Tbx5 Stoichiometry on Epigenetic Status of Direct Cardiac Reprogramming

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Reprogramming from fibroblasts into induced cardiomyocytes (iCMs) offers alternative strategies for cardiac disease modeling and cardiac regeneration. Cellular reprogramming is closely associated with global re-patterning of the epigenetic landscape. The reprogramming cells must overcome the epigenetic barriers to acquire the target cell-like epigenetic pattern. We have recently demonstrated that stoichiometry of iCM reprogramming factors Gata4 (G), Mef2c (M) and Tbx5 (T) has profound impact on reprogramming efficiency and quality, yet the underlying mechanism remains unclear. Here, we demonstrate that the optimal stoichiometry combination MGT resulted in a reduced binding of the repressive marker histone 3 lysine 27 trimethlytion (H3K27me3) and an enhanced binding of the active marker histone 3 lysine 4 trimethlytion (H3K4me3) on the cardiac gene loci, compared to the least optimal combination GTM. We found that this epigenetic change occurred as early as day 3 when cardiac marker genes started to be induced in the reprogramming fibroblasts. Additionally, we determined the DNA methylation status in two cardiac gene loci during MGT and GTM induced reprogramming. Subsets of the CpG islands in these promoters were significantly more demethylated in MGT than GMT-transduced cells. We thus propose these CpGs islands as the “regulatory” CpGs critical for iCM reprogramming. In conclusion, we demonstrated that stoichiometry of G, M, T influences the epigenetic status of the iCM cells, suggesting a potential mechanism of how stoichiometry of G,M,T influences reprogramming.

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Global RNA Splicing Regulation in Cardiac Maturation

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Background: The complexity of transcriptome and proteome is contributed by alternative splicing of mRNA. Altered mRNA splicing is implicated in both development and disease. However, the change of alternative mRNA splicing during cardiomyocytes maturation is unknown, and the regulatory mechanisms remain unexplored.
Methods and Results: Using deep RNA-Sequencing, we identified global alternative splicing changes associated with both cardiac development and pathological remodeling in mouse heart. Further, we identified a highly conserved splicing regulator-RBFox1 to be significantly induced during zebrafish, mouse and human cardiac maturation. RBFox1 expression was also detected in cardiomyocytes derived from both mouse and human embryonic stem cells but at much lower levels comparing to adult heart. In zebrafish embryos, inactivation of RBFox1 caused cardiomyocyte maturation defects. Expression of RBFox1 in cultured neonatal cardiomyocytes was sufficient to promote maturation by reducing fetal marker gene expression while increasing calcium handling gene expression including RyR and promoting sarcomere organization. Deep RNA-Sequencing analysis showed that RBFox1 expression promoted alternative splicing in genes involved in calcium cycling, blood vessel development and muscle contraction. Finally, we identified a highly conserved mutually exclusive alternative splicing event of transcription factor MEF2 to be a direct downstream target of RBFox1. Expression of individual MEF2 splicing variants led to different cardiac developmental phenotypes in zebrafish, indicating their different transcriptional activities.
Conclusion: Our study provided the first comprehensive analysis of mRNA splicing regulation in heart during post-natal development and heart failure, and identified RBFox1 as a key regulator for alternative RNA splicing during cardiomyocytes maturation. Further exploration of RBFox1 mediated RNA splicing regulation in heart may yield novel insight to the underlying mechanisms of cardiac maturation and new approach to improve cell based therapy for heart diseases.
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Enhancing Myocardial Repair With CardioChimeras

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Dual cell transplantation of cardiac progenitor cells (CPCs) and mesenchymal stem cells (MSCs) after infarction enhances myocardial repair and performance in large animal models relative to delivery of either cell population individually. However, a single stem cell to support both direct and indirect mechanisms of myocardial repair has yet to be identified. CardioChimeras (CCs), a progenitor cell formed by fusion between CPCs and MSCs were analysed for reparative potential after myocardial infarction (MI) relative to individual parents cell or combined parent cell delivery. Two representative CCs, CardioChimera 1 (CC1) and CardioChimera 2 (CC2) were used for this study. CC1 and CC2 improved left ventricular anterior wall thickness (AWT) at 4 weeks, but only CC1 treatment preserved AWT at 18 weeks relative to no cell treatment (PBS). Ejection fraction was enhanced at 6 weeks post injury in CC1 and CC2 groups, which was maintained in CC1, CC2 and CPC + MSC combined groups up to 18 weeks. Infarct size was decreased by 5% in CC1 and CC2 hearts, whereas CPC + MSC and CPC parent groups remained unchanged when comparing 4 to 12 week change in scar size. MSC and PBS groups displayed marked increases in infarct size (10-15%). CC1 and CC2 showed enhanced engraftment potential by 3-fold relative to CPC + MSC and CPC hearts. In contrast, MSCs were detected at low levels (0.04%). CC1 and CC2 discovered within the myocardium expressed early commitment marker cardiac troponin T relative to controls. CC1 and CC2 treatment increased capillary density within the infarct, indicating that cell persistence facilitates paracrine mediated vasculature stabilization and/or formation. CCs merge the application of distinct cells into a single entity for cellular therapeutic intervention in the progression of heart failure. CCs represent a tractable cellular system that improves upon combinatorial cell therapy approaches and supports myocardial regeneration.
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Nuclear Calcium/Calmodulin-dependent Protein Kinase II Signaling Enhances Cardiac Progenitor Cell Survival and Cardiac Lineage Commitment

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Ca2+/Calmodulin-dependent protein kinase II (CaMKII) signaling in the heart regulates cardiomyocyte contractility and growth in response to elevated intracellular Ca2+. The δB isoform of CaMKII is the predominant nuclear splice variant in the adult heart and regulates cardiomyocyte hypertrophic gene expression by signaling to the histone deacetylase HDAC4. However, the role of CaMKIIδ in cardiac progenitor cells (CPCs) has not been explored. During developmental growth endogenous CPCs display primarily cytosolic CaMKIIδ, which localizes to the nuclear compartment of CPCs after myocardial infarction injury. CPCs undergoing early differentiation in vitro increase levels of CaMKIIδB in the nuclear compartment where the kinase may contribute to the regulation of CPC commitment. CPCs modified with an established lentiviral based constructs to overexpress CaMKIIδB (CPCeδB) have reduced proliferative rate compared to lentiviral transduction of CPCs with eGFP alone (CPCe). Additionally, stable expression of CaMKIIδB promotes distinct morphological changes such as increased cell surface area and increased length of cells compared to CPCe. CPCeδB are resistant to oxidative stress induced by H2O2 relative to CPCe, whereas a knockdown of CaMKIIδB using small hairpin RNA resulted in an up regulation of cell death compared to scrambled treated controls. Dexamethasone treatment to promote cardiac differentiation increased cardiomyogenic markers cardiac troponin T and α-smooth muscle actin measured by RT-PCR and immunoblot analyses in CPCeδB compared to control CPCe. Therefore, CaMKIIδB may serve as a novel

modulatory protein to enhance CPC survival and commitment into the cardiac and smooth muscle lineage.

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Increasing in vivo Apoa1/HDL Levels Negates the Cardiotoxic Effects of Doxorubicin, and Involves Signalling Through SR-BI, PI3K, and AKT1

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Doxorubicin (DOX) is a clinically used anti-tumor drug, though the use of DOX is limited by its potent cardiotoxic side effect that can lead to heart failure. HDL protects isolated cardiomyocytes against DOX induced apoptosis, though whether this effect translates in vivo has yet to be determined. Here we assess whether ApoA1/HDL overexpression can protect mice in vivo against DOX induced cardiotoxicity, and explore the intracellular signalling mechanisms involved in protection.

Mice overexpressing human ApoA1 (ApoA1tg/tg) and ApoA1+/+ mice were treated chronically with DOX, and effects on cardiac function and cardiomyocyte health were assessed. Over expression of human ApoA1 in mice corresponded to ~2.5 fold increase in plasma HDL-C as compared to ApoA1+/+ mice. Following 5 weekly injections of 5mg/kg DOX, ApoA1+/+ mice displayed cardiac dysfunction as evidenced by reduced left ventricular developed pressure, and reduced rate of pressure development. In contrast, left ventricular function was maintained following DOX treatment in ApoA1tg/tg mice. Histological analysis revealed reduced cardiomyocyte cross-sectional area and increased cardiomyocyte apoptosis following DOX treatment in ApoA1+/+ mice. ApoA1tg/tg mice, on the other hand, were protected against DOX induced cardiomyocyte atrophy and apoptosis. Interestingly, pAKT:tAKT was reduced in ApoA1+/+ by treatment with DOX, but the ratio was maintained in ApoA1tg/tg mice.

We evaluated the roles of SR-BI, PI3K, and AKT1/2 in the signalling cascade of HDL in neonatal mouse cardiomyocytes and human immortalized ventricular cardiomyocytes. Through inhibition of AKT and PI3K, and knockdown or knockout of SR-BI, AKT1, and AKT2, we demonstrated that SR-BI, PI3K and AKT1 are required for HDL mediated protection against DOX induced cardiomyocyte apoptosis.

Our results provide evidence for ApoA1 mediated protection against DOX cardiotoxicity in vivo and demonstrate the roles of SR-BI, PI3K, and AKT1 as mediators in the protective effect.

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Microrna-19b Enhances Cardiac Cell Survival After Ischemic Injury

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Ischemia/reperfusion (I/R) injury in cardiac tissue results in substantial loss of cardiomyocytes, leading to a functional decline. The release of several cytokines increases cardiomyocyte survival after ischemic injury by activating the janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway. However, this pathway is inhibited by Suppressor of cytokine signaling (SOCS)1 and SOCS3, which act as endogenous negative regulators of cardiomyocyte survival by blocking JAK activity. MicroRNAs (miRNAs) are small, non-coding RNAs that can repress gene expression by binding to recognition sequences within target genes. In a bioinformatic screen, we identified conserved binding sites in SOCS1 and 3 for miR-19b. Using a luciferase reporter assay, we were able to confirm direct binding of miR-19b to the 3'-UTR of both SOCS1 and 3 *in vitro*. In addition, we found that miR-19b downregulates SOCS1 in cardiac-like cells and isolated neonatal cardiomyocytes. *In vivo*, we observed a downregulation of miR-19b in both rodent and human cardiac tissues after ischemic injury, which corresponds to an upregulation of both SOCS1 and 3.

Since inhibition of SOCS1 and 3 could enhance cardiac JAK-STAT signaling, miRNA-based inhibition could lead to an increase in cardiomyocyte survival. Here we show that cardiomyocyte-specific overexpression of miR-19b lowers SOCS1 and SOCS3 expression and enhances JAK-STAT signaling during ischemia reperfusion, which corresponds to a decrease in the pro-apoptotic proteins Bad, Bax and P53. Additional genes that are decreased by miR-19b during ischemic injury are Rnf11 and TNFAIP3, both repressors NF-kB signaling. To further explore the cardioprotective effects of miR-19b, we are currently investigating the therapeutic benefits of administering synthetic miR-19b mimics through intracardiac injection after I/R injury in mice. Our study indicates a conserved mechanism by which miR-19b targets both SOCS1 and 3 and to increase the activation of JAK/STAT signaling to decrease cardiomyocyte apoptosis. Administration of miR-19b mimics might be of therapeutic interest to enhance cardiomyocyte cells survival and preserve heart function in the setting of ischemic injury.

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Diabetic Ketoacidosis Compromises Cardiac Mitochondrial Quality Control in Humans

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Mitochondrial injury plays a key role in the pathogenesis of diabetic cardiomyopathy, a risk factor for heart failure in diabetic populations. Dysfunctional mitochondria are eliminated through several coordinated mitochondrial quality control mechanisms including the autophagy-lysosome degradation pathway, a process termed mitophagy. Diabetic ketoacidosis (DKA) is a potentially life-threatening complication of type 1 diabetes. We have shown that adolescents and young adults with uncomplicated DKA mount a robust systemic inflammatory response which is associated with diastolic cardiac abnormality. However, it remains unknown if cardiac abnormality in patients with DKA is correlated with altered mitochondrial quality control processes. In the present study, using immunofluorescent labeling and confocal microscopic imaging, we examined a series of proteins involved in autophagy, mitophagy and mitochondrial dynamics in paraffin-embedded autopsy heart tissues from young people without or with fatal DKA. We found that the expression levels of beclin 1, microtubule-associated protein light chain 3 (LC3) and lysosome-associated membrane protein (LAMP) 1/2 are not changed, but that of p62 is increased in the heart of DKA patients, suggesting a decreased autophagy. Also, the expression levels and localization of Pink1, parkin and Rab9, three potential regulators of mitophagy, are

markedly changed, indicating an altered mitophagy in the DKA heart. In addition, mitochondrial fusion and fission proteins (Mitofusin 2, Opa1, Drp1 and Fis1) are all altered to varying degrees in the DKA heart, which is accompanied by increased mitochondrial fragmentation, oxidative injury and apoptosis (TUNEL positive cells). Together, these findings demonstrate that the mitochondrial quality control mechanisms are not operating normally in the heart of patients with DKA. Therefore, therapeutic strategies that aim to improve the efficiency of the mitochondrial quality control may have the potential to reduce diabetic cardiac injury and heart failure in young type 1 diabetic patients.

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Transient Receptor Potential M2 is Involved in Secondary Myocardial Injury Induced by Nonlethal Mechanical Trauma in Rats

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Background: It is an imperative task to identify the mechanisms responsible for post-traumatic secondary myocardial injury. Our previous experiments showed that mechanical trauma (MT) could induce secondary myocardial injury via oxidative stress. The transient potential receptor M2 (TRPM2) channel has emerged as an important Ca^{2+} signaling mechanism in a variety of cells, contributing to cellular functions that include cytokine production, cell motility and cell death. However, the role of TRPM2 channel in nonlethal mechanical traumatic cardiac damage remains unclear. The aim of the present study was to investigate whether TRPM2 channel is involved in myocardial injury in rats subjected to nonlethal MT. **Methods and results**: Western blot was used to quantify TRPM2 protein levels in Ventricular myocytes of adult male Sprague Dawley rats. Up-regulation of TRPM2 channel protein was observed in the following 12h after MT. It was observed that plasma harvested from MT rats increased cytosolic Ca^{2+} concentration dose-dependently in H9c2 cells. To verify the role of TRPM2 further, we administered TRPM2 blockers flufenamic acid (FFA, 100uM) and clotrimazole (CLZ, 30uM) respectively to inhibit Ca^{2+} influx, which leads to attenuated intracelluar Ca^{2+} overload and apoptosis induced by MT plasma in H9c2 cells. Those two TRPM2 blockers also improved cardiac dysfunction induced MT in rats. When we used TMB-8 (inhibitor of sarcoplasmic reticulum Ca^{2+} store) to inhibit calcium store mobilization, intracellular Ca^{2+} level, apoptosis and cardiac dysfunction were also ameliorated. However, the administration of KBR-7943 (inhibitor of Na/Ca exchanger) did not reverse the pathological process following MT. **Conclusion**: These results demonstrate that post-trauma pathological phenomena is associated with TRPM2 closely via a redox-sensitive signal transduction pathway (mainly via MT-initiated Ca^{2+} influx, even calcium overload pathway) .We propose that treatments like blockage of TRPM2 channel-associated Ca^{2+} influx and mobilization, may shed light on the novel therapeutic strategy in reducing cardiac injury and post-trauma multiple organ failure.

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Disruption of TRAF2-TAK1-NF-κB Signaling Axis Triggers K-48 Linked Poly-Ubiquitylation of RIP1 and Necrotic Cell Death in Doxorubicin Cardiotoxicity

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The anthracycline doxorubicin (Dox) is a highly effective anti-tumour agent, however, its use is limited by its severe cardiotoxic effects that manifests as heart failure. The decline in cardiac performance induced by doxorubicin remains poorly defined. A critical survival role for the canonical IKKβ -mTOR-NF-κB signaling pathway has been demonstrated in ventricular myocytes. In this report, we demonstrate that, Dox impairs IKKβ-mTOR- NF-κB signaling in ventricular myocytes accompanied by mitochondrial perturbations including mPTP, loss of mitochondrial membrane potential and ROS production. IKKβ- NF-κB signaling involves TRAF 2 mediated ligation of K63- ubiquitin chains to RIP1 (Receptor Interacting Protein 1) which serves as scaffold for recruitment of ubiquitylated Tak1 complexes and phosphorylation-dependent activation of IKKβ -NF-kB signaling. Interestingly, ventricular myocytes treated with dox demonstrated reduction in expression levels of TRAF2 and TAK1, in vivo and in vitro. This was accompanied by a decline in K63- and concomitant increase in K-48 linked polyubiquitination on RIP1, impaired NF-kB activation and necrotic cell death of cardiac myocytes. Interestingly, inhibiting the kinase activity of RIP1 with Necrostatin-1, (Nec1) suppressed necrotic cell injury induced by dox but not NF-kB activation. Concordant with these findings was a marked increase in necrotic cell death in cardiac myocytes defective for IKKB signaling or MEF cells deficient for p65 treated with dox. Notably, mitochondrial perturbations, including PT-pore opening , ROS production, calcium uptake, LDH, Tn(T) and HMGB-1 release and necrotic cell injury induced by dox were completely abrogated by restoring NF-kB signaling in cardiac myocytes or Nec-1. Herein, we provide novel evidence that K-48 linked poly ubiquitylation of RIP1 provides a functional switch that impairs NF-kB activation and signals necrosis in cells treated with dox. Interventions that modulate NF-kB activity may prove beneficial in mitigating the cardiotoxic effects of dox.

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Bnip3 Provokes ROS Production and Maladaptive Autophagy by Displacing Uncoupling Protein3 (UCP3) From Cytochrome c Oxidase of Respiration Chain Complex in Cardiotoxicity

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Doxorubicin is known for its cardiotoxic effects and inducing cardiac failure, however, the underlying mechanisms remain cryptic. Earlier we established the inducible - death protein, Bcl-2-like Nineteen-Kilodalton- Interacting - Protein 3 (Bnip3) to be crucial for disrupting mitochondrial function and inducing cell death of cardiac myocytes. Whether Bnip3 underlies cardiotoxic effects of doxorubicin toxicity is unknown. Herein we demonstrate a novel signaling pathway that functionally links activation and preferential mitochondrial targeting of Bnip3 to the cardiotoxic properties of doxorubicin. Perturbations to mitochondria including increased calcium loading, ROS, loss of $\alpha\Psi m$ and mPTP opening were observed in cardiac myocytes treated with doxorubicin. In mitochondria, Bnip3 forms strong association with Cytochrome c oxidase subunit1 (COX1) of respiratory chain and displaces uncoupling protein 3 (UCP3) resulting in increased ROS production, decline in maximal and reserved respiration capacity and cell viability. Impaired mitochondrial function was accompanied by an accumulated increase in autophagosomes and necrosis demonstrated by increase release of LDH, cTnT and loss of nuclear High

Mobility Group Protein 1 (HMGB-1) immunoreactivity. Interestingly, pharmacological or genetic inhibition of autophagy with 3-methyl adenine (3-MA), or Atg7 knock-down suppressed necrotic cell death induced by doxorubicin. Loss of function of Bnip3 restored UCP3-COX complexes, mitochondrial respiratory integrity and abrogated necrotic cell death induced by doxorubicin. Mice germ-line deficient for Bnip3 were resistant to doxorubicin cardiotoxicity displaying normal mitochondrial morphology, cardiac function and survival rates comparable to vehicle treated mice. The findings of the present study demonstrate that doxorubicin provokes maladaptive autophagy and necrotic cell death of ventricular myocytes that is mutually dependent and obligatorily linked to Bnip3.
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Polyphenolic - Ellagic Acid Suppresses Mitophagy-Induced Necrotic Cell Death During Doxorubicin Cardiotoxicity
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Reactive oxygen species (ROS) play a major role in cardiac dysfunction during myocardial ischemia. ROS production has been linked to oxidative stress injury and mitochondrial perturbations including permeability transition pore opening (mPTP), loss of mitochondrial membrane potential (ΔΨm) and necrotic cell death. Previously we identified the inducible Bcl-2 protein, Bnip3 as critical regulator of mitochondrial function and cell death of ventricular myocytes. Polyphenolic compounds including ellagic acid from pomegranate, have strong anti-oxidant properties. The effects of ellagic acid on oxidative stress injury in the heart has not been explored. In this report, we provide new compelling evidence that ellagic acid suppressed mitochondrial ROS production, loss of ΔΨm and necrotic cell death of cardiac myocytes induced by doxorubicin (DOX) or hypoxia. We further show mechanistically that the cytoprotective effects of ellagic acid were related to the transcriptional repression of Bnip3. In contrast to vehicle treated cells, cells treated with DOX or hypoxia displayed a marked increase in Bnip3 expression and mitochondrial association, concordant with increased ROS, mPTP, and loss of ΔΨm. Consistent with these mitochondrial defects there was a marked increase in mitophagy as confirmed by the dual emission Mitokeima probe that detects autophagic degradation by labelling mitochondria containing autophagosomes fused with lysosome. Mitophagy was accompanied by a marked increase in LDH release, loss of nuclear HMGB1 immunostaining and cell death. Interestingly, cells treated with ellagic acid were resistant to mitochondrial and the cytotoxic effects of DOX displaying reduced ROS production, mitophagy and were indistinguishable from vehicle treated control cells with respect to cell viability. Notably, Dox-induced Bnip3 expression was dramatically reduced in cells treated with ellagic acid. Hence, the findings of the present study demonstrate that ellagic acid suppresses mitochondrial perturbations and cell death of cardiac myocytes by mechanism that links to the repression of mitochondrial Bnip3.
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Fibroblast Growth Factor 21 Prevents Diabetic Cardiomyopathy by Attenuating Cardiac Lipotoxicity via Erk1/2-dependent Signaling Pathway in Mice
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The role of FGF21 plays in the development and progression of diabetic cardiomyopathy (DCM) has not been addressed. Here we demonstrated that type 1 diabetes decreased FGF21 levels in the blood, but up-regulated cardiac fgf21 expression about 40 fold at 2 months and 3-1.5 fold at 4 and 6 months after diabetes, which indicated a cardiac specific FGF21 adaptive up-regulation. To define the critical role of FGF21 in DCM, type 1 diabetes was induced in FGF21 knock out (FGF21KO) mice. At 1, 2 and 4 months after diabetes onset, no significant differences between FGF21KO and wild type (WT) diabetic mice in blood glucose and triglyceride levels were observed. But FGF21KO diabetic mice showed earlier and more severe cardiac dysfunction, remodeling and oxidative stress, as well as greater increase in cardiac lipid accumulation than WT diabetic mice. Mechanistically, FGF21 reduced palmitate-induced cardiac cell death, which was accompanied by up-regulation of cardiac Erk1/2, p38 MAPK and AMPK phosphorylation. Inhibition of each kinase with its inhibitor and/ or siRNA revealed that FGF21 prevents palmitate-induced cardiac cell death via up-regulating the Erk1/2-dependent p38 MAPK/AMPK signaling pathway. In vivo administration of FGF21, but not FGF21 plus ERK1/2 inhibitor, to diabetic mice significantly prevented cardiac cell death and reduced inactivation of Erk1/2, p38 MAPK and AMPK, and prevented cardiac remodeling and dysfunction at late-stage. Our results demonstrate that cardiac FGF21 decompensation may contribute to the development of DCM and FGF21 may be a therapeutic target for the treatment of diabetic cardiac damage via activation of Erk1/2-P38 MAPK-AMPK signaling.
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Psychometric Testing of Instruments for Use in Women's Cardiovascular Recovery Globally
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Worldwide, 3.4 million women die each year from cardiovascular disease. After experiencing a cardiovascular event, a woman's physical health, the women's likelihood of being treated with coronary artery bypass graft surgery, likelihood for referral for cardiac rehabilitation are less favorable than men. An established conceptual model depicts psychosocial stressors and the influence of behavioral risk factors on the pathogenesis of atherosclerosis and the occurrence of CV events. The woman's social stressors of role quality and behavioral risk factors of low self-efficacy for physical activity are associated with physical and mental health outcomes following cardiovascular crises. The study aimed to evaluate the reliability of the translated versions (Japanese, Ukrainian, Tagalog, Hispanic and Arabic) of the worker, partner, mother Role Quality and the Self Efficacy of Lifestyle Physical Activity indices among 282 women (aged 35-92 years) representing seven cultures. The study was performed in a multi-center, multicultural context. Translations followed an established process. Results showed reliability was strong (coefficient alphas of 0.93 and 0.88). These instruments underwent first-time confirmatory factor analyses with acceptable, though

borderline fit, thus providing valuable input for strengthening in future studies. Understanding a woman's role quality and self-efficacy for lifestyle physical activity provides valuable information that assists health-care professionals to co-develop with the woman an individualized plan to reduce role stress and to initiate increased physical activity following cardiovascular episodes.

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Extracellular Matrix Coating Enhances Nanoparticle Uptake by Lung Epithelial Cells

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Introduction: Rapid uptake of drug-loaded nanoparticles (NPs) by lung cells is critical for effective pulmonary delivery of therapeutic agents because of the rapid pulmonary clearance mechanisms. We tested the possibility that coating NPs with extracellular matrix (ECM) derived from lung tissue enhances nanoparticles uptake by lung cells. **Methods and Results**: Fresh adult porcine lung tissue obtained from a local slaughterhouse was decellularized using detergent (sodium dodecyl sulfate) and then enzymatically digested into a soluble solution. The double emulsion method was utilized to fabricate core-shell poly(lactic-co-glycolic) (PLGA) nanoparticles loaded with bovine serum albumin (BSA) for protein release studies, 6-coumarin for cellular uptake studies, or human erythropoietin receptor (hEPOR) cDNA co-expressing green fluorescent protein (GFP) for *in vivo* gene expression studies. The ECM was coated onto the nanoparticle surface by physical adsorption using the ECM solution (100 µg/ml). There is no significant difference in the diameter, blood compatibility or cell toxicity between coated and uncoated NPs. ECM-coated NPs showed slower protein release rate than uncoated NPs as the ECM coating hindered protein diffusion into the solution. ECM-coated NPs showed significantly higher cellular uptake by human lung epithelial cells than collagen-coated or uncoated NPs. In addition, ECM-coated and uncoated NPs loaded with hEPOR-GFP cDNA were aerosolized and delivered by inhalation into rat lung. Following single inhalation using uncoated NPs, GFP expression in lung tissue progressively increased for up to 21 days. Using the ECM-coated NPs EPOR expression peaked at 14 days, then declined thereafter. **Conclusions:** Coating NPs with lung-derived ECM markedly enhances NP uptake by lung cells, delays the release of encapsulated protein or DNA, and shortens the duration of peak tissue gene expression compared to uncoated NPs. This NP formulation may be useful where more precise timing of delayed payload release is desired.

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Biodegradable Elastomeric Polyurethane Scaffolds Mechanically Matching With Native Heart Muscle

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Introduction: Biodegradable cardiac patches need to be mechanically matching with native heart muscle, in order to provide appropriate mechanical support to rapidly restore heart functions and promote tissue remodeling for myocardial infarction (MI) management. Here, we utilized chemical molecular design to develop biodegradable elastomers with low initial modulus and then process them into porous scaffolds mechanically matching with native heart muscle. **Methods and Results:** We synthesized various amorphous copolymers including poly (δ-valerolactone-co-ε-caprolactone) (PVCL) and poly (ether ester) triblock copolymers with various molecular weights and poly(ethylene glycol) (PEG) molecular weights (PVCL-PEG-PVCL). The polyurethanes were then synthesized from PVCL or PVCL-PEG-PVCL as a soft segment, hexamethylene diisocyanate (HDI) as a hard segment and putrescine as a chain extender. The polyurethane products were presented as PU-PEGx-VCLy, where x and y refer to molecular weights of PEG and PVCL, respectively. Five polymers including PU-VCL_{2k}, PU-VCL_{6K}, PU-PEG_{1K}-VCL_{1K}, PU-PEG_{1K}-VCL_{6K} and PU-PEG_{2K}-VCL_{6K} were obtained. All polymers gradually degraded in phosphate buffer solution and enzyme solution. The 3T3 fibroblasts can grow and proliferate on all polymer film surface within 5 day culture, indicating the polymers have good cellular compatibility. PU-VCL_{6K}, PU-PEG_{1K}-VCL_{6K} and PU-PEG_{2K}-VCL_{6K} were further processed into porous scaffolds using thermally induced phase separation (TIPS). The PU-PEG_{2K}-VCL_{6K} scaffold at wet state had 0.19 ± 0.08 MPa initial modulus, which has no significant difference from initial modulus (0.19 ± 0.04 MPa) of the native porcine heart muscle. But the tensile strength of this scaffold is lower than that of heart muscle, which requies to be improved in the future. **Conclusions:** A new family of biodegradable elastic polyurethanes was synthesized and processed into porous scaffolds. The scaffolds showed promising mechanical match with heart muscle. These biodegradable polyurethane scaffolds would find opportunities to be used as a cardiac patch for heart infarction treatment.

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4-PBA Prevents TAC-induced Myocardial Hypertrophy and Interstitial Fibrosis by Attenuating ER Stress in Mice

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Background: Recently, 4-phenylbutyric acid (4-PBA) has been recognized as a potent ER stress inhibitor and a histone deacetylase inhibitor, but its therapeutic effect in cardiovascular diseases is still not fully understood. Our previous study indicated that attenuation of ER stress by administration of low dosage of 4-PBA (20 mg/kg/d) prevented post-infarction-induced cardiac rupture and remodeling through modulating both cardiac apoptosis and fibrosis in the mouse model of myocardial infarction. However, little is known whether the administration of 4-PBA is effective for hypertrophic heart disease. The aim of this study is therefore to test the therapeutic effect of 4-PBA on pressure-overload induced myocardial hypertrophy.

Methods and Results: Transverse aortic constriction (TAC) was used to produce pressure-overload in

C57BL/6 male mice for 4 weeks. After surgery, 4-PBA (20mg/kg/d) or 0.9% NaCL was intraperitoneally injected daily. At the end of 4 weeks, the survivals were underwent high-resolution echocardiographic imaging observation. The results showed that the left ventricular posterior wall thickness at end systole (LVPWs) and left ventricular posterior wall thickness at end diastole (LVPWd) were increased in TAC group. Administration of 4-PBA ameliorated this hypertrophic effect. Autopsy also confirmed the anti-hypertrophic effect of 4-PBA. Masson-staining found that there was no difference in perivascular fibrosis between TAC and TAC+4-PBA groups. However, myocardial interstitial fibrosis and collagen deposition were significant decreased by 4-PBA. We finally detected the ER stress and histone acetylation using western blotting. Our results showed that 4-PBA, at the dosage of 20 mg/kg/day, decreased the expression of CHOP and had no effect on histone 3 acetylation.
Conclusion: These findings indicate that attenuating ER stress by 4-PBA maybe a promising therapeutic strategy to prevent pressure-overload induced myocardial hypertrophy and interstitial fibrosis.
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High-density Lipoprotein, a Feed-forward Forechecking Loop
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Background: The protective effect of HDL is projected into the cholesterol back-transport of breakdown products of lipid metabolism to the liver. This is a feed-back circuit which controls cholesterol exit. Cholesterol entry could be safeguarded in a feed-forward forechecking loop at the proteoglycan (PG) receptor sites (syndecan, perlecan). HDL is the counter partner in the system of internalization and diffusion control for lipoprotein entry into the vascular wall. Based on stereochemical and chiral conformity with its PG receptor and the much higher negative charge density, HDL owns by far a higher affinity compared with LDL. **Methods:** Flow-dependent isometric tension, intracellularly recorded membrane potential and cAMP-cGMP were measured in segments of 25 coronaries from heart transplantations. **Results and Discussion:** Compared to Krebs solution, LDL (100 mg/dL) impaired flow-dilatation and caused a relative contraction by 18.5% (Table). In contrast, HDL (50 mg/dL) and HDL+LDL stimulated flow-dilatation by 31.1% and 41.4%, respectively ($p < 0.96$). Thus, the contractile effect of LDL was absent in the Krebs-HDL-LDL solution. The same effects were apparent in the membrane potential and cAMP-cGMP-concentrations of the VSM cells. These results are confirmed by ellipsometry measurements, where nanoplaque formation by LDL is suppressed by preincubation with HDL [Siegel, Malmsten, Ermilov: Adv Coll Interface Sci 205 (2014) 275-318]. **Conclusion:** LDL-cholesterol entry into the vascular wall is effectfully controlled by HDL in a feed-forward forechecking loop (HDL 4× higher affinity constant to the PG receptor). The interaction between both is dominated by competition.

Solution	T_2 [g]	T_{100} [g]	ΔT [g]	p*
Krebs + LDL	1.815	1.569	0.246	< 0.0007
Krebs	1.776	1.474	0.302	
Krebs + HDL	1.670	1.274	0.396	< 0.0004
Krebs + HDL + LDL	1.704	1.277	0.427	< 0.0001

*tested against Krebs solution

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Mitral Valve Interstitial Cells With the Serotonin Transporter (SERT)-LL Polymorphism are More Efficiently Activated by Serotonin
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Prolapse of the Mitral Valve (MV) leaflet is a hallmark of several cardiac disorders such as Myxomatous MV Disease (MMVD) or ischemic MV regurgitation (MR), and is only treated surgically. Elevated levels of serotonin (5HT) have been associated with valvulopathies. Ongoing studies in our laboratory suggest that 5HT signaling plays an important role in the progression of MMVD. In addition, a 44 base polymorphism in the promoter region of the 5HT transporter (SERT) gene, designated as short (S) or long (L) allele, has been reported with Mendelian distribution, with LL resulting in increased 5HT reuptake compared to SS.
We hypothesized that patients with MMVD have enhanced 5HT signaling due to a high frequency of SERT-LL. DNA was extracted from 152 MMVD surgical patients, genomic fragment analysis performed to determine allelic frequencies, and a chi-square statistical test completed. We show that surgical patients with MMVD have a higher than expected frequency of SERT-LL (34% (52 of 152) vs. 25%). Notably, the frequency of SERT-LL is particularly enhanced (51% (21 of 41) vs. 25%) ($p=0.009$) in MMVD patients under 60 years old. Based on these findings, we tested whether MV interstitial cells (MVICs) expressing SERT-LL have increased susceptibility to 5HT-mediated activation. We demonstrate that SERT-LL MVICs respond to 5HT with a 4-fold increase in smooth muscle actin (SMA) mRNA expression, a marker of VIC activation, compared to a less than 1-fold increase in SERT-LS and SERT-SS cells. The SMA response is abrogated by specific pharmacological inhibitors of the 5HT receptors 5HTR2A and 5HTR2B, and SERT, as well as siRNA knockdown. Studies of 5HTR expression in cells of each genotype are in progress.
Due to the enhanced frequency of the SERT-LL genotype among MMVD patients, particularly within the younger subset, and increased susceptibility of SERT-LL MVICs to 5HT, we postulate that the SERT-LL genotype may contribute to an increased risk of rapid disease progression by increasing the efficiency of 5HT signaling and uptake in MVICs resulting in extracellular matrix production leading to prolapse and impaired valve function. The SERT-genotype may constitute a novel means of identifying patients who may benefit from pharmacotherapy that alter 5HT-related mechanisms.
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Prostaglandin E2 Reduces Cardiac Contractility via EP3 Receptor
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Prostaglandin E2 (PGE2) EP receptors EP3 and EP4 are present in the heart and signal via decreased and increased cAMP production, respectively. Previously we reported that cardiomyocyte-specific EP4 KO mice develop a phenotype of dilated cardiomyopathy with

reduced ejection fraction. We thus hypothesized that PGE2 decreases contractility via EP3. To test this hypothesis, the effects of PGE2 and the EP1/EP3 agonist sulprostone (sulp) were examined in the mouse langendorff preparation and in adult mouse cardiomyocytes (AVM) using the IonOptix cell contractility system. Isolated hearts of 18-20 wk old male C57Bl/6 mice were mounted and equilibrated for 10 min, then perfused with PGE2 (10^{-6}mol/l) or sulp (10^{-6}mol/l) for 30 min. Values at the end of equilibration were set to 100%. Compared to vehicle, PGE2 decreased +dp/dt (77.8±3% vs 96.7±3%, p<0.01) and left ventricular developed pressure, LVDP (77.2±2% vs 96.8±3%, p<0.001). Sulp decreased +dp/dt (75.9±2% vs 96.7±3%, p<0.001), -dp/dt (72.2±1% vs 85.7±1%, p<0.01) and LVDP (70.9±1% vs 96.8±3%, p<0.001). The effects of both PGE2 and sulp were reversed by the EP3 antagonist, L789,106 (10^{-6}mol/l). Myocyte contractility was evaluated on the IonOptix system with pacing at 1Hz. Treatment with PGE2 (10^{-9}M) for 10 min reduced contractility as measured by peak height (3.69 ± 0.48% for vehicle vs 2.00 ± 0.22% for PGE2, p < 0.05), departure velocity (-171.9 ± 22.9 um/sec for vehicle vs -106.3± 12.5 um/sec for PGE2, p < 0.05) and return velocity (87.7 ± 16.3 um/sec for vehicle vs 36.7 ± 6.6 um/sec for PGE2, p < 0.05) with similar effects noted for sulp. Sulp reduced change in peak height (4.79 ± 1.15% for vehicle vs 1.81 ± 0.37% for sulp, p < 0.05), departure velocity (-169.1 ± 35.8 um/sec for vehicle vs -59.4 ± 10.3 um/sec for sulp, p < 0.05) and return velocity (86.5 ± 23.8 um/sec for vehicle vs 16.9 ± 14.7 um/sec for sulp, p < 0.05). We then examined the acute effects of PGE2 and sulp on expression of phosphorylated phospholamban (PLN) and SERCA using Western blot. Treatment of AVM for 15min with either PGE2 or sulp decreased expression of phosphorylated PLN corrected to total PLN, by 67% and 43%. SERCA2a expression was unaffected. In conclusion, PGE2 and sulp reduce contractility via the EP3 receptor through effects on PLN.

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STIM1 Increases Calcium Stores in the Sarcoplasmic Reticulum of Adult Feline Ventricular Myocytes

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Background: STIM1 is a Sarcoplasmic Reticulum (SR) membrane resident protein implicated in sensing and maintaining SR Ca2+ levels. The role of STIM1 in the regulation of SR Ca2+ stores in the normal and diseased heart is not well described. Previous reports confirm that STIM1 is present at low levels in the healthy adult heart, but expression levels increase after cardiac injury.
Objective: To determine if increased STIM1 expression after cardiac injury may be involved in the disturbed Ca2+ cycling present within diseased cardiomyocytes. Results: We used adenovirus to express either STIM1 or red fluorescent protein (RFP) in freshly isolated adult feline ventricular myocytes (AFMs). After 48 hours in culture, STIM1 induced cell death in 60% of myocytes versus only 5% in RFP controls. Addition of nifedipine rescued the cell death caused by STIM1, but block of transient receptor potential canonical (TRPC) channels was unable to improve viability. AFMs expressing STIM1 exhibited increased fractional shortening and Ca2+ transient amplitude, which was associated with increased SR load. Interestingly, high SR load levels caused by STIM1 were not reduced by nifedipine addition. We also found that baseline L-type channel current amplitude was significantly reduced by 20%, but no difference in current amplitude was found after Bay K8644 addition.
Conclusions: In this study, we found that STIM1 caused Ca2+ overload leading to cell death. This process was L-type channel dependent, but TRPC channels were not involved. STIM1 also caused increased SR load at rest, which was not altered by nifedipine revealing that STIM1 mediated Ca2+ influx does not require L-type channel activity but Ca2+ influx through L-type channels is essential for STIM1 induced cellular death. Furthermore, STIM1 reduced L-type current activity but did not alter channel availability. These data show that after cardiac injury, STIM1 is likely to play a role in regulating cell survival as well as excitation-contraction coupling.

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Pseudo-knockin Mice Expressing JPH2-A399S Develop Cardiac Hypertrophy by Magnetic Resonance Imaging

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Background: Dysfunctional intracellular Ca2+ handling has been implicated in adverse cardiac remodeling leading to hypertrophy and failure. Recent evidence has linked mutations in the Ca2+ handling protein Junctophilin-2 (JPH2) with the development of hypertrophic cardiomyopathy (HCM). However, the mechanism remains unknown. Objective: To use advanced in vivo imaging modalities in conjunction with biochemical techniques to determine the mechanism of hypertrophic remodeling in a murine model hosting a novel JPH2 mutation. Methods and Results: 1. Pseudo-knockin (PKI) Mice: transgenic mice with the JPH2-A399S mutation (or WT JPH2) containing inducible shJPH2 were dosed with tamoxifen to knock down the combined levels of JPH2 to near WT expression. 2. MRI: A399S and WT PKI controls were imaged at 2 months post injection at which time body weight was similar for mutants (29.8±.81g) and controls (31.8±1.29g). Intragate (Bruker) was used to obtain FLASH cine images and EKG-gated tagged images were obtained to determine strain. MRI post-processing and measurements were performed using Amira and Diagnosoft software. A399S PKI mice exhibited significantly increased left ventricular mass (2.96±.21g/kg; n=4) compared to controls (2.27±.08g/kg; n=3) and max diastolic septal thickness (1.39±.05mm; n=9 versus 0.97±.00mm; n=4; P<0.01). Mutants trended toward decreased septal strain (-8.97±1.04; n=4) compared to the control (-10.28; n=1) indicative of reduced regional contractility 3. Biochemistry: stress markers were measured by qPCR. Average BNP was increased over 3 fold. Larger sample size is needed to reach significance. Western blot showed no significant change (p=.78) in phosphorylated Ca2+/calmodulin-dependent protein kinase II, which is often activated by aberrant Ca2+ signaling. Conclusions: Our data show that the JPH2-A399S mutation leads to septal hypertrophy in PKI mice. This suggests that defects in JPH2 are sufficient to induce pathological cardiac remodeling. Despite the role of JPH2 in Ca2+ handling, this form of hypertrophy does not appear to be mediated by traditional Ca2+ signaling. Further studies will focus on alternative Ca2+-dependent pathways to elucidate the molecular mechanisms of these hypertrophic changes.

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Administration of 17β-Estradiol in C57Bl/6N Female Mice Leads to Cardiac Atrophy and Dysfunction via a β-Catenin Mechanism

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The steroid hormone 17β-estradiol (E2) regulates several biological processes. In contrast to its anti-hypertrophic effects under pressure overload, we recently found that E2 induced physiological hypertrophic growth in healthy C57Bl/6J mice but not C57Bl/6N mice. Here, we aimed at the characterization of the effects of E2 in C57Bl/6N mice and tested the hypothesis that β-catenin mediates these E2 effects. Following ovariectomy, 2-month-old C57Bl/6N wild-type and cardiac-specific β-catenin-deleted (β-cat$^{\Delta ex2-6}$) mice were randomized to an E2-containing or soy-free (control, CON) diet (n = 7-13/group). Cardiac function was examined by echocardiography following established procedures. The 3-month physiological dose of E2 led to a higher relative uterus weight compared with CON (P < 0.001) in both WT and β-cat$^{\Delta ex2-6}$ mice. The relative heart weight was significantly reduced by E2 compared with CON in WT mice (P < 0.001), while there was no significant effect in β-cat$^{\Delta ex2-6}$ mice. Cardiomyocyte cross-sectional area was also significantly decreased by E2 (n = 5-7/group; P < 0.001) compared with CON in WT mice, while there was no significant effect in β-cat$^{\Delta ex2-6}$ mice. Echocardiography revealed a significant decrease in septum width (P < 0.001) and posterior wall thickness (P < 0.01) in E2 treated WT mice compared with CON, while there was no significant effect in β-cat$^{\Delta ex2-6}$ mice (n = 8/group). These E2-induced structural changes in WT mice were accompanied by a significant decrease in cardiac function, namely a 23% decrease in fractional shortening compared with CON (P < 0.05), while there was no significant effect in β-cat$^{\Delta ex2-6}$ mice. Immunoblotting revealed a significant increase in the levels of the ubiquitin ligase and key regulator of proteasome-dependent protein degradation muscle-specific RING finger protein 1 (MuRF1) by E2 compared with CON in WT mice (P < 0.05), while there was no significant effect in β-cat$^{\Delta ex2-6}$ mice. Although we hypothesized increased autophagic activity, we found no effect on the autophagy-related protein LC3 in WT or β-cat$^{\Delta ex2-6}$ mice. In conclusion, our surprising findings show that E2 leads to cardiac atrophy and dysfunction in C57Bl/6N mice via a β-catenin mechanism seemingly in an autophagy-independent manner.

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Nrip Deficiency Leads to Dilated Cardiomyopathy

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Previously, we demonstrate a gene, nuclear receptor interaction protein (NRIP, also named DCAF6 or IQWD1) as a Ca2+- dependent calmodulin binding protein that can activate calcineurin phosphatase activity. Here, we found that α-actinin-2 (ACTN2), is one of NRIP-interacting proteins from the yeast two-hybrid system using NRIP as a prey. We further confirmed the direct bound between NRIP and ACTN2 using in vitro protein-protein interaction and in vivo co-immunoprecipitation assays. To further map the binding domain of each protein, the results showed the IQ domain of NRIP responsible for ACTN2 binding, and EF hand motif of ACTN2 responsible for NRIP bound. Due to ACTN2 is a biomarker of muscular Z-disc complex; we found the co-localization of NRIP and ACTN2 in cardiac tissues by immunofluorescence assays. Taken together, NRIP is a novel ACTN2-interacting protein. To investigate insights into in vivo function of NRIP, we generated conventional NRIP-null mice. The H&E staining results are shown in the hearts of NRIP KO mice are enlarged and dilated and the cell width of NRIP KO cardiomyocyte is increased. The EM of NRIP KO heart muscles reveal the reduction of I-band width and extension length of Z-disc in sarcomere structure; and the echocardiography shows the diminished fractional shortening in heart functions. Additionally, the calcium transient and sarcomere contraction length in cardiomyocytes of NRIP KO is weaker and shorter than wt; respectively. In conclusion, NRIP is a novel Z-disc protein and has function for maintenance of sarcomere integrity structure and function for calcium transient and muscle contraction.

S. Chen: None.

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Simultaneous Optical Pacing and Optical Voltage Mapping in Optogenetic Neonatal Rat Ventricular Myocyte Cultures

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Optogenetics is an emerging technology allowing remote and precise control of cell activity in living tissues. Despite its rapid advancements, application of this innovative technology to cardiovascular research is still limited, in part due to shortage of optogenetic cardiac tissue models and compatible imaging methods. The present study aimed to develop an optogenetic culture model using neonatal rat ventricular myocytes (NRVM) expressing light-gated Channelrhodopsin-2 (ChR2) and characterize activation spread during optical stimulation using optical mapping of membrane potential (Vm). Primary NVRM cultures were infected with lentivirus containing ChR2 gene. Cultures were paced electrically or optically with pulses of blue (470 nm) LED light. Activation spread was simultaneously mapped using Vm-sensitive dye (RH-237) and a photodiode mapping system. Results showed that ChR2 could be readily transduced to NRVMs by the lentiviral method; however, high-level ChR2 expression was associated with substantial cell toxicity. Lower ChR2 expression, achieved by administration of bromodeoxyuridine, had minor effects on cell morphology and function while allowing optical pacing at frequencies of 0.5-3 Hz. Simultaneous Vm mapping showed that conduction velocity, APD80, and dV/dtmax were similar in optogenetic and control cultures. Finally, the optogenetic cultures could be optically paced at multiple sites, leading to significantly reduced overall activation time. In summary, we demonstrated that ChR2 expression can cause cell toxicity in NRVM cultures but the toxicity can be mitigated allowing optical pacing and simultaneous optical activation mapping without significant impairment of electrophysiological function. This optogenetic cardiac culture model expands the availability of optogenetic tools for cardiac research.

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CD47 Regulates Aberrant Ca2+ Channel Current in Cardiac Myocytes

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Background: Left ventricular (LV) heart failure (HF) affects over 5 million Americans with 50% of HF patients dying within 5 years of diagnosis. Therapeutics, while only relieving symptoms and have multiple side effects, have not completely resolved this

process. HF is associated with cardiac myocyte cytosolic Ca2+ overload due to impaired sarcoplasmic reticulum (SR) and/or membrane Ca2+ handling. We recently found that a widely expressed cell surface receptor CD47 and its high affinity ligand, thrombospondin-1 (TSP1), are expressed on cardiac myocytes. We further showed that activated CD47 increases isolated neonatal cardiac myocyte cytosolic Ca2+ levels in part through SR Ca2+ channels. We postulated that TSP1-CD47 signaling may in part play a role in LV HF secondary to dysregulation of Ca2+, though the specific Ca2+ dynamics through which TSP1-CD47 signaling controls cardiac Ca2+ remains to be determined.
Methods: Isolated hearts from mutated mice lacking CD47 (CD47-/-) and wild type (WT) mice with and without 4 weeks of transverse aortic constriction (TAC) were studied in ex vivo perfused heart Langendorff using dual optical mapping of Cai transients and action potential or voltage measurements. Isolated cardiac myocyte experiments were performed using whole cell voltage-clamp analysis.
Results: In TAC-stressed WT murine hearts studied in the Landendorff system, tetracaine (an inhibitor of SR ryanodine receptor2) blunted arrhythmia induced by electrical stimulation. Isoproterenol-induced Ca2+ transient irregularities in hearts from WT mice were ameliorated in CD47-/- mice. Further, hearts from WT mice treated with peptide 7N3, a TSP1 mimetic, showed induced Ca2+ transient irregularities and elicited early after depolarization which was also attenuated with K2O1 (ryanodine receptor2 stabilizer) treatment. Voltage-clamp studies of isolated adult mouse cardiac myocytes showed that 7N3 peptide increased the L-type Ca2+ channel current (ICa,L) whereas the scrambled control peptide did not alter ICa,L.
Conclusion: These data demonstrate that CD47 activation may be a trigger for aberrant myocyte Ca2+ handling typically associated with HF. Targeting of TSP1-CD47 signaling may open a new therapeutic avenue to be further studied in HF.
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Discovery and Characterization of the Novel Muscle-Specific Membrane Protein Smco1
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Mutations in numerous membrane proteins cause debilitating myopathies. The discovery of novel muscle-specific, membrane proteins would likely provide insight into mechanisms of disease and potentially yield new therapeutic targets. Through bioinformatics screening for muscle-specific membrane proteins with unknown function, we identified C3orf43 or single-pass membrane protein with coiled-coil domains 1 (Smco1). Consistent with bioinformatics predictions, Smco1 is expressed exclusively in cardiac and skeletal muscle. We demonstrate with chromatin immunoprecipitation and luciferase promoter assays that Smco1 is a Mef2-regulated gene with robust expression occurring shortly after birth. Immunofluorescent analysis demonstrates Scmo1 localizes to the cardiomyocyte sarcolemma and intercalated disks. Talen-mediated disruption of Smco1 in mice results in stunted postnatal growth, cardiac hypoplasia and skeletal muscle myopathy as early as postnatal day 15. While studies are on going to determine the function of Smco1, our findings reveal an essential role of Smco1 in striated muscle structure and function. The identification of heart- and muscle-specific membrane proteins will likely illuminate the mechanisms of muscular membrane diseases.

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Different MicroRNA Expression Profiles in Murine Aorta and Carotid Arteries
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Atherosclerosis, a major cause of death, affects vascular walls diffusely. Some vascular beds are, however, preferentially involved. Small noncoding RNAs, microRNAs, have emerged as key regulators of gene expression in either physiological or pathophysiological processes. Here, we investigated whether different vascular beds, commonly affected by atherosclerosis, present specific microRNA expression profiles. To this end, aorta (Ao) and carotid (Ca) arteries from two male Wistar rats (200 g) were dissected, total RNA was extracted with Trizol, and the small RNA fraction was enriched using magnetic beads. Sequencing was performed using RNA-Seq on Ion Torrent PGM platform and data were analyzed using CLC Genomics Workbench software. We identified 372 and 305 microRNAs in Ca and Ao, respectively - the arteries shared 280 microRNAs. The majority of the 20 most expressed microRNAs were similar between the arteries. Of these top 20, 90% in the Ao were also among the most expressed in Ca, and in Ca 65% were also among the most expressed in Ao. Both mechano and platelet-microRNAs were identified in both arteries. Note that overexpression of mechano-miR-21 is reported to attenuate lipid accumulation and to reduce inflammation, preventing atherosclerosis. We detected mechano-miR-21, however, only in the Ao. We also found that murine aorta and carotid arteries express differently 29 microRNAs ($p<0.05$). Among the 29 microRNAs, 8 (28%) have been related to atherosclerosis. Of these, 6 were more expressed in Ca (miR-181, miR-9a-1, miR-9a-2, miR-511, miR-146, miR-27), and 2 were more expressed in Ao (miR-144 and miR-322). Note that 3 of the 6 more expressed microRNAs in Ca are related to atherosclerosis, that is: miR-181 regulates cell stress and proliferation; miR-511 regulates cholesterol synthesis, and miR-27 is a known marker of the disease. Taken together, our results suggest that specific microRNA expression profiles may play a role in the vascular behavior during the atherosclerotic process.
E. Curty-Costa: None. **L. Hoffmann:** None. **R. Silva:** None. **T.P. Ürményi:** None. **D.S. Faffe:** None.

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Differentiation of Human Multipotent Vascular Stem Cells Contributes to Vascular Diseases
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Vascular disease, such as neointimal hyperplasia and atherosclerosis, involves in migration and proliferation of vascular cells in blood vessel wall. It is generally accepted that the de-differentiation of vascular smooth muscle cells (SMCs) contributes to vascular diseases. However, previous studies suggest that resident multipotent vascular stem cells (MVSCs) are also involved in neointima formation. In this study, we

isolated MVSCs from the medial layer of human carotid artery and thoracic aorta by explant cultures, characterized these cells, and identified the origin of these vascular cells populated in lesion areas. Human MVSCs could be isolated from healthy and diseased blood vessels, were cloneable, and were positive for stem cell markers SOX10 and SOX17 but not SMC markers α-actin and calponin-1. MVSCs were able to differentiate into neural cells, SMCs and other mesenchymal lineages in vitro. In addition, we examined the location of MVSCs in diseased vascular wall by immunohistochemistry. The majority of cells within tunica media were calponin-positive cells, whereas a small population of cells was proliferative and double positive for SOX10 and Ki67 in the border between tunica intima and media. The number of SOX10+ cells in atherosclerotic lesions was more than that in healthy blood vessels, and some of these cells were double positive for SOX10 and chondrogenic markers. Furthermore, we performed in situ PCR and proximity ligation assays to stain for the epigenetic markers of SMC lineage. The results suggested that SOX10+ cells in cell cultures and tissue sections were not derived from de-differentiated SMCs. In conclusion, these findings support that MVSCs contributes to human vascular diseases.

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Investigating Early Cardiac Development in Patients With Hypoplastic Left Heart Using Human Induced Pluripotent Stem Cells

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Hypoplastic left heart (HLH) is one of the most severe forms of congenital heart disease, characterized by hypoplasia of the left ventricle, ascending aorta and aortic and mitral valves. Although our knowledge of the clinical aspects of HLH is improved, little is known about the underlying genetic causes, which lead to disease, particularly at the cellular level. In the current study, we have generated an in vitro model of HLH using induced pluripotent stem (iPS) cells to model early development with the aim to uncover genetic factors, which may cause disease. iPS cell lines were generated from three HLH patients as well as both parents, thus providing controls that are as genetically similar to the patients as possible. To study early cardiac development in vitro, we used a novel, fast, reproducible and efficient directed differentiation protocol. Briefly, by modulation of WNT, TGF-β, and SHH signalling pathways in an accurate time and dose-dependent manner, iPS cells were efficiently differentiated into beating cardiomyocytes, which appeared within 7 days. To investigate differences during early cardiac development, samples were collected at different time points after initiation of differentiation and gene expression patterns (qPCR) and cellular populations (flow cytometry) were examined. Results show that after 15 days, more than 80% of cells from both patients and parents were cTNT+, indicating the high success of the cardiomyocyte differentiation protocol in both groups. Flow cytometry analysis at an early developmental time point (day 10) showed higher percentages of NKX2.5+ and MLC2a+ cells in cultures derived from the control group compared to patients. At later stages of differentiation (day 20) however, a higher population of NKX2.5+ and MLC2a+ cells was found in the patient group. Gene expression analysis corroborated this result by revealing higher expression of cardiac progenitor markers such as HAND1 and GATA4 in early stages, followed by MYH6 in later stages in patients vs. controls. We also found that Ca flux properties of cardiac cells derived from patients and parents are different. Together our findings suggest that both timing and cardiogenesis in HLH iPS cells is perturbed resulting in an alteration of the gene expression network.

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Telomere and Mitochondrial Dysfunction in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive disease that is result of mutations in the dystrophin gene and is the most common myopathic disease in humans with a prevalence of one in every 3500 males. Dystrophin is crucial for the formation of a dystrophin-glycoprotein complex (DGC), which connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix in both skeletal and cardiac muscles. In the heart, loss of dystrophin leads to increased fibrosis and death in the third decade of life due to dilated cardiomyopathy. A conundrum in studying and developing therapies for DMD has been the lack of a mouse model that fully recapitulates the clinical phenotype, as mice that lack dystrophin (mdx model), unlike patients, exhibit only mild skeletal muscle defects, essentially no cardiac defects and have a relatively normal lifespan. Our lab reasoned that the difference in the manifestation of the disease in mice and humans could be telomere length, as mice have substantially longer telomeres than humans. We created a novel mouse model with shortened telomere lengths (similar to humans) that fully recapitulates the skeletal muscle (Cell. 2010;143:1059-1071; the mdx/mTRKO model) and cardiac muscle phenotype of DMD (Nat Cell Biol. 2013; 15:895-904; dilated cardiomyopathy). Interestingly, we observed a relative 45% reduction in cardiomyocyte telomere length in our mdx/mTRKO animals (3 animals per group, N = 300-400) as well as patient samples (4 DMD patient samples, N = 40-95). Here we present new evidence of mitochondrial dysfunction and telomere dysfunction.

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Systemic Illness in Ece1 Ablated Adult Mice

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Endothelin converting enzyme-1 (ECE1) catalyzes the conversion of inactive big endothelin 1 (ET1) to active ET1. Homozygous *Ece1* knock out (KO) mice die *in utero* or at birth, displaying multiple abnormalities including mandibular hypoplasia and cardiac outflow tract malformations, in spite of the presence of ample tissue ET1. However, increased ECE1 activity and circulating and/or tissue ET1 are associated with many adult cardiovascular diseases, including idiopathic pulmonary fibrosis (IPF), a chronic and fatal lung disease. There is an apparent paradox between the need for ET1 in development and its harmful effects in adult disease. Therefore, our lab developed a conditional *Ece1* KO mouse, in which *Ece1* is ablated following tamoxifen (tam) treatment. We hypothesized that ECE1 serves to localize ET1 signals to specific cell populations and is essential in normal adult physiology.

We studied the following groups: mice given vehicle rather than tam, mice lacking tam-inducible Cre recombinase, mice harboring a normal *Ece1* allele (*Ece1*$^{+/flox}$), and the experimental animals (Cre *Ece1*$^{-/flox}$). Mice were treated with vehicle or tam at 8-9 weeks of age. Cre *Ece1*$^{-/flox}$ mice showed 85-100% mRNA knock-down efficiency 8 weeks after tam treatment. By 17 weeks of age, Cre *Ece1*$^{-/flox}$ mice have tachypnea, decreased activity, and weight loss, requiring euthanasia for humane considerations. They display depleted adipose tissue mass compared to controls. By two weeks after treatment, Cre *Ece1*$^{-/flox}$ mice had lower blood pressure relative to controls, which persisted until euthanasia at 17-20 weeks old (p=0.004). Between 17-20 weeks of age, most of Cre *Ece1*$^{-/flox}$ mice develop pectus excavatum, enlarged right hearts and have reduced stroke volume and cardiac output as analyzed by echocardiography. Histological examination revealed eosinophilic crystalline pneumonia and increased collagen in the lung and heart. These findings are consistent with development of IPF in the experimental mice. Our findings show that *Ece1* ablation in post-natal animal results in a severe cardiorespiratory disease, suggesting that ectopic activation of ET1 by other tissue proteases is the primary mechanism underlying the association of increased ET1 signaling in disease states.

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Identification of Novel Alternate Splicing Events in Humans With RBFOX2 Mutations and Hypoplastic Left Heart Syndrome

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Alternative splicing (AS) of protein isoforms is an integral mechanism for cardiac development. RNA Binding Protein, Fox-1 Homolog (C. Elegans) 2 (RBFOX2) is an RNA binding protein preferentially expressed in muscle and neuronal cells and regulates tissue-specific alternate exon splicing in ~2,100 target genes by binding the conserved RNA sequence motif (U)GCAUG. RBFOX2 was recently implicated in the pathogenesis of abnormal cardiac and cerebral development via loss-of-function studies in zebrafish and mouse. However, convincing evidence remains incomplete, as the full complement of RBFOX2 target genes and differential exon usage (DEU) in human cardiovascular cell lines are incompletely defined. We identified de novo mutations in RBFOX2 from four human cases of congenital heart disease (CHD) with hypoplastic left heart syndrome (HLHS) via whole exome sequencing. To test the hypothesis that RBFOX2 mutations alter DEU in known target genes, we performed RNA-seq on ductus arteriosus tissue from human CHD cases with and without RBFOX2 mutation. Analysis of RNA-seq for DEU was performed with DEXSeq. To limit the effect of differential gene expression, we restricted analysis to subjects with high global gene expression correlation (r2 > 0.9, case=1 vs. control=5). DEU in known RBFOX2 target genes were highly enriched compared to all known genes (115/2,100 vs. 589/26,310, p=5.78e-15). A high percentage of the DEU genes (60.0%, 69/115) have high heart expression (HHE) in the developing mouse (mean expression of four cardiac chambers at e14.5). DEU genes with HHE include VCL, TPM1, FN1, ACTN1, and CALD1. Functional annotation clustering reveals enrichment for several actin, cytoskeletal, and contractile Gene Ontology terms, suggesting a possible role in the epithelial-mesenchymal transition (EMT) developmental processes active during early cardiac formation. We also identified enrichment from genes implicated in CHD by allelic specific expression or de novo mutations (44/1,263 vs. 589/26,310, p=0.007). These results are the first in humans to identify differentially expressed exons associated with RBFOX2 mutations in CHD and suggests RBFOX2-mediated alternate splicing may influence EMT pathways implicated in the pathogenesis of HLHS.

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Loss of the Cardio Protection Inferred by S-nitrosylation of GRK2 At Cysteine 340 Leads to Decreased Cardiac Performance and a Hypertensive Phenotype With Aging

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In the next 35 years, the global population of individuals above 60 years of age will double to approximately 2 billion. In the aged population, cardiovascular diseases are known to occur at a higher prevalence ultimately leading to increased mortality. G protein-coupled receptors (GPCRs) have been identified as vital regulators of cardiac function. GPCR kinases (GRKs) are important in cardiac GPCR regulation through desensitization of these receptors. GRK2 is highly expressed in the heart, and has been widely characterized due to its upregulation in heart failure. Studies from our lab have shown that elevated GRK2 levels in ischemia-reperfusion (I/R) injury result in a pro-death phenotype. Interestingly, cardio-protection can be inferred via S-nitrosylation of GRK2 at cysteine 340. Further, we have generated a knock-in GRK2 340S mouse, in which cysteine 340 was mutated to block dynamic GRK2 S-nitrosylation. GRK2 340S mice are more susceptible to I/R injury. Given that GRK2 340S mice are more susceptible to oxidative stress, and there is a nitroso-redox imbalance in senescence, it is possible that these mice are more likely to exhibit decreased cardiac performance as they age. Therefore, we hypothesize that with age GRK2 340S knockin mice will develop an overall worsened cardiac phenotype compared to control wild-type (WT) mice. To test this hypothesis, 340S and WT mice were aged for a year, and cardiac function was evaluated via echocardiography. Aged 340S mice exhibited significantly decreased ejection fraction and fraction shortening relative to aged WT controls. Prior to tissue harvesting, in-vivo hemodynamics was conducted via Millar catheterization. At baseline, aged 340S mice exhibited increased systolic blood pressure compared to aged WT mice. At the conclusion of this protocol, mice were sacrificed and heart weight (HW), body weight (BW), and tibia length (TL) measured to evaluate cardiac hypertrophy. Aged 340S mice exhibited significantly increased HW/BW and HW/TL ratios, indicative of cardiac hypertrophy, relative to aged WT controls. Taken together, these data suggest that with age, loss of the cardio protection inferred by S-nitrosylation of GRK2 at leads to decreased cardiac performance, and an overall worsened cardiac phenotype.

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Clonal Analysis of Cardiac Cell Generation During Cardiac Development and Injury

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Accumulating evidence supports limited regenerative potential of the mammalian heart. However, this endogenous regenerative capacity significantly decreases with age and it is not sufficient to replenish the lost myocardium following injury in the adult. Both resident cardiac stem/progenitor cells and mature cardiomyocytes have been proposed to contribute to cardiac tissue generation. Understanding the molecular and cellular mechanisms governing cardiac cell formation is imperative towards the development of novel therapeutic strategies for cardiac regeneration. Hypothesis: Cardiac growth occurs primarily through progenitor cells and to a lesser extent through cardiomyocyte proliferation. Nkx2.5+ cells are the predominant population driving cardiac growth during development and may represent candidate progenitors for cardiac regeneration. Moreover, a subset of "proliferating" αMHC+ cardiomyocytes may also contribute to cardiac growth during early embryonic development and the first week of life. Materials and methods: We performed clonal analysis using a multicolor reporter system (Rainbow) that allows labeling of single cells with one of three fluorescent proteins and retrospective analysis of their progeny. Rainbow mice were crossed to βactinCreER, αMHCCreER, Nkx2.5CreER and Rosa26CreER mice. Tamoxifen was administered at E9.5 or E12.5 and analysis was performed at P1, P7, P15 and P30. Results: We observed significant clonal expansion in βactinCreER;Rainbow and Nkx2.5CreER;Rainbow hearts while αMHCCreER;Rainbow hearts exhibited clones comprising of smaller cell number. We also found that αMHC positive cells lose their proliferative capacity from E9.5 to E12.5, whereas cells expressing Nkx2.5 continue to clonally expand during that same time period. Finally, we demonstrated that αMHC+ cardiomyocyte proliferation is reactivated following myocardial injury soon after birth. Conclusion: Our data support that clonal dominance of progenitor cells promotes cardiac development, while cardiomyocyte proliferation contributes to a lesser extent early in development and postnatally.

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Mir-21 Contributes to Cardiac Aging by Targeting Pten

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Background—Aging is among predominant risk factors for cardiovascular diseases and it also contributes to a significantly worse outcome in patients with acute myocardial infarction. MicroRNAs (miRNAs, miRs) have been indicated in aging. However, the regulatory mechanisms of miRNAs which govern the aging progress are poorly understood.
Objective—To identify miRNAs responsible for cardiac aging besides miR-34 family members.
Methods and Results—miRNA arrays were used to determine the dysregulated miRNAs in cardiac aging using cardiac ventricles from mice at 8 weeks and 15months of age. Besides miR-34 family members, miR-21 was also found to be elevated 3-fold in ventricles from mice aged 15 months compared to mice aged 8 weeks. Over-expression of miR-21 in the neonatal rat cardiomyocytes (NRCM) shortened the telomere length, attenuated and strengthened the activity of telomerase and senescence-related beta-galactosidase, respectively, which was similar to the aging caused by doxorubicin (DOX). Using the quantitative PCRs and Western blotting, phosphatase and tensin homologue (PTEN) gene was found to be negatively regulated by miR-21 in NRCM. Moreover, knockdown of PTEN using siRNA mimics the effects of miR-21 in pro-aging in NRCM. Interestingly, knockdown of miR-21, as well as up-regulation of PTEN, repressed NRCM aging induced by DOX.
Conclusions—These findings suggest that ectopic up- or down-modulation of miR-21 may be an important regulatory mechanism governing cardiac aging by targeting PTEN. Inhibition of miR-21 represent a novel therapy for cardiac aging.

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GATA4-Dependent Control of Myocyte Protein Quality Mediated by HSPB7

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The transcription factor GATA4 plays essential roles in heart development, including regulation of cardiomyocyte stress response. GATA4 depletion leads to defective cardiogenesis while GATA4 overexpression is protective in stress models including myocardial ischemia and cardiotoxin administration. However, the molecular basis for GATA4 function remains poorly understood. We discovered that expression of small heat shock protein beta 7 (HSPB7) is regulated by GATA4 and that HSPB7 depletion causes defects in cardiomorphogenesis. The goal of our study is to determine the cellular and molecular mechanism of action of HSPB7 in cardiomyogenesis. Through proteomic mass spectrometry and co-immunoprecipitation, we identified Filamin C (FLNC), a large sarcomeric protein, as an HSPB7 binding partner. FLNC mutations lead to pathological aggregate formation and progressive myopathy. Previous work suggests HSPB7 may prevent damaged protein aggregation through autophagic pathways, and our studies in zebrafish embryos suggest HSPB7 depletion alters the morphology and cytoskeletal architecture of ventricular cardiomyocytes, in particular the trabeculae. We hypothesize that HSPB7 facilitates myocyte function by processing damaged FLNC to prevent aggregation. Preliminary data suggest that BAG3 is also a part of this chaperone complex, linking HSPB7 to the process of Chaperone Assisted Selective Autophagy. Interestingly, disturbing autophagic pathways in the chick embryo precipitates dextrocardia, a phenotype observed in HPSB7 morphant embryos. We also generated a spectrum of defined HSPB7 mutant alleles using TALEN technology. Four such alleles have been bred to homozygosity including a suspected N-terminal truncation that deletes a putative regulatory polyserine stretch. Although homozygous mutants do not exhibit an overt cardiac phenotype, we hypothesize that loss of this polyserine domain may result in a hypomorphic HSPB7 protein. Exposure of mutant embryos to various stressors, including increased temperature (32°C) and the autophagy inducer rapamycin (1μM), led to an increase in phenotypes we have observed in HSPB7 morphants, including dextrocardia and the incidence of defects in cardiac morphology.

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Thymosin β4 Targets Wisp1 to Protect Cardiomyocytes in AngII- induced Cardiac Hypertrophy

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ABSTRACTS

Background: Thymosin beta-4 (Tβ4) is a ubiquitous protein with many properties relating to cell proliferation and differentiation that promotes wound healing and modulates inflammatory mediators. However, the role of Tβ4 in cardiomyocytes hypertrophy is currently unknown. The purpose of this study is to dissect the cardio-protective mechanism of Tβ4 in Ang II induced cardiac hypertrophy.
Methods: Rat neonatal cardiomyocytes with or without Tβ4 pretreatment were stimulated with Ang II and expression of cell sizes, hypertrophy marker genes and Wnt signaling components was evaluated by quantitative real-time PCR, western blotting and fluorescent microscopy. Selected target gene Wisp-1 was either overexpressed or silenced by siRNA transfections in neonatal cardiomyocytes and effect of Tβ4 in Ang II-induced cardiac hypertrophy was evaluated.
Results: Pre-treatment of Tβ4 resulted in reduction of cell sizes, hypertrophy marker genes and WNT-associated gene expression and levels induced by Ang II in cardiomyocytes. Tβ4 pretreatment also resulted in an increase in the expression of antiapoptotic proteins and reduction of Bax/BCl_2 ratio in the cardiomyocytes. Wisp-1 overexpression promotes cardiac hypertrophy and was reversed by pretreatment with Tβ4. Knocking down of Wisp1 partly rescue the cells from hypertrophic response after Tβ4 treatment.
Conclusion: This is the first report that demonstrates the effect of Tβ4 on cardiomyocytes hypertrophy and its capability to selectively target Wisp-1 in neonatal cardiomyocytes thus preventing cell death, thereby, protecting the myocardium. Wisp-1 promotes the cardiac hypertrophy which was prevented by Tβ4 treatment.
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PPAR-γ Targeted by MicroRNA-130a Regulates Angiotensin II-Induced Cardiac Fibrosis
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AIMS: Cardiac fibrosis which occurs due to disruption of extracellular matrix network resulted in the accumulation of excess collagens and other matrix components leading to myocardial dysfunction. Angiotensin II (Ang II), a critical effector of this system has been implicated in the development of hypertension-induced cardiac fibrosis. In recent years, miRNAs have identified as an attractive targets for therapeutic intervention in various disease pathologies including cardiac fibrosis. However, the exact effect and underlying mechanism of miRNAs in cardiac fibrosis remains unclear. Here, we sought to investigate and test our hypothesis that miR-130a plays a critical role in the development of myocardial fibrosis by restoring PPARγ level.
METHODS AND RESULTS: We have identified a panel of novel miRNAs *via* miRNA array in Ang II infused mice heart. Among them, we found that miR-130a was upregulated both in pressure overload and Ang II infused models targeting PPARγ. Overexpressing miR-130a in cardiac fibroblast promoted the pro-fibrotic gene expression (collagen I/III, fibronectin and CTGF) and myofibroblasts differentiation. Inhibition miR-130a reversed the process and weakened these activities. Using luciferase-linked constitutive and dominant negative constructs of PPARγ, we determined the underlying mechanism of cardiac fibrosis occurred *via* targeting PPARγ. The *in vivo* inhibition of miR-130a by subcutaneous injections of LNA-based anti-miR-130a in mice subjected to Ang II infusion significantly reduced the severity of cardiac fibrosis, hypertrophy. The protective mechanism is associated with restoration of PPARγ level, reduction of pro-fibrotic genes and apoptosis; reversion of myofibroblasts differentiation and improved cardiac function.
CONCLUSIONS: Our findings provide evidence that miR-130a plays a critical role in the progression of cardiac fibrosis by directly targeting PPARγ, and that inhibition of miR-130a reversed the cardiac fibrosis. We conclude that miR-130a may be a new marker for cardiac fibrosis and inhibition of miR-130a would be a promising strategy in the treatment of cardiac fibrosis.
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PP1, a Derivative of Salubrinal, Ameliorate Ventricular Function after Myocardial Infarction in Rats
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Objective: salubrinal, an inhibitor of ER-stress, has potent effect on cell injury. In this study, we investigated the effect of salubrinal and its derivative-PP1 on chronic heart failure induced by myocardial infarction (MI).
Methods: Male wistar rats were randomly divided into 5 groups:i) sham-operated; ii) vehicle (MI+ DMSO); iii) MI + salubrinal; iv) MI + PP1; v) MI + kaptopril. After 6 weeks treatment, transthoracic echocardiographic and hemodynamic parameters were evaluated. The cardiac tissue sections were subjected to TUNEL to assess the level of apoptosis. The expression of mRNA and protein involved in apoptosis, autophagy, ER stress was analyzed using real-time reverse transcription-polymerase chain reaction and western blotting, respectively.
Results: The VW/BW and LVW/BW were reduced by salubrial and PP1.The LVEDD, LVESD were significantly decreased while the EF and FS were greatest increased by salubrinal and PP1. The hemodynamic parameters including CO, SV, LVEDV, LVESV, LVEDP, LVESP, +dp/dtmax, -dp/dtmax, +dv/dtmax, -dv/dtmax, Ea, Tau were ameliorated. By calculating the apoptotic index, we found that salubrinal and PP1 have a great anti-apoptotic effect. The mechanism analysis demonsted that salubrinal and PP1 have a cell protection effect through downregualtion of PERK, GRP78, chop, caspase12 and upregulation of p62 , LC3II, ATF4 as detected by real-time PCR and western blotting.
Conclusions: The current study suggested that salubrinal and PP1 have a cell protection effect involed in ER stress related autophay and apoptosis.This may implicate that salubrinal and PP1 may be a potent compound for the treatment of chronic heart failure.
C. Liu: None. **Y. He:** None. **K. He:** None.

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Effect of Salubrinal and PP1 on Cardiomyocytes Protection after Myocardial Infarction in Rats
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Objective: salubrinal, an inhibitor of ER-stress, has potent effect on cell injury. In this study, we investigated the effect of salubrinal and its dervative-PP1 on cell protection aganinst myocardial infarction (MI).
Methods: Male wistar rats were randomly divided into 5 groups:i) sham-operated; ii) vehicle (MI+ DMSO); iii) MI + salubrinal; iv) MI + PP1; v) MI + metoprolol. After 24 hours treatment, heart tissue was havested and stained by 2,3,5-triphenyltetrazolium chloride (TTC). Serum was havested for the detection of CK-MB, cTNT, CRP. The changes in histomophology were observed

using hematoxylin and eosin (HE) staining. The expression of mRNA and protein involved in apoptosis, autophagy, ER stress was analyzed using real-time reverse transcription-polymerase chain reaction and western blotting, respectively.
Results: Salubrinal and PP1 decreased the infarction area and the level of CK-MB, cTNT, CRP. The mechanism analysis demonsted that salubrinal and PP1 have a cell protection through a ER-stress related pathway, showing upregualtion of PERK, eIF2α, ATF4, GRP78, GADD34, p62 , LC3IIand down regualtion of chop, caspase12 as detected by real-time PCR and western blotting.
Conclusions: The current study suggested that salubrinal and PP1 have a cell protection effect involed in ER stress related autophay and apoptosis.This may implicated that salubrinal and PP1 may be a potent compound for the treatment of MI.
C. Liu: None. **Y. He:** None. **K. He:** None.

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Lack of EDA Attenuates Adverse Cardiac Remodeling Due to Pressure Overload
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Background Heart failure due to pressure overload is also characterized by excessive fibrosis. Fibrosis is defined by excessive deposition of extracellular matrix. After injury the splice variant of fibronectin (FN), EDA-FN, is transiently upregulated and plays a role in a range of fibrotic diseases. We have previously shown that after myocardial infarction EDA ameliorates adverse cardiac remodeling. Nonetheless, the role of EDA-FN in the pressure-overloaded heart has not yet been elucidated. In this study we investigated the function of EDA in adverse cardiac remodeling due to pressure overload.
Methods EDA knock-out (KO) mice and wild type (WT) underwent trans aortic constriction (TAC) with a 27 gauge needle. Cardiac function and geometry was assessed using echo at day 7 and 42. At day 42 immunohistochemistry was performed to assess fibrosis (αSMA, picrosirius red) and cardiomyocyte hypertrophy (WGA). Zymography was executed to determine MMP activity. To study fibroblast function in the absence of EDA in vitro collagen contraction assays were carried out.
Results mRNA expression of EDA is already increased after 7 days of TAC and remains upregulated at least until 42 days after TAC. Heart/body weight ratio at 42 days is lower in EDA KO mice compared to WT mice (7±0.2 mg/g versus 11±0.6 mg/g). EDA KO mice show improved cardiac function after 42 days of TAC, shown by a decrease in EDV (80±16 µl versus 122±21 µl) and ESV (49±14 µl versus 104±19 µl)
At 42 days of TAC there are less myofibroblasts present in the EDA KO hearts, however this does not lead to less fibrosis. WGA staining does not show any difference in cell size. Zymography shows that MMP activity is reduced in the EDA KO mice. In vitro, there is no intrinsic difference in myofibroblast transdifferentiation between the WT and EDA KO.
Conclusion/discussion There are less myofibroblasts present. Furthermore, there is a decrease in MMP activity, however there is no difference in fibrosis. It might be that there is a reduced matrix turnover which prevents dilation of the heart. We will study this process in more detail in the future to elucidate the mechanism of action of EDA in the failing heart.
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First Induced Heart Failure Model in Adult Zebrafish by Chronic Isoproterenol Treatment
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Impaired β-adrenergic signal transduction is a common molecular alteration found in heart failure (HF). Chronic catecholamine release and overstimulation of β-adrenergic receptors (β-ARs) in the failing heart results in their desensitization, largely mediated through upregulation of the G-protein coupled receptor kinase 2 (GRK2), and consequently to the progression into HF. Chronic activation of β-ARs by isoproterenol (iso) infusion efficiently induces HF in mice, while therapeutic targeting of GRK2 in HF animal models preserves cardiac function, highlighting their significance in HF progression.
Zebrafish represents an established model to evaluate genetic causes of HF and to screen for novel therapeutic targets. However, the contribution of the β-A system in zebrafish models of HF is not known. We here systematically analyzed the effect of iso on heart function in larval and adult zebrafish.
Larvae first responded to iso with 3 days of age (d). Here, β-AR stimulation resulted in the activation of conserved signaling components and in the induction of common stress responsive genes. Chronic β-AR stimulation for 5 days induced signs of HF accompanied by similar expression changes seen in mammals. As heart phenotypes are usually not analyzed in larval zebrafish beyond 3d, our data implicates that previous studies neglected a possible impact of β-AR signaling, particularly important when screening for therapeutic components.
Adult zebrafish recently emerged as an attractive cardiac model, especially for regenerative medicine. Echocardiography revealed that in adult zebrafish hearts, iso robustly enhanced cardiac function. Chronic β-AR stimulation for 14 days efficiently induced HF symptoms. Consistent with mammals, we found reduced expression of β-ARs and elevated expression of GRK2 and ANP. Additionally, these fish develop essential characteristics accompanied with HF, including increased cell death and elevated inflammation.
In conclusion, we show that β-AR function in zebrafish is comparable to that in mammals. Further, we present the first iso-induced HF model in adult zebrafish, thereby introducing adult zebrafish as a particularly valuable model to study the pathogenesis of HF and to test for novel therapeutic strategies to treat HF.
M. Kossack: None. **H.A. Katus:** None. **P. Most:** None. **D. Hassel:** None.

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Dicarbonyl Stress and Diabetic Heart Failure: The Role of Endothelial Cells
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Dicarbonyl stress (DS) caused by the accumulation of α-oxoaldehyde metabolites, like methylglyoxal (MG), leads to detrimental DNA and protein modifications. Under normal conditions, MG is detoxified by glyoxalase-1 (GLO1) and -2 enzymes, but this system fails in diabetes. While the role of DS in diabetic cardiomyopathy through changes in cardiomyocyte function has been well described, this study aimed to link DS with the development of endothelial dysfunction (ED) and early heart failure in diabetes.
Transgenic mice that over-express GLO1 in endothelial cells (ECs) but not in cardiomyocytes, and their wild-

type (WT) littermates were treated with STZ to induce hyperglycemia (WT-diabetic and GLO1-diabetic mice) or vehicle (non-diabetic controls). Hyperglycemia increased the circulating levels of ED markers in WT-diabetic (E-selectin 1.5-fold, ICAM 1.4-fold, and VCAM 1.1-fold), but not GLO1-diabetic mice. The number of vWF+ ECs in WT-diabetic hearts was reduced 2-fold compared to other groups, whereas GLO1 over-expression preserved capillary density. Cell death, determined by TUNEL staining, was greater in the hearts of WT-diabetic mice compared to all other groups. GLO1 over-expression resulted in reduced inflammation: TNF-α protein expression was increased in both diabetic groups (≥2-fold), but significantly less so in GLO1-diabetic mice (p=0.03). The preservation of ECs in GLO1-diabetic mice was associated with delayed signs of heart failure. At 4wk of hyperglycemia, WT-diabetic mice had reduced heart function compared to all other groups (p=0.04). At 8wk, cardiac function in GLO-diabetic mice was greater than in WT-diabetic mice, but both were reduced compared to non-diabetic controls (p=0.02; p=0.4). A possible mechanism for EC survival in GLO1 mice despite the presence of inflammation was examined in vitro using human aortic ECs. ECs exposed to high glucose or MG for 24h had increased apoptosis induced by TNF-α compared to cells treated only with TNF-α (by 2- and 3-fold, respectively), suggesting that reduced MG protects ECs from TNF-α mediated death.
Taken together, these results suggest that DS in diabetes increases inflammation and ED, leading to the loss of ECs in the heart, which contributes to the development of heart failure.
B. Vulesevic: None. **B. McNeill:** None. **F. Giacco:** None. **M. Brownlee:** None. **R. Milne:** None. **E. Suuronen:** None.

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Role of Follistatin 315 in Regulating Cardiac Hypertrophy in Physiology and Pathophysiology
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Heart failure is characterized by initial compensatory changes, including the myocyte hypertrophy, chamber dilation, and matrix remodeling, that proceed until progressive dysfunction produces end stage heart failure and mortality. Recently, the roles of secreted factors in the heart that could regulate pathological hypertrophy, including follistatin (FST) and related molecules, have been examined by various investigators. FST is a molecule that blocks secretion of follicle-stimulating hormone from the pituitary and regulates members of the transforming growth factor beta (TGF-β) family including myostatin. Here we tested the effects of a particular FST isoform, FST288, on heart function in mice. The gene encoding FST produces three isoforms that differ in biological activities and cell surface binding capabilities. The FST315 isoform contains all six exons, and proteolytic cleavage of the FST315 C-terminal tail results in production of FST303. The lack of exon 6, which codes for the acidic C-terminal tail of the putative full-length protein, results in FST288. The missing acidic C-terminal tail region found in soluble FST315 allows FST288 to bind cell surface heparin-sulfated proteoglycans, accounting for the differential actions of these FST isoforms. Since mice that are null for the FST gene die embryonically, we used genetically modified mice that express only the FST288 isoform to test the role of FST315 in adult heart. Examination of these animals suggests that the loss of FST315 expression has limited effects on the heart at the resting state. When these mice are subjected to pressure overload through transverse aortic constriction (TAC) surgery they appear to be resistant to the compensatory cardiac hypertrophy present in wild type mice by 4 weeks post surgery. Both cardiac structure (examined by histology) and function (as measured by echocardiography and pressure/volume loops) following TAC are improved in the genetically modified mice when compared to wild type mice. This response is likely due to modification of the myostatin signaling pathway, one of the major targets of FST315. Overall, our data illustrates that FST315 is an important contributor to the progression of pressure overload induced cardiac hypertrophy.
Z. Xu: None. **A.D. Blazek:** None. **E. Beck:** None. **J. Alloush:** None. **J. Li:** None. **A. Schneyer:** None. **S. Agarwal:** None. **T. Hewett:** None. **N. Weisleder:** None.

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Cardiac-specific Deletion of HuR Reduces Pathological Hypertrophy and Ventricular Remodeling Following Transverse Aortic Constriction
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The RNA binding protein HuR (Human antigen R) interacts with specific AU-rich domains in target mRNAs and is highly expressed in many cell types, including cardiomyocytes. However, the role of HuR in cardiac physiology is largely unknown. Our results show that HuR undergoes cytoplasmic translocation, indicative of its activation, in hypertrophic mouse cardiac myocytes at 8 weeks post-transverse aortic constriction (TAC). To determine the role of HuR in the development of cardiac hypertrophy, we have created a novel mouse model of cardiac myocyte-specific deletion of HuR. While cardiac-specific HuR deletion mice do not show an overt basal phenotype, they have preserved ejection fraction and reduced ventricular remodeling (hypertrophy and chamber dilation) compared to wild-type littermates at 8 weeks following TAC. Furthermore, HuR activation in the hypertrophic heart is strongly co-localized with regions of fibrosis. To this end, we show that HuR knockdown reduced hypertrophy and pro-fibrotic TGF-β gene expression in a phenylephrine treated neonatal rat ventricular myocyte (NRVM) model of hypertrophy. Thus, our results suggest that HuR activation in cardiac myocytes promotes pathogical hypertrophy and initiation of cardiac fibrosis via myocyte expression of TGF-β. These findings are significant as we have identified HuR as a novel mediator of pathological hypertrophy and suggest a pro-fibrotic role as a potential mechanism for this effect.
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miRNA-21 Targets Left Ventricular Peroxisome Proliferator-activated Receptor Alpha in a Rat Model of Type 4 Cardiorenal Syndrome
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Type 4 cardiorenal syndrome (CRS4) is a condition in which chronic kidney disease (CKD) contributes to cardiovascular pathology including cardiac dysfunction, left ventricular (LV) hypertrophy, atherosclerosis, and heart failure. We have used a rat model of CKD, the 5/6 nephrectomy (5/6 NX), to study molecular mechanisms that mediate the development of cardiac pathology in CRS4. We previously reported that the upregulation of microRNA miR-21-5p (miR-21) in the left ventricle (LV) was accompanied by pathological remodeling and a drop in fractional shortening in adult male Sprague Dawley rats 7 weeks after 5/6 NX. Systemic knockdown of miR-21 in 5/6 NX rats with

LNA-modified anti-miR-21 improved cardiac function, however it did not reduce the modest fibrosis observed with our 5/6 NX model or upregulate miR-21 targets identified in other models of cardiac pathology, suggesting a novel cardiac target for miR-21 in this model. Through next-generation mRNA sequencing of LV tissues from anti-miR-21 treated rats, and subsequent Ingenuity Pathway Analysis, we have found cardioprotective alterations in genes related to cardiac hypertrophy, metabolism, immune and inflammatory signaling, and atherosclerosis. Suppression or reduction of miR-21 target peroxisome proliferator-activated receptor alpha (PPARa), a master regulator of fatty acid oxidation, has been reported to be involved in all of these processes. Translational suppression of PPARa through miR-21 has been confirmed in other tissues, but not in the myocardium. The average LV PPARa protein expression level was significantly reduced (-37.7 ± 5.4%) in the 5/6 NX model and restored by miR-21 knockdown (Western blot; n=5-6/group; mean ± SEM). Immunohistochemistry revealed that the pronounced alterations in PPARa expression occurred within cardiomyocytes (CMs) in these samples. Transfection of neonatal CMs with pre-miR-21, significantly reduced PPARa protein expression within 48 hours (-23.0 ± 0.9%; n=3/group). These data indicate that PPARa suppression by miR-21 occurs within LV in 5/6 NX model of CKD and that miR-21 can regulate PPARa in CMs. This regulation may be relevant in other models of chronic cardiac disease where increased miR-21 and suppression of PPARa have been independently reported.

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Cardiac Troponin T Isoform Switching in Early Childhood Tropomyosin-linked Dilated Cardiomyopathy

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An oft-noted component of sarcomeric DCM is the observation that patients within families carrying the same primary mutation exhibit significant phenotypic variability. This lack of a distinct link between genotype and phenotype has complicated clinical management. In a recent study of two unrelated multigenerational families with the tropomyosin (Tm) mutation Asp230Asn (D230N), a striking "bimodal" distribution of severity was observed. In these families, many children (<1 year) with the mutation presented with a severe form of DCM that led to sudden, often fatal CHF while adults developed a mild to moderate DCM in mid-life. Of note, children who survived the initial presentation often recovered significant systolic function into young adulthood. A potential hypothesis to explain this improvement despite the continued presence of the mutant Tm, is that the phenotype is modified by other thin filament isoforms. Thus we propose that the age-dependent remodeling seen in children with D230N Tm is a result of temporal isoform switches involving a closely linked Tm binding partner cardiac Troponin T (cTnT). Our initial biophysical studies (Regulated-IVM) revealed a decreased Ca2+ sensitivity in filaments containing D230N Tm that is more severe in the presence of fetal TnT (cTnT1), suggesting a modulatory role for cTnT1. Cardiac performance, assessed via 2D echo, in our novel D230N Tm x cTnT1 double transgenic (DTg) mouse model found a significantly reduced % FS for DTg (17%) mice as compared to D230N Tm (21%) littermates. This reduction in %FS was seen at 4 months but not 2 suggesting a progressive cardiomyopathy. Current efforts aim to model the early phase of this "bimodal" phenotype and assess the potential for disease reversibility using a cardiac specific inducible cTnT1 transgenic mouse model. Furthermore, we propose that modulation by cTnT1 could represent a more general mechanism for the progressive remodeling seen in human heart failure. Preliminary in vitro studies with human tissue found that RNA levels of cTnT1 are significantly higher in failing hearts as compared to non-failing. Thus these data suggest an isoform dependent mechanism for the "bimodal" phenotype in patients carrying D230N Tm that could translate to other sarcomeric cardiomyopathies.

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Absence of Fibulin-2 Attenuates Cardiac Hypertrophy and Fibrosis in Transgenic Mice Over-expressing Circulating Transforming Growth Factor-beta

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Background: TGF-β is a potent growth factor that induces myocardial hypertrophy and fibrosis. However, the interaction between circulating TGF-β and local myocardial TGF-β in inducing cardiac hypertrophy is not well understood. Fibulin-2 is an ECM protein that mediates TGF-β activation during cardiac hypertrophy in response to chronic angiotensin (Ang) II infusion. We tested the hypothesis that fibulin-2 mediates systemic TGF-β-induced cardiac hypertrophy.
Materials & Methods and Results: We created double mutant mice by crossing TGF-β over-expressing transgenic mice (TG) and fibulin-2 knockout mice (KO). TG are known to produce excessive mature porcine TGF-β from the liver. TG/WT developed significant myocardial hypertrophy at 8 weeks compared with non-TG (NTG) groups. Hypertrophy in KO/TG was significantly attenuated compared with TG/WT. Myocardial TGF-β mRNA up-regulation was significantly higher in TG/WT than in TG/KO or NGT groups, so was Smad2 activation, but myocardial TGF-β bioactivity measured by PAI-1 promoter/luciferase bioassay was comparable among all four groups. Serum carrier-bound TGF-β (total TGF-β) in WT/TG was significantly higher than that in KO/TG and NTG groups, but free TGF-β level was equally elevated in TG groups compared with NTG groups. The difference in hypertrophy between WT/TG and KO/TG may be attributed to increased myocardial TGF-β mRNA and elevated serum total TGF-β in WT/TG, not directly to bioactive myocardial TGF-β or serum free TGF-β. Endogenous TGF-β mRNA levels in kidney and liver were equally increased in TG groups compared with NTG groups, and were same in all 4 groups in lung, suggesting fibulin-2 was not involved in TGF-β-induced TGF-β synthesis in these organs.
Conclusion: Systemic TGF-β-induced-endogenous TGF-β up-regulation was mediated by fibulin-2 in the myocardium. After secretion, endogenous myocardial TGF-β may be either sequestrated into insoluble ECM or directly released into circulation as a soluble carrier-bound form. It is circulating endogenous TGF-β secreted from the heart that is primarily responsible for myocardial hypertrophy. Heart is a major source of circulating endogenous TGF-β in the TG/WT, which assumes an endocrine role in inducing myocardial hypertrophy.

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Conditional Knockout of Activin Like Kinase-1 (ALK-1) Leads to Heart Failure Without Maladaptive Remodeling

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Activin like kinase 1 (ALK1) mediates signaling via the TGFb family of ligands. ALK1 activity promotes endothelial proliferation and migration. Reduced ALK1 activity is associated with arteriovenous malformations. No studies have examined the effect of global ALK1 deletion on indices of cardiac remodeling. We hypothesized that reduced levels of ALK1 promote maladaptive cardiac remodeling. Methods: We employed an ALK1 conditional knockout mice (cKO) harboring the ROSA26-CreER knock-in allele whereby a single dose of intraperitoneal tamoxifen triggered ubiquitous Cre recombinase mediated excision of floxed ALK1 alleles. Tamoxifen treated wild-type (WT-Tam; n=5) and vehicle treated ALK1-cKO mice (cKO-Veh; n=5) served as controls for tamoxifen treated ALK1-cKO mice (cKO-Tam; n=15). Results: ALK1 cKO-Tam mice demonstrated reduced 14-day survival compared to cKO-Veh controls (33% vs 100%, respectively, $p<0.01$). Seven days after treatment, ALK1 cKO mice began to exhibit reduced left ventricular (LV) fractional shortening, progressive LV dilation, and gastrointestinal bleeding. After 14 days total body mass was reduced, but LV and lung mass increased in cKO-Tam not cKO-Veh mice. Peak LV systolic pressure, contractility, and arterial elastance were reduced, but LV end-diastolic pressure and stroke volume increased in cKO-Tam, not cKO-Veh mice. LV ALK1 mRNA and protein levels were reduced in cKO-Tam, not cKO-Veh mice. LV levels of other TGFb-family ligands and receptors (ALK5, TBRII, BMPRII, Endoglin, BMP7, BMP9, and TGFB1) were unchanged between groups. Cardiomyocyte area and LV levels of BNP were increased in cKO-Tam mice, but LV levels of b-MHC, SerCA, and calcineurin were unchanged. No increase in cardiac fibrosis Type I collagen, CTGF, or PAI-1 levels were observed between groups. No differences were observed for any variable studied between cKO-Veh and WT-Tam mice. Conclusion: Global deletion of ALK1 is associated with the development of high output heart failure without maladaptive remodeling. Future studies exploring the functional role of ALK1 in cardiac remodeling are required.

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Desmin Preamyloid Oligomers in Experimental Heart Failure and Cultured Cardiac Myocytes

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Background: Heart Failure (HF) is one of the main causes of morbidity and mortality in westernized countries but the molecular mechanisms underlying its development are still unclear. A paradigm-shifting view focuses on the accumulation of preamyloid oligomers (PAOs), similar to those observed in Alzheimer disease, as a potential mechanism of cardiac toxicity. We reported that differential desmin phosphorylation at serines (S) 27 and 31 could drive the formation of PAOs in the heart, in the absence of genetic mutations. We sought to establish the identity of the molecular seed triggering the nucleation of cardiac PAOs in an experimental model of HF and in cultured cardiac cells. Methods: Mice were subjected to transverse aortic constriction (TAC) for 4 weeks (FS% = 29.3±2.6, $P=0.0001$). Alternatively, neonatal rat ventricular myocytes were transduced with lentiviral vectors carrying alanine (A) or phospho-mimetic aspartate (D) desmin double mutants at S27 and S31, fused with GFP. Protein homogenates were subjected to western blot analysis with fluorescent co-staining using the A11 anti-PAOs and anti-desmin antibodies. Transduced cells were also subjected to live imaging to assess phenotype. Results: Co-western blot analysis of both TAC mice and phospho-mimetic mutant cells revealed the colocalization of PAOs with desmin modified (potentially cleaved) forms. Preamyloid oligomers and a desmin fragment were both increased in TAC mice vs. controls (2.8-fold, $P=0.023$ and 1.8-fold, $P=0.038$, respectively). The DD mutant, mimicking the doubly phosphorylated desmin that we hypothesized is the physiological form, showed a healthier phenotype in terms of number of spontaneously contracting cells ($P=0.041$) and incorporation of GFP-desmin at the Z-discs ($P=0.0027$), whereas the mono-phosphomimetic mutant (AD) resulted in the increase of desmin positive aggregates ($P=0.0014$). Conclusions: This preliminary evidence suggests that desmin modified forms represent the seed triggering the formation of cardiac PAOs, in the absence of genetic mutations. The accumulation of desmin PAOs could therefore represent an overarching mechanism underlying the deterioration of cardiac function in HF.

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Discovery of a Novel Pharmaceutical Class as Potential Heart Failure Treatment

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Utilizing an unbiased *in vivo* phenotypic chemical screening platform in zebrafish embryos, our laboratory has identified a number of novel compounds with high selectivity for a wide range of cellular targets, including kinases (CK2a, DRAK2, DYRK2 and bone morphogenetic protein receptors), GPCRs (lysophosphatidic acid receptor 1, and extracellular proton sensor), p300 histone acetyltransferase, and phosphodiesterase-4 (PDE4). While the compounds we discovered have therapeutic implications for a wide range of diseases, our translational work has focused on addressing the cardiovascular diseases. Heart failure (HF) is a leading cause of disability and mortality in US, affecting about 6 million Americans, and the incidence of heart failure is anticipated to increase substantially in the coming decades. Yet, current HF pharmaceuticals are palliative, and the outlook for HF drug pipeline is uncertain. Within this backdrop, we recently discovered eggmanone, an extraordinarily selective PDE4 inhibitor which has no known off-target. An usual feature of eggmanone is that it increases cAMP levels specifically in distinct cellular microdomains, without raising the total cellular cAMP content. In isolated mouse cardiomyocytes and human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), eggmanone increases cardiac contractility by targeting a discrete myocyte microdomain without causing significant changes in myocyte calcium cycling. Importantly, eggmanone enhances systolic function in mice with failing hearts without increasing the heart rate. These results raise the exciting possibility that a microdomain-specific PDE4 inhibitor like eggmanone may be useful as an inotropic therapy for HF which avoids the pitfalls of traditional PDE inhibitors, whose

utility has been limited by proarrhythmia, tachyphylaxis and cardiotoxicity.
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miR-19 Deficiency Impairs Cardiac Repolarization in Zebrafish
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The most common outcome of heart failure (HF) is sudden cardiac death which results mostly from prolonged action potential duration (APD) and arrhythmias. During the pathogenesis and progression of HF, a vast number of signaling pathways are altered. microRNAs are small noncoding RNAs that post-transcriptionally finetune gene expression. Interestingly, several microRNAs are dysregulated during HF, suggesting a potential involvement in the development and progression of the disease. Here, we identified miR-19 as an important regulator of heart function. Zebrafish lacking miR-19 developed severe bradycardia and reduced cardiac contractility. While the mammalian genome encodes for two isoforms of miR-19, zebrafish express four members (19a-d). We found that the reduction of miR-19b specifically is sufficient to cause bradycardia and reduced cardiac contractility. Imaging of ventricular APs from whole hearts revealed that APD is significantly prolonged and repolarization is impaired in miR-19b deficient zebrafish. By qRT-PCR experiments we showed that the expression of several cardiac ion channels is altered. Moreover, miR-19b deficiency results in increased sensitivity to an AV-Block, which is a characteristic feature of long QT-Syndrome in zebrafish. In conclusion, we identified miR-19b as a novel and essential modulator of the electrical activity of the heart and establish miR-19b as a potential candidate gene causative for human long QT syndrome.
A. Benz: None. **H.A. Katus:** None. **D. Hassel:** None.

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Endostatin Promotes Proliferation and Migration of Rat Myofibroblasts Isolated From the Infarcted Area After Myocardial Infarction
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Background: Endostatin, a 20 kDa non-collagenous fragment of type XVIII collagen, is known as an endogenous anti-angiogenic factor. In the heart tissues of experimental heart failure models, such as cardiac hypertrophy and myocardial infarction, the expression levels of endostatin increase. Proliferation, migration and collagen synthesis of myofibroblasts are important processes during the tissue remodeling in injury sites after myocardial infarction. However, the effects of endostatin on cardiac myofibroblasts have not been clarified. We investigated the effect of endostatin on the functions of myofibroblasts isolated from infarcted myocardial tissues of rat.
Methods and Results: Left ventricular myocardial infarction was induced by ligation the left anterior descending coronary artery of male Wistar rats. The infarcted myocardial tissues were harvested 2 weeks after the operation and placed on the culture plate with serum-containing medium. Migrated cells from the tissues were isolated and used as myofibroblasts. High expression of α-smooth muscle actin, vimentin and type I collagen in these cells were confirmed by immunofluorescence staining. Cell counting assay was performed to determine a cell proliferation. Endostatin (100-3000 ng/ml, 48 h) increased the proliferation of myofibroblasts. Boyden chamber assay was performed to measure a cell migration. Endostatin (300-3000 ng/ml, 24 h) stimulated the migration of myofibroblasts. Western blotting was performed to measure a secretion of type I collagen. Endostatin (100-3000 ng/ml, 24 h) had no influence on it.
Conclusions: These data suggest that endostatin might promote scar formation after myocardial infarction through the activation of proliferation and migration of myofibroblasts.
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Reduction of Diastolic Dysfunction by Treatment of a Combination of Agonists of Adenosine Receptor in Spontaneously Hypertensive Rats Submitted to Myocardial Infarction
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Introduction. This work investigated the cardioprotective actions of the combination of a positive inotropic agent (LASSBio-294) and a potent vasodilator (LASSBio-897) in spontaneously hypertensive rats (SHR) submitted to myocardial infarction (MI).
Methods. Twenty four SHR (180-200 g) were randomly divided in sham-operated (SO) and infarcted groups (MI) and each group subdivided in two: treatment with vehicle (DMSO) or with LASSBio-294 + LASSBio-897 (5mg/kg each, p.o.) during 8 weeks. After treatment period, the animals were submitted to echocardiography to determine the anterior wall thickness (AWT), ejection fraction (EF), fractional shortening (FS) and the ratio of early and late transmitral filling velocity (E/A). In addition, the following hemodynamic parameters were evaluated: mean blood pressure (MBP), left ventricular end diastolic pressure (LVEDP), left ventricular end-systolic pressure (LVESP) and LV contractility and relaxation (dp/dt_{max}). Hypertrophy was measured using the relation between heart weight to body weight (HW/BW). The volume fraction of collagen (%) was determined by measuring the area of H&E stained tissue within a given field.
Results. MI induced in SHR promoted a decrease in AWT; EF; FS and E/A from 2.0 ± 0.4 to 1.6 ± 0.9 mm; from 53.1 ± 7.5 to 25.3 ± 6.4 %; from 40.0 ± 0.9 to 25.3 ± 11.0 %; and from 1.4 ± 0.1 to 0.9 ± 0.1, respectively. Treatment with the combination of drugs, increased AWT to 2.5 ± 0.6 mm; EF to 73.2 ± 1.0 %; FS to 43.5 ± 6.6%; and E/A to 1.3 ± 0.1. Increase of LVEDP from 4.6 ± 0.3 to 30.0 ± 3.6 mmHg and duplicated oxygen consumption were observed in MI-SHR. The negative dP/dt was reduced from 6152 ± 1015 to 3957 ± 1225 mm Hg/s. After treatment, all hemodynamic parameters were restored to values similar to SO group. Mean blood pressure which was increased after MI from 168. 2 ± 18.6 to 197.7 ± 10.7 returned to 137.0 ± 19.3 mm Hg after treatment. Increased deposition of colagen from 15.1 ± 3.9 to 24.0 ± 0.9 % induced by MI was prevented with treatment with the combination of drugs (12.9 ± 3.8 %). Conclusion: Oral administration of the combination of LASSBio-294 and LASSBio-897 could be considered promising in preventing cardiac dysfunction in SHR submitted to MI.
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Novel Multi-target Compound, Agonist of Adenosine Receptor and Inhibitor of Phosphodiesterase 5, Ameliorates Monocrotaline-induced Pulmonary Hypertension in Rats

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Aims: Pulmonary hypertension (PH) is a disease that results in right ventricular (RV) dysfunction and premature death. This work investigated the effects of LASSBio-1386, a compound with dual target, activation of the adenosine A_{2A} receptor and inhibition of phosphodiesterase 5 in rats with monocrotaline (MCT)-induced PH. **Methods and Results:** Protocols were approved by Animal Care and Use Committee at Universidade Federal do Rio de Janeiro. Male Wistar rats received a single intraperitoneal injection of MCT (60 mg/kg) for PH induction. Experimental groups were: control, MCT + vehicle (DMSO), MCT + LASSBio-1386 (100 μmol/kg/day p.o.) and MCT + Sildenafil (100 μmol/kg/day p.o.). Animals were treated with vehicle or drug for 14 days after the onset of disease (n = 6 per group). Treadmill test and transthoracic echocardiography were performed to access exercise capacity and cardiac function, respectively. Right ventricular systolic pressure (RVSP) and ratio between RV and body weight (RV/BW) were analyzed. Exercise capacity (m.kg) was reduced from 1336.2 ± 82.2(control) to 479.7 ± 75.5 (MCT+ vehicle) and recovered to 1357.0 ± 87.8 and 1221.0 ± 96.8 after treatment with LASSBio-1386 and sildenafil, respectively. Pulmonary acceleration time (PAT) (ms) was reduced from 45.3 ± 0.8 (control) to 21.9 ± 0.3 in MCT + vehicle group ($P < 0.05$) and restored to 42.9 ± 0.7 in MCT + LASSBio-1386 group, but it was only partially restored to 37.8 ± 1.3 (ms) in MCT + Sildenafil-treated group. RVSP (mmHg) was increased from 27.1 ± 0.7 (control) to 52.9 ± 1.5 in the PH rats and was reduced to 29.1 ± 1.0 and to 31.8 ± 0.6 after treatment with LASSBio-1386 and sildenafil. PH induced an increase of RV/BW (mg/g) from 0.62 ± 0.03 (control) to 1.81 ± 0.20 which was reduced to 0.70 ± 0.07 after administration of LASSBio-1386. Sildenafil treatment did not reverse the RV hypertrophy in PH rats (RV/BW = 1.52 ± 0.10 mg/g). **Conclusions:** LASSBio-1386 was effective to prevent RV dysfunction and exercise intolerance indicating important implications for ongoing clinical evaluation of multi-target drugs for the treatment of PAH.

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Soy Isoflavone Protects Myocardial Ischemia/reperfusion Injury Through Increasing Endothelial Nitric Oxide Synthase and Decreasing Oxidative Stress in Ovariectomized Rats

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Rationale There is a special role for estrogens in preventing and curing cardiovascular disease in women. Soy isoflavone (SI), a soy-derived phytoestrogen, is a group of biologically active plant substances with chemical structures which are similar to that of an endogenous estrogen-estradiol.
Objective We ought to elucidate possible mechanism of SI to improve myocardial ischemia/reperfusion (MI/R) injury in ovariectomized rats.
Methods and Results Female SD rats were underwent bilateral ovariectomy or sham ovariectomy. One week later, rats were randomly divided into several groups and began to feed soy-free chow: sham ovariectomy operation (control group), ovariectomy with MI/R or ovariectomy with sham MI/R. Other ovariectomy rats were given different doses of SI dissolved in 0.5% carboxymethycellulose (CMC-Na) by gavage. Additional ovariectomy rats were administrated with the same volume of CMC-Na by gavage or 50 μg/kg·d of 17β-estradiol (E_2) by subcutaneous injection. After four-week treatment, they were exposed to 30 minutes of left coronary artery occlusion followed by 6 or 24 hours of reperfusion. SI treatment decreased body weight, increased estradiol level and uterus weight. Isoflavone administration significantly reduced myocardial infarct size, improved left ventricle function and restored endothelium-dependent relaxation function of thoracic aortas after MI/R in ovariectomized rats. SI also decreased creatine kinase and malonaldehyde in plasma and attenuated oxidative stress in the myocardium. Meanwhile, SI increased phosphatidylinositol 3 kinase (PI3K) / Akt/ endothelial nitric oxide synthase (eNOS) signal pathway.
Conclusion Soy isoflavone protects myocardial ischemia/reperfusion injurys in ovariectomized rats through increasing PI3K/Akt/eNOS signal pathway and decreasing oxidative stress.

L. Xie: None. **G. Meng:** None. **Y. Ji:** None.

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Hydrogen Sulfide Prevents Myocardial Hypertrophy in a Klf5-dependent Manner

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Rationale H_2S is a gasotransmitter that regulates multiple cardiovascular functions. Krüppel-like transcription factor (KLF) exerts diverse functions in the cardiovascular system.
Objectives The aim of present study was to investigate the effect of hydrogen sulfide (H_2S) on myocardial hypertrophy.
Methods and results Myocardial samples of 22 patients with left ventricle hypertrophy were collected and underwent histological and molecular biological analysis. Spontaneously hypertensive rats (SHR) and neonatal rat cardiomyocytes were studied for functional and signaling response to GYY4137, a H_2S-releasing compound. Expression of cystathionine -lyase (CSE), a main enzyme for H_2S generation in human heart, decreased in human hypertrophic myocardium, while KLF5 expression increased. In SHR treated with GYY4137 for 4 weeks, myocardial hypertrophy was inhibited as evidenced by improvement in cardiac structural parameters, heart mass index, size of cardiac myocytes and expression of atrial natriuretic peptide (ANP). Levels of oxidative stress and phosphorylation of mitogen-activated protein kinases were also decreased after H_2S treatment. H_2S diminished expression of the KLF5 in myocardium of SHR and in neonatal rat cardiomyocytes rendered hypertrophy by angiotensin II (Ang II). H_2S also inhibited ANP promoter activity and ANP expression in Ang II-induced neonatal rat cardiomyocyte hypertrophy, and these effects were suppressed by KLF5 knockdown. KLF5 promoter activity was increased by Ang II stimulation, and this was reversed by H_2S. H_2S also decreased activity of specificity protein-1 (SP-1) binding to the KLF5 promoter and attenuated KLF5 nuclear translocation by Ang II stimulation.
Conclusion H_2S attenuated myocardial hypertrophy, which might be related to inhibiting oxidative stress and decreasing ANP transcription activity in a KLF5-dependent manner.

G. Meng: None. **L. Xie:** None. **Y. Ji:** None.

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Defining the Role of Transforming Growth Factor, Beta-induced in Adverse Ventricular Remodeling

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Heart failure (HF) is the final culmination of many forms of ongoing cardiovascular diseases, the later of which remains the leading cause of death in the United States. HF is characterized by ventricular remodeling and decreased wall compliance due to excess extracellular matrix (ECM) deposition, chronic inflammation and loss of myocytes. Treatments that target cardiac remodeling and preserve heart function are lacking, thus new therapeutic strategies are needed. The matricellular protein, Transforming growth factor, beta-induced (BigH3), is a member of the fasciclin-domain-containing family that includes periostin, stabilin 1 and stabilin 2. These ECM proteins are involved in cell-cell adhesion and ECM stabilization. BigH3 is re-expressed in the adult heart after injury, suggesting that it could play a role in ventricular remodeling. Here, we generated an inducible, cardiac-specific transgenic mouse model expressing BigH3. We observed that cardiac specific over-expression of BigH3 was sufficient to induce hypertrophy with age. BigH3 and periostin have been proposed to be functionally redundant due to similarities in structure. To test this, mice deficient for both BigH3 and periostin were generated. While periostin null mice are more prone to cardiac rupture and death following myocardial infarction injury, periostin/BigH3 double null mice showed significantly less survival following MI due to myocardial wall rupture, although BigH3 null mice alone showed no difference from controls. Taken together, these data suggest that BigH3 may have functions that are independent of periostin, in addition to redundant functions, suggesting that BigH3 could be a potential therapeutic target.

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The Role of Fibroblast-specific Canonical Tgfβ Signaling in Cardiac Fibrosis

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Heart failure is a progressive disease characterized by cardiomyocyte loss, interstitial fibrosis, and chamber remodeling. During physiological conditions cardiac fibroblasts contribute to the homeostatic maintenance of myocardial structure as well as the maintenance of biochemical, mechanical and electrical properties of the heart. Injury and/or cytokine stimulation activate fibroblasts which transdifferentiate into myofibroblasts. These newly formed cells secrete extracellular matrix (ECM) for wound healing and tissue remodeling through their contractile activity. Fibrosis mediated by these cells can initially be a beneficial response that acutely scarifies areas after an infarct to prevent wall rupture. However, during chronic disease states such as heart failure, persistent recruitment and activation of fibroblasts leads to excessive deposition of ECM that results in stiffening and pathological remodeling of the ventricles. During chronic heart disease, cardiomyocytes, immune cells and fibroblasts secrete the cytokine transforming growth factor-TGFβ, which activates fibroblasts and promotes their conversion to myofibroblasts. Manipulation of TGFβ by losartan, which antagonizes angiotensin II (AngII) and aspects of TGFβ signaling, has shown some anti-fibrotic effects in cardiovascular remodeling. Also deletion of Tgfbr1 (type I TGFβ receptor) in cardiomyocytes or a TGFβ blocking antibody reduced the fibrotic response after pressure overload. However heart failure was not improved because deleterious TGFβ signaling in fibroblasts persisted. We therefore utilized a novel fibroblast-specific inducible Cre-expressing mouse line (Periostin-MerCreMer) to examine the canonical (Smad2/3) TGFβ signaling within fibroblasts to determine how these cells and their activation mediate disease in heart failure. Our data indicate that fibroblast-specific deletion of Smad3 but not Smad2 was sufficient to significantly inhibit myocardial fibrosis. Smad2/3 double nulls were also generated and analyzed, as were TGFBR1 and TGFBR2 loxp targeted mice, also crossed with the Postn-MerCreMer knockin allele to achieve specificity in activated fibroblasts.

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Ischemic Heart Failure is Exacerbated in CD39-null Mice

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Background: CD39 (ectonucleoside triphosphate diphosphohydrolase) is a nucleotidase expressed on endothelial cells, vascular smooth muscles cells, and leukocytes. CD39 plays a key role in vascular homeostasis, hydrolyzing extracellular ATP and ADP. CD39 has been shown to be important in models of ischemic preconditioning and cardiac ischemia reperfusion. However, the effect of CD39 activity on functional recovery of heart after myocardial infarction (MI) has not been evaluated.
Hypothesis: Genetic ablation of CD39 expression exacerbates post-myocardial infarction cardiac function and fibrosis.
Methods: Wild-type (WT) and CD39-null mice were subjected to coronary artery ligation. Cardiac function and protein evaluation of fibrotic markers was performed at day 28 post-MI.
Results: Evaluation at Day 28 post-MI revealed that while mice of both genotypes had similarly reduced ejection fraction and equally compromised contractile function (dP/dtmax), there was a more pronounced negative effect on lusitropy (dP/dtmin) and increased left ventricular end-diastolic pressure in CD39-null mice. Therefore, cd39 gene ablation associates with the development of worsening cardiac performance. Histological analysis revealed increased collagen deposition and abundance of alpha-smooth muscle actin (αSMA) positive interstitial cells in the CD39-null hearts compared to WT hearts. To quantify these findings immunoblot analysis for collagen and αSMA was performed. We found that collagen and αSMA were increased at Day 28 post-MI, in CD39-null hearts compared to WT hearts.
Conclusion: CD39 ablation has detrimental effects on post-MI recovery, resulting in diminished cardiac performance and increased fibrosis.

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Effects of Long-term Angiotensin-II Infusion on Cardiac and Renal Fibrosis are Blunted in TNFR1-deficient Mice

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Background: Brief systemic infusion of Angiotensin-II (Ang-II) to wild-type (WT) mice initiates the development of cardiac interstitial fibrosis. Genetic deletion of tumor necrosis factor receptor 1 (TNFR1)

obviates this development and concurrently inhibits Ang-II-induced cardiac remodeling and dysfunction. We now investigated long-term effects of Ang-II on the heart, kidney, and cardiorenal function.
Methods: WT and TNFR1-KO mice were infused with 1.5 ug/kg/min Ang-II for 1 and 6 weeks (no uninephrectomy or high-salt diet). Heart, kidney, and serum were isolated and evaluated by histology, cytometry, qPCR, and ELISA techniques. Cardiac function was determined by 2D-echocardiography, systolic blood pressure by tail-cuff plethysmography.
Results: Brief infusion of Ang-II to WT mice did not evoke a fibrotic response in the kidney. However, after 6 weeks, WT kidneys developed minimal, but significant interstitial collagen deposition which was supported by upregulation of collagen-I, collagen-III, and alpha-smooth muscle actin gene activation. This fibrotic development was associated with the appearance of myeloid fibroblast precursors, pro-inflammatory M1 and pro-fibrotic M2 cells, and myofibroblasts. Transcriptional expression of pro-inflammatory and pro-fibrotic genes was also increased. These changes were not seen in Ang-II-infused TNFR1-KO kidneys. In WT hearts, despite the disappearance of myeloid cells, cardiac fibrosis persisted throughout the 6-week infusion. WT hearts developed clear evidence of accelerated cardiac hypertrophy and remodeling associated with impaired systolic function. Again, these changes were not seen in Ang-II-infused TNFR1-KO hearts. By contrast, both WT and TNFR1-KO mice responded identically with similar elevations of systolic blood pressure, and serum blood urea nitrogen and creatinine levels.
Conclusions: Ang-II-infusion induced an immediate fibrotic response in the heart while fibrosis in the kidney developed slowly. The cardiac fibrosis was accompanied by progressive adverse remodeling and worsening of function over time. TNFR1-KO mice were protected from the Ang-II-induced cardiac and renal fibrosis, despite similar increases in blood pressure and renal dysfunction.
M. Mayr: None. **C. Duerrschmid:** None. **D.B. Lee:** None. **G. Medrano:** None. **G.E. Taffet:** None. **M.L. Entman:** None. **S.B. Haudek:** None.

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MiR-125b Regulates Myofibroblast Transition and Cardiac Fibrosis
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Transforming growth factor-β (TGF-β)-induced fibroblast-to-myofibroblast transition (FMT) is a critical determinant of cardiac fibrosis. However, the contribution of microRNAs leading to TGF-β-induced FMT and cardiac fibrosis are not well-understood. Our results elucidate that blocking the canonical TGF-β pathway protects from FMT in primary cultures of human cardiac fibroblasts and that miR-125b is significantly upregulated during cardiac FMT. Furthermore, we observed significant upregulation of miR-125b in fibrotic human myocardium and two murine models of cardiac fibrosis. Importantly, we discovered that miR-125b is sufficient to induce cardiac FMT. In contrast, the knockdown of miR-125b using an antagomir approach attenuated TGF-β-induced FMT. In silico analysis and biochemical analysis revealed that miR-125b directly targets multiple anti-fibrotic mediators including p53 and apelin. In addition, miR-125b also plays a potent role in the regulation of fibroblast proliferation, an important cause of cardiac fibrosis. Finally, miR-125b was successfully inhibited in vivo by the systemic delivery of locked nucleic acid (LNA) targeted against miR-125b both in the presence and absence of Angiotensin II (Ang II). These results demonstrated that LNA-125b protected against Ang II-induced proliferation and fibrosis in the mouse heart in vivo. We conclude that TGF-β-induced miR-125b is an important regulator of both fibroblast proliferation and FMT, and miR-125b inhibits key anti-fibrotic mediators to promote cardiac fibrosis. We propose that miR-125b may serve as a novel therapeutic target for the preventative therapy for fibrosis.
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Transforming Growth Factor-β Induces the Cardiac Fibrosis and Remodeling in Guanylyl cyclase/Natriuretic Peptide Receptor-A Gene-Disrupted Null Mutant Mice
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Genetic disruption of guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA) gene (Npr1) in mice exhibits high blood pressure, cardiac hypertrophy, fibrosis, and remodeling leading to congestive heart failure. The objective of this study was to determine the mechanisms regulating the development of fibrosis in Npr1 gene-disrupted mice hearts. The Npr1 null mutant (Npr1-/-, 0-copy), heterozygous (Npr1+/-, 1-copy), and wild-type (Npr1+/+, 2-copy) mice were administered by oral gavage with transforming growth factor-β1 (TGF- β1) receptor inhibitor GW788388 (1mg/kg/day) for 28 days. The heart tissues were isolated and used for quantification of fibrotic markers by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot analyses. Together, systolic blood pressure (SBP), heart weight-to-body weight (HW/BW) ratio, left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVEDS), and percent fractional shortening (FS) were analyzed. The Npr1-/- null mutant mice hearts displayed 6-fold induction of fibrosis compared with wild-type (WT) Npr1+/+ mice. Furthermore, the increased expression of fibrotic markers as observed, including connective tissue growth factor (CTGF, 5-fold), α-smooth muscle actin (α-SMA, 4-fold) and TGF-β receptor I (TGF-βRI, 4-fold), TGF-β receptor II (TGF-βRII, 3.5-fold) and Smad2/3 proteins in Npr1-/- mice hearts compared with WT control mice. However, treatment with TGF-β receptor antagonist, GW788388, significantly prevented the cardiac fibrosis and down-regulated the expression of fibrotic markers and Smad proteins in Npr1-/- mice compared to vehicle-treated WT controls. The results of the present study suggest that the activation of cardiac fibrosis in Npr1-/- mice is mainly triggered through TGF-β mediated Smad-dependent pathways.
K.N. Pandey: None. **U. Subramanian:** None.

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Nrf2 Protects From Intermittent Hypoxia-induced Cardiomyopathy via Metallothionein-dependent and Independent Mechanisms
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Background: We recently reported that cardiac expression of the antioxidant metallothionein (MT) was increased by acute expsorue to intermittent hypoxia

(IH), and protected from chronic IH-induced cardiomyopathy. However, how IH stimulates MT in the heart remains unclear. Here we tried to define whether nuclear factor erythroid 2-related factor 2 (Nrf2), a critical redox-balance controller in the body, protects from IH-induced cardiomyopathy and its relationship with MT, and whether Nrf2 and MT are indispensable for sulforaphane (SFN) prevention of IH-induced cardiomyopathy.
Methods and Results: Mice were exposed to IH for 3 days to 8 weeks. Like MT, cardiac Nrf2 expression was significantly increased in response to 3-day IH, but decreased in response to 4- or 8-week IH. Mice with cardiac overexpression or global deletion of the Nrf2 gene (Nrf2-TG or Nrf2-KO) were completely resistant or susceptible to IH-induced cardiomyopathy. Cardiac protection from IH by endogenous Nrf2 and MT are indispensable each other. Mechanistically 4-week exposure to IH significantly decreased cardiac Nrf2 binding to the promoter of MT, lowing its transcription and translation. MT stimulated Nrf2 function via activation of PI3K/Akt/GSK-3β/Fyn signaling pathway in a feedback manner. Furthermore, Nrf2 inducer SFN prevented IH-induced cardiomyopathy in both WT and MT-KO mice, but not Nrf2-KO mice.
Conclusions: These results suggest that a reciprocal regulation between Nrf2 and MT is necessary for an efficiently antioxidant response to and protection from IH. However, the protective effect of SFN on IH-induced cardiomyopathy is Nrf2-dependent, instead of MT.
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Activation of Nrf2 by Sulforaphane via the AKT/GSK-3β/Fyn Pathway Prevents Angiotensin II-induced Cardiomyopathy
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Aims: Sulforaphane (SFN) as a nuclear factor erythroid 2-related factor 2 (Nrf2) activator protects the heart from, and deletion of the Nrf2 gene exaggerates, the effects of diabetes. Angiotensin II (Ang II) plays a critical role in the development of diabetic cardiomyopathy; therefore, whether SFN prevents Ang II-induced cardiomyopathy through activation of Nrf2 was examined.
Methods and Results: The chronic cardiac effects of Ang II with and without SFN were examined in wild-type mice, transgenic Nrf2 knockout (Nrf2-KO) mice, and mice in which cardiac tissue overexpressed Nrf2 (Nrf2-TG). The signaling pathways of SFN-mediated Nrf2 activation were examined in H9C2 cells. Administration of a subpressor dose of Ang II to WT mice induced cardiac oxidative stress, inflammation, remodeling and dysfunction, all of which could be prevented by SFN treatment, which also up-regulated Nrf2 expression and activation. Nrf2-TG mice showed resistance and Nrf2-KO mice displayed resistance to Ang II-induced cardiomyopathy. Meanwhile, the ability of SFN to protect against Ang II-induced cardiac damage was lost in Nrf2-KO mice. Up-regulation and activation of Nrf2 by SFN is accompanied by activation of AKT, inhibition of glycogen synthase kinase (GSK)-3β, and increased nuclear accumulation of Fyn. In vitro up-regulation of Nrf2 by SFN in H9C2 cells was abolished and nuclear Fyn accumulation was increased when cells were exposed to a PI3K inhibitor or GSK-3β-specific activator.
Conclusion: Nrf2 plays a central role in the prevention of Ang II-induced pathological effects, and SFN can prevent Ang II-induced cardiomyopathy through activation of Nrf2 partially via the AKT/GSK-3β/Fyn pathway.
Y. Xin: None. **Y. Bai:** None. **X. Jiang:** None. **S. Zhou:** None. **Y. Wang:** None. **K.A. Wintergerst:** None. **Y. Tan:** None. **T. Cui:** None. **L. Cai:** None.

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Beta-catenin Loss-of-function in Cardiac Fibroblasts Attenuate Cardiac Fibrosis after Transverse Aortic Constriction in Mice
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Background: Cardiac fibrosis increases myocardium stiffness, impairs cardiac function and contributes to heart failure. Activated fibroblasts produce excessive extracellular matrix leading to fibrosis and impaired cardiac function. Pathologically activated canonical Wnt signaling has been implicated in the pulmonary-, renal-, dermal- and liver fibrosis as well as in scarring after myocardial infarction. We hypothesize that Wnt/β-catenin signaling contributes to cardiac fibroblast (CF) activation and β-catenin loss-of-function (βLOF) in CFs reduces fibrosis and preserves cardiac function.
Methods and Results: The role of Wnt/β-catenin signaling in CF activation was studied using in vitro CF culture and in vivo genetic manipulation. Postnatal (P)0, P8 and P60 CFs were isolated and cultured. Wnt1 treatment significantly activated CFs, while Wnt inhibition (XAV) completely blocked Wnt1-induced CF activation as determined by αSMA staining. Interestingly, XAV also partially abrogated CF activation induced by TGFβ. In vivo, PeriostinMerCreMer (Pn) or Tcf21MerCreMer (T21) mice were crossbred with βLOF and ROSAmTmG. At baseline, CFs were labeled by T21 but minimal activity was observed with Pn. Accumulation of CFs labeled by Pn or T21 Cres was observed in myocardium 8 weeks after TAC. Inducible CF-specific βLOF transgenic mice (Pn-βLOF or T21-βLOF) were subjected to transverse aortic constriction (TAC) or sham surgeries. βLOF was induced post-surgery via tamoxifen. In sham-operated mice, no difference in cardiac function or morphology was observed between βLOF and control mice. In mice subjected to TAC, cardiac function, as measured by echocardiography, was significantly improved, and the interstitial and peri-vascular fibrosis, as well as heart weight to tibia length ratios, were significantly reduced in the βLOF group, compared to controls, 8 weeks after TAC. Thus, induction of βLOF in CFs after TAC leads to improved cardiac performance, decreased fibrosis, and reduced maladaptive cardiac remodeling.
Conclusions: βLOF in CFs inhibits myo-fibroblast activation, reduces cardiac fibrosis and preserves cardiac function in TAC induced cardiac injury.
F. Xiang: None. **M. Fang:** None. **K. Yutzey:** None.

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Mnsodtg Mice Exhibit Improved Heart and Mitochondrial Function During Chronic Chagas Disease
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Background: We observed that mitochondrial reactive oxygen species (mtROS) plays very important roles in the pregression of chagesic disease (CD). In this study, we utilized genetically-modified mice to scavenge mtROS to investigate the impact of improved ROS scavenging capacity on heart function in CD.
Methods and Results: C57BL/6 mice (wild-type, MnSODtg, MnSOD+/-) were infected with Trypanosoma cruzi(Tc). Chronically infected mice (≥120dpi) exhibited a substantial decrease in heart tissue MnSOD gene expression, protein level, enzyme activity and antioxidant level; decrease of heart dysfunction via lower of SV, CO, EF, FS and LVPW,s, and increase of ESV/EDS and LVID;s; enhancement of hypertrophy by

increase of IVS, LV mass and areas duo to augmentation of collagen expressions. One of our novel observations was that sarco/endoplasmic reticulum Ca2+-ATPase (SERCA2) lost its role of maintenance of low cytoplasm free calcium and mediated calcium uptake to intracellular store in Tc-induced chronic chagasic disease. Studies of fresh heart slices using O2K confirmed that Tc diminished heart mitochondrial function like decrease of oxygen flux and respiratory control ratio (RCR), which were caused by enhancements of ROS. Myocardial mitochondrial damage was pronounced and associated with a >x% decline in mitochondrial oxygen flux in chronically infected wild-type and MnSOD transgenic mice. Imaging of intact heart for cardiomyocytes and collagen by the nonlinear optical microscopy techniques showed significant increase in collagen (>x0-fold) in chronically infected wild-type mice; while MnSODtg mice exhibited a basal increase in collagen that did not change during chronic phase. Chronically infected MnSODtg mice exhibited a marginal decline in Tc-induced heart function, heart hypertrophy, mitochondrial dysfunction Conclusions: Overexpression of MnSOD inhibited Tc-induced oxidative damage od heart tissue. , suggesting that enhancing the mitochondrial ROS scavenging capacity was beneficial in controlling the inflammatory and oxidative pathology, and cardiac remodeling responses that are hallmarks of chronic Chagas disease.
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Modulation of Macrophage Function via Metabolism by Trypanosoma cruzi
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Chagas heart disease is an inflammatory cardiomyopathy which presents with mononuclear infiltrates in the interstitium and myocardial fibrosis in the chronic phase. Incomplete clearance by macrophages of the etiologic agent, *Trypanosoma cruzi*, is a significant cause of chronic disease development in approximately 30% of those serologically positive for the blood-borne parasite. The differential metabolic status, anaerobic glycolysis and mitochondria-dependent oxidative phosphorylation, are respectively associated with pro-inflammatory (M1) and anti-inflammatory (M2) functional activation of macrophages. Reactive oxygen species (ROS) have been shown to be an intracellular signal for glycolysis while peroxisome proliferator-activated receptors (PPARs) that enhance fatty acid oxidation provide transcription control of macrophage functional state. In our studies using diverse *T. cruzi* isolates, we showed that SylvioX10 (virulent), but not TCC (non-virulent), isolates are able to differentially control extracellular and intracellular ROS levels in macrophages. We found in macrophages infected with SylvioX10, the nuclear expression of PPAR-α was increased by 18 hours post-infection, and mitochondrial metabolic activity was similar to that of not-infected and M2 controls; which indicates anti-inflammatory function of macrophages, and therefore prohibiting *T. cruzi* clearance. In our ongoing studies, we are examining the impact of PPAR-α inhibitors in modulating the metabolic gene expression profile, functional phenotype and parasite survival in macrophages. Our data will provide the first indication that host macrophages have deficient pro-inflammatory capacity due to sub-optimal glucose oxidation, and enhancing the metabolism that supports *T. cruzi* clearance will provide a valuable basis for a strategy to arrest Chagas disease progression.
S. Koo: None. **N.J. Garg:** None.

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Expression of the Transcription Factors PPAR Alpha and Gamma, and Their Target Genes in the Heart of Obese Mice
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The Peroxisome-Proliferator Activated Receptors (PPARs) have been identified as key regulators of energy metabolism by sensing fatty acid availability and activating the transcription of enzymes involved in fatty acid and glucose metabolism. In the heart, PPARs have been proven to participate in the development of diseases, such as cardiac hypertrophy and diabetic cardiomyopathy, conditions that have been associated to obesity. Furthermore, the use of PPARs activator drugs is frequent for the treatment of obesity-dependent diseases, such as hypoglycemiant (fibrates) and anti-diabetic (thiazolidinediones) drugs. Therefore, the objective of this project was to characterize the expression of PPARs and some of their target genes involved in fatty acid and glucose metabolism in the heart of obese mice. Mice (C57BL/6) were fed with either a standard chow diet (control group) or a high fat diet (obese group) for three months. Hearts were weighed prior to quantifying protein expression of two PPARs (alpha and gamma), and both mRNA and protein expression of three enzymes (phosphofructokinase (PFK), glycerol-3-phosphate dehydrogenase (GPD1) and glycerol-3-phosphate acyltransferase (GPAT)). Protein levels were measured by western blot, while mRNA levels were quantified by real-time PCR. The obese group presented 41% increase in body weight compared with control mice. However, no change was observed in obese hearts weight compared with control group, meaning that hypertrophy had not been developed yet. The expression of PPAR-alpha was increased 42% in obese mice compared with the control group, suggesting a possible activation of the fatty acid oxidation pathway. PPAR-gamma together with GPD1 and GPAT showed no differences at any level; however, PFK presented 90% increase in mRNA level and 54% increase in protein level in obese mice. Interestingly, decrease in PFK has been reported in any altered PPAR-alpha condition, contrary to our results. These results suggest that there is a dysregulation of the heart's energetic pathways mediated by PPARs in earlier stages of obesity.
A. Ruiz-Velasco: None. **A. Rios:** None. **B. Escalante:** None.

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A Novel Mitochondrial Targeted Fusion Protein Containing Endonuclease III Protects the Heart Against Myocardial Infarction and Pressure Overload Heart Failure
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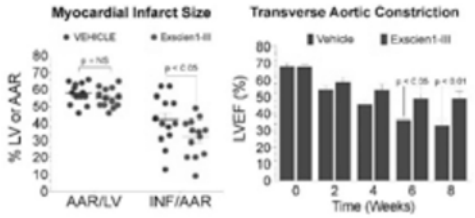
Background: Oxidative stress is a primary cause of mitochondrial DNA (mtDNA) damage and plays a role in myocardial cell death. mtDNA repair enzymes are crucial for mtDNA repair and cell survival. We tested the efficacy of a novel, mitochondrial targeted fusion protein that traffics Endonuclease III (Exscien1-III) in murine models of myocardial ischemia/reperfusion (MI/R) injury and transverse aortic constriction (TAC) heart failure (HF). We previously demonstrated that Exscien1-III administered at R reduced myocardial infarct size and preserved left ventricular ejection fraction (LVEF) following MI/R. We hypothesized that delayed administration of Exscien1-III would promote mtDNA repair and protect the myocardium against MI/R and TAC heart failure.
Methods: Male C57/BL6J (10-12 wks) were subjected

to 45 min of MI and 24 hrs of R. Exscien1-III (4 mg/kg, i.p., n=13) or vehicle (VEH, n=13) was administered 30 min after R. Male C57/BL6J were subjected to TAC (27 g needle) and Exscien1-III (4 mg/kg/d, i.p., n=10) or VEH (n=6) were administered starting at 3 wks post TAC. Echocardiography was performed at baseline and following TAC to assess LVEF.
Results: Exscien1-III reduced myocardial INF/AAR by 24% ($p < 0.05$ vs. VEH). Exscien1-III preserved LVEF (49.1 ± 4.0% vs. 32.9 ± 3.2%, $p < 0.01$) and reduced LV dilation (LVEDD/LVESD; 3.8/2.8 vs. 4.4/3.4, $p < 0.05$) at 8 wks compared to vehicle.
Conclusion: These results demonstrate that delayed administration of Exscien1-III significantly attenuates myocardial cell death and preserves LV function in acute MI and HF. Studies are currently underway to define the molecular mechanisms involved in Exscien1-III induced cardioprotection.



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Exercise Prevents Cardiac Injury and Improves Mitochondrial Biogenesis in Advanced Diabetic Cardiomyopathy With Pgc-1α and Akt Activation

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Background/Aims: Diabetic cardiomyopathy (DCM) represents the major cause of morbidity and mortality among diabetics. Exercise has been reported to be effective to protect the heart from cardiac injury during the development of DCM. However, the potential cardioprotective effect of exercise in advanced DCM remains unclear. **Methods:** Seven-week old male C57BL/6 wild-type or db/db mice were either subjected to a running exercise program for 15 weeks or kept sedentary. Cardiac function, myocardial apoptosis and fibrosis, and mitochondrial biogenesis were examined for evaluation of cardiac injury. **Results:** A reduction in ejection fraction and fractional shortening in db/db mice was significantly reversed by exercise training. DCM induced remarkable cardiomyocyte apoptosis and increased ratio of Bax/Bcl-2 at the protein level. Meanwhile, DCM caused slightly myocardial fibrosis with elevated mRNA levels of collagen I and collagen III. Also, DCM resulted in a reduction of mitochondrial DNA (mtDNA) replication and transcription, together with reduced mtDNA content and impaired mitochondrial ultrastructure. All of these changes could be abolished by exercise training. Furthermore, DCM-associated inhibition of PGC-1α and Akt signaling was significantly activated by exercise, indicating that exercise-induced activation of PGC-1α and Akt signaling might be responsible for mediating cardioprotective effect of exercise in DCM. **Conclusion:** Exercise preserves cardiac function, prevents myocardial apoptosis and fibrosis, and improves mitochondrial biogenesis in the late stage of DCM. Exercise-induced activation of PGC-1α and Akt signaling might be promising therapeutic targets for advanced DCM.

Key words: diabetic cardiomyopathy, exercise, mitochondria, PGC-1α, Akt

H. Wang: None. **Y. Lu:** None. **W. Sun:** None. **J. Xiao:** None. **X. Kong:** None.

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Loss of Mir-155 Attenuates Sepsis Induced Cardiac Dysfunction

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Objective: Sepsis induced cardiac dysfunction is featured by inflammation and metabolic repression. miR-155 is a typical multifunctional miRNA and loss of miR-155 has been shown to protect the heart from pathological cardiac hypertrophy while increased miR-155 could promote the formation of foam cell in atherogenesis. However, the role of miR-155 in sepsis induced cardiac dysfunction is unclear.
Methods: E.coli lipopolysaccharide (LPS) (5mg/kg) was administered to C57BL/6 mice to create a sepsis-induced cardiac dysfunction model. Cardiac function was assessed by echocardiography 5-6 h post-LPS administration. Heart tissues were collected within 7-9 h after LPS treatment for the analysis of gene expressions. Tail vein injection of miR-155 antagomir (80mg/kg/d) or miR-155 agomirs (30mg/kg/d) for 3 consecutive days were used to decrease or increase miR-155 expressions in heart.
Results: LPS induced a reduction of 15% in fractional shortening (%FS) and 25% in ejection fraction (%EF). Expression of miR-155 was increased by 2 fold in sepsis-induced cardiac dysfunction mouse model. Over-expression of miR-155 agomirs led to a decrease of 5% in FS and 10% in EF as compared to scramble controls. Aggravation of LPS induced cardiac dysfunction by miR-155 agomir was not associated with alteration in inflammation or cardiac metabolism. However, miR-155 agomir increased LPS- induced myocardium apoptosis and also elevated the ratio of Bax/Bcl-2 at the protein level. Intravenous injection of cholesterol-modified antisense oligonucleorides antagomirs of miR-155 markedly rescued the LPS induced heart failure and apoptosis. Western bloting indicated that miR-155 overexpression in *vivo* led to a significant inhibition of Pea15a while miR-155 knock-down caused a significant upregulation of Pea15a, indicating that Pea15a was a potential target gene of miR-155. Interestingly, plasma miR-155 levels were also found to be significantly increased in critically ill patients with sepsis compared to healthy controls.
Conclusion: This study demonstrates that miR-155 regulates sepsis induced cardiac dysfunction and Pea15a is a potential targer gene of miR-155. Loss of miR-155 represents a novel therapeutic method for sepsis induced cardiac dysfunction

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Microrna-21* Controls Sepsis-induced Cardiac Dysfunction

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Background: Sepsis-induced cardiac dysfunction is charactered by cardiac contractility dysfunction, myocardial inflammation and cardiac metabolism abnormal. Dysfunction of microRNAs (miRNAs, miRs) contributes to a variety of human diseases. However, their roles in sepsis-induced cardiac dysfunction are unclear.
Methods and Results: Cardiac dysfunction was induced by E.coli lipopolysaccharide (LPS) administration in mice and 8 dysregulated miRNAs were identified by miRNA arrays. Among them, miR-21* was found to be increased most obviously as determined by quantitative reverse transcription polymerase chain reactions. Inhibition of miR-21* in vivo by antagomir attenuated the reduction of factional shortening (FS) and ejection fraction (EF) induced by LPS administration while forced over-expression of miR-21* in vivo by agomir accelerated LPS-induced cardiac dysfunction. Besides that, S100A8 and S100A9, two genes related to cardiac contractility were also found to be regulated in vivo by injection of miR-21* agomirs and antagomirs. Interestingly, cardiac inflammation indictors such as TNF-α and IL-6 and cardiac metabolism regulators including PPAR family, CD36, FATP, GLUT1, GLUT4, PDK4 were not changed by miR-21* in vivo. These data indicate that miR-21* controls sepsis-induced cardiac dysfunction by direct affecting cardiac contractility instead of cardiac inflammation and metabolism. SORBS2 was identified as a target gene of miR-21* and it was decreased by miR-21* agomir and increased by miR-21* antagomir in vivo. In consist with this, circulating levels of miR-21* were also increased in patients with sepsis compared with healthy controls.
Conclusion: miR-21* controls sepsis-induced cardiac dysfunction by regulating SORBS2. Inhibition of miR-21* represents a novel therapeutic strategy for sepsis-induced cardiac dysfunction.
H. Wang: None. **Y. Bei:** None. **J. Shi:** None. **W. Sun:** None. **H. Liu:** None. **X. Li:** None. **J. Xiao:** None. **X. Kong:** None.

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MitoTimer Mouse: a Novel Tool for the Study of Mitochondrial Turnover in vivo

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In order to study mitochondrial turnover at the level of a single mitochondrion, our laboratory has developed the MitoTimer protein. Timer is a mutant of DsRed fluorescent protein developed by Terskikh et al. The Timer protein transitions from green fluorescence to a more stable red conformation as it matures over a span of 48 h. Furthermore, the protein is very stable under physiological conditions, insensitive to variations in ionic strength, and changes in pH between 7.0 and 8.0. Notably, Timer maturation from green to red is significantly slowed in deoxygenated buffer, suggesting that molecular oxygen plays a part in fluorophore maturation. We fused the Timer protein with the mitochondrial signal sequence from the cytochrome c oxidase subunit VIII (COX8) to target the protein to the inner membrane of the mitochondria, and further cloned the protein into a construct with a cardiac-restricted α-myosin heavy chain promoter. This construct was used to create the α-MHC MitoTimer mice. Surprisingly, initial analysis of the hearts from these mice reveals a remarkable degree of heterogeneity in the ratio of red-to- green fluorescence of MitoTimer in cardiac tissue. Furthermore, individual mitochondria within cardiomyocytes display a higher red-to-green fluorescence, implying a block in import of newly synthesized MitoTimer that would be caused by the lack of a high membrane potential, indicative of older, dysfunctional mitochondria. Initial studies suggest that these mice represent an elegant tool for the investigation of mitochondrial turnover in the heart.
A.B. Stotland: None. **J. Ramil:** None. **R.A. Gottlieb:** None.

90

Akt Mediated Mitochondrial Dysfunction Involves Mitochondrial Endothelial Nitric Oxide Synthase Translocation

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Pulmonary arterial hypertension (PH) is a fatal disease characterized by uncontrolled pulmonary vascular cell proliferation. Mitochondrial dysfunction (MD) of pulmonary endothelial cells (EC) was shown to be one of the primary events implicated into proliferative, apoptosis resistant cell phenotype. However, the particular molecular mechanisms responsible for MD remain unclear. The development of PH in patients is associated with severe nitrosative stress, leading to post-translational protein modifications. Thus, we have recently found that Akt is susceptible to nitration of tyrosine Y350 residue. We hypothesize that nitration of Akt induces activation of Akt signaling and contributes to the development of MD. Nitrosative stress in EC was initiated by eNOS uncoupler ADMA or peroxynitrite donor SIN-1 and resulted in significant Akt nitration and activation (1.5±0.1 fold control; p=0.007; N=3-4), as well as activation of anti-apoptotic (BAD phosphorylation), and proliferative (mTOR phosphorylation) signaling cascades. Increased Akt signaling induced phosphorylation of eNOS at serine S615 (1.47±0.08 fold control, p=0.005, N=3) and S1177 (0.22±0.04 vs. 0.47±0.07, p=0.029, N=3). Phosphorylation of eNOS resulted in its translocation to mitochondria (3± 0.3 fold control; p=0.003; N=3) which, in turn, significantly decreased basal mitochondrial respiration (oxygen consumption rate, pmol/min: untransfected cells 1022±96 vs. mitochondrial targeted phospho-mimetic eNOS mutants S615D 102±9 and S1177D 117±7, p<0.001, N=4-6), perhaps due to previously reported inhibitory effect of NO on mitochondrial respiratory chain. Finally, we have created an anti-oxidant conjugated “shielding” peptide that, by shielding the Akt nitration site, is capable to prevent Akt activation. Indeed, pre-treatment with shielding peptide (100μg/ml, 30min) completely abolished SIN-1 induced nitration of Akt in EC. We conclude that Akt nitration may contribute to proliferative/apoptosis resistant EC phenotype through pathological activation of Akt signaling and Akt mediated mitochondrial eNOS translocation. Besides, our novel shielding peptide based therapeutic strategy opens new avenues in MD prevention.
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Interdependence of Mitochondrial Fission and Mitophagy in Adult Mouse Hearts

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Canada; Gerald W Dorn II, Washington Univ Sch of Med, Saint Louis, MO

The role of Drp1-mediated mitochondrial fission in normal hearts is controversial. Cardiomyocyte mitochondria are hypo-dynamic, but mitochondrial dynamism factors are abundantly expressed. Cardiac-specific Drp1 gene deletion (KO) provokes mitochondrial enlargement, MPTP-dependent cardiomyocyte loss, and dilated cardiomyopathy (Song et al Cell Metab, 2015). We postulated that Drp1-dependent mitochondrial fission is essential for triage and elimination of damaged cardiomyocyte mitochondria by Parkin-mediated mitophagy. Others described no benefit of Parkin KO in mice with perinatal cardiac Drp1 KO (Kageyama et al EMBO J, 2014), but these Parkin KO mice have little basal phenotype due to germ-line compensation. Here, we assessed the individual and interactive roles of Parkin and Drp1 in adult mouse hearts by conditionally ablating each gene (Cre-Lox at 8 wks), separately and in combination. Parkin KO hearts appeared normal; as reported, Drp1 KO caused lethal cardiomyopathy after 6-7 wks. Cardiac-specific Parkin KO concomitant with Drp1 KO ameliorated the underlying cardiomyopathy by: 1. Increasing survival (94% vs 56%; P<0.0001); 2. Enhancing cardiac contractility (LV FS 36.2±4.0 vs 23.2±1.7%; P=0.01); 3. Decreasing adverse remodeling (LV r/h 5.0±0.5 vs 6.3±0.4; P=0.05); 4. Reducing cardiomyocyte necrosis (1.9±0.5 vs 5.0±1.2%; P=0.05) and replacement fibrosis (33.3±5.3 vs 13.0±1.5%; P=0.02); 5. Attenuating mitochondrial deficiency (5.7±0.4 vs 4.7±0.6 µg/mg; P=0.20). Deleting Parkin did not affect mitochondrial enlargement or respiratory function in Drp1 KO cardiomyocytes, but normalized mitochondrial-associated LC3 and p62, mitophagy markers increased in cardiac Drp1 KO. These studies show how Drp1-mediated mitochondrial fission and Parkin-mediated mitophagy interact to maintain the quality of cardiac mitochondria: Interrupting mitochondrial fission (Drp1 KO) prevents segregation of damaged mitochondrial components into daughter organelles normally targeted for mitophagy; mitophagy thus ultimately consumes fission-defective parent organelles. Parkin KO suppresses generalized mitophagy, postponing (but not preventing) the Drp1 KO cardiomyopathy.

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The Mitochondrial Calcium Uniporter is Necessary for Metabolic Matching of Contractile Demand During Acute Sympathetic Stress

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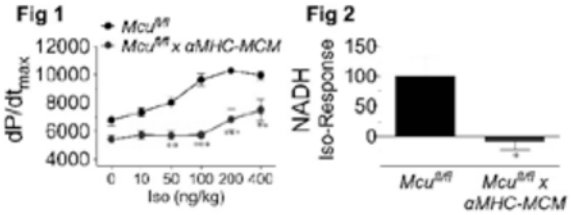
Contractility is mediated by a variable flux in intracellular calcium (Ca^{2+}), which is proposed to be integrated into mitochondria to regulate cardiac energetics. Moreover, $_{m}Ca^{2+}$-overload is known to activate the mitochondrial permeability transition pore (MPTP) and induce cell death. Recent studies have reported that the *Mcu* gene encodes the channel-forming portion of the mitochondrial calcium uniporter (MCU) and is required for $_{m}Ca^{2+}$ uptake. To examine the role of $_{m}Ca^{2+}$ in the heart, we generated a conditional, cardiac-specific knockout model and deleted *Mcu* in adult mice (*Mcu-cKO*). Loss of *Mcu* protected against myocardial ischemia-reperfusion (IR) injury by preventing the activation of the MPTP. In addition while we found no baseline phenotype, *Mcu*-cKO mice lacked contractile responsiveness to beta-adrenergic receptor stimulation as assessed by invasive hemodynamics (Fig 1) and in parallel were unable to activate mitochondrial dehydrogenases and increase cardiac NADH levels. Further experimental analyses in isolated adult cardiomyocytes confirmed a lack of energetic responsiveness to acute sympathetic stress (isoproterenol failure to mediate an increase in NADH, Fig 2), supporting the hypothesis that the physiological function of the MCU in the heart is to modulate Ca^{2+}-dependent metabolism during the 'fight or flight' response.



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Genetic Deletion of Slc8b1, the Mitochondrial Sodium/Calcium Exchanger, Causes Sudden Cardiac Death and Overexpression Protects Against Myocardial Infarction and Pressure-Overload Heart Failure

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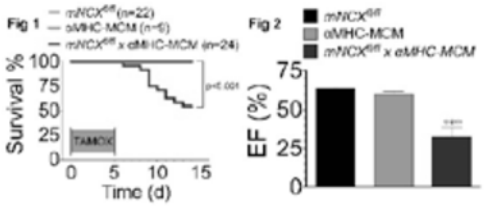
Mitochondrial calcium ($_{m}Ca^{2+}$) signaling is critical for both energy production and the activation of cell death pathways in the heart. Further, $_{m}Ca^{2+}$ overload is hypothesized to be a significant contributor to the development and progression of heart failure (HF). The mitochondrial sodium/calcium exchanger (mNCX) is hypothesized to be the primary mechanism of $_{m}Ca^{2+}$ efflux, but to date no study has genetically confirmed its identity or function in an *in vivo* system. To investigate the role of mNCX in HF, we generated mutant mice with loxP sites flanking exons 5-7 of the candidate gene, *Slc8b1 (also known as NCLX)*, and crossed them with a tamoxifen (tamox)-inducible cardiac-specific Cre mouse to delete mNCX in the adult heart (mNCX-cKO). Cardiomyocytes isolated from mNCX-cKO mice displayed a significant reduction in $_{m}Ca^{2+}$ efflux rate and Ca^{2+} uptake capacity. Tamoxifen-induced ablation of mNCX resulted in sudden death with only 54% of mice surviving 8d post-tamoxifen treatment (Fig 1). Assessment of mNCX-cKO hearts 2d post-tamox revealed significant remodeling characterized by dilation and a decrease in %EF (Fig 2). Next, we generated a conditional, cardiac-specific mNCX overexpression mouse model (mNCX-Tg) to evaluate if increased $_{m}Ca^{2+}$ efflux would alter the progression of HF. mNCX-Tg and controls were subjected to both myocardial infarction (LCA ligation) and pressure-overload induced HF (transverse aortic constriction). mNCX-Tg mice displayed preserved LV function, structure and a reduction in HF indices in both models. For the first time we show that mNCX is essential for $_{m}Ca^{2+}$ efflux in cardiomyocytes and that mNCX represents a novel therapeutic target in HF.



T.S. Luongo: None. **M. Nwokedi:** None. **J.P. Lambert:** None. **E. Gao:** None. **A.C. Carpenter:** None. **M. Madesh:** None. **J.D. Molkentin:** None. **J.W. Elrod:** None.

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The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart

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Ca^{2+} is central to cardiac contraction, metabolism and survival. Physiologically, Ca^{2+} fluxes drive both enhanced cardiac function and ATP supply through mitochondrial Ca^{2+} loading. Under pathologic conditions however, mitochondrial Ca^{2+} overload, such as after ischemic injury, can trigger mitochondrial permeability transition pore (MPTP) opening leading to cardiomyocyte death. Mitochondrial Ca^{2+} uptake under both physiologic and pathologic conditions is thought to be mediated by the mitochondrial Ca^{2+} uniporter (MCU). Despite considerable focus on delineating MCU regulation and the importance of mitochondrial Ca^{2+} to metabolism and cell survival, there is no clear consensus regarding MCU's function in the heart. To test the functional role of MCU in the heart, we generated a genetic mouse model with the inducible and cardiomyocyte-specific deletion of the *Mcu* locus. Heart mitochondria from cardiac-specific *Mcu*-deleted mice displayed impaired acute Ca^{2+} uptake, blunted Ca^{2+}-stimulated ATP production, and defective Ca^{2+}-mediated MPTP opening. Mice lacking *Mcu* in the adult heart were protected from acute ischemia-reperfusion injury, indicating that MCU is required for acute mitochondrial Ca^{2+} uptake and that the MCU-mitochondrial Ca^{2+}-MPTP axis plays a key role in cardiomyocyte death. Interestingly however, basal mitochondrial Ca^{2+} levels were unaltered in hearts of *Mcu*-deleted mice and *Mcu*-deleted mice did not display any overt cardiac phenotype with up to 1 year of aging or greater defects after 8-weeks of chronic cardiac pressure overload. In contrast, *Mcu* deletion inhibited acute increases in cardiac metabolism and function during several minutes of short-term beta-adrenergic stimulation, a defect that eventually recovered over the next 30-60 min as mitochondrial Ca^{2+} loading gradually recovered. Moreover, Mcu-deleted mice failed to run adequately on a treadmill in the first few minutes, but if given a 40 minute warmup ramping period they were now able to attain the same maximal performance as WT controls. These results suggest that the MCU is not required for long-term mitochondrial Ca^{2+} homeostasis but instead serves as a "fight-or-flight" mediator to acutely match changes in cardiac workload with ATP production.

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Pharmacological Characterization of Adenosine Effects on Spontaneously Beating Human-IPSC-Derived Cardiomyocytes

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Purified human-Induced Pluripotent Stem Cell-derived cardiomyocytes (hIPSC-CMs: iCells®, Cellular Dynamics) form electrically coupled, spontaneously beating cell monolayers (syncytia) upon plating and continuous culture. In this study, we investigate the pharmacology of the hIPSC-CM adenosine (ADO) response via the use of selective A1 and A2A-receptor antagonists and equilibrative nucleoside transporter type-1 (ENT1) inhibitors. After a 14-day culture in 96-well impedance electrode-plates (ACEA Biosciences), the spontaneous iCell beating rates are highly stable from well to well, with a mean beat period of 1.7 sec at 37°C, and a coefficient of variation (CV) < 1% . Extracellularly applied ADO inhibits the spontaneous beating rate of iCells with an IC_{50} = 2.2 µM, and a saturation value of near 50% inhibition. ADO also destabilizes the beat period (CV EC_{50} = 1.2 μM) with a maximum CV of 20% at saturating ADO concentrations. The ADO IC_{50} is shifted to 248 µM upon pre-incubation of the iCells with 1 µM of an A1-selective antagonist DPCPX (A1 K_i=3.9 nM), but not the A2A-selective antagonist ZM241385 (A2A K_i=1nM). The duration of the depressive effect of ADO on beating rate is transient, even at super-saturating ADO concentrations, and depends on the well ADO concentration. The ENT1 inhibitor Draflazine (ENT1 pK_i=9.5) pre-applied at 0.1 µM increases ADO sensitivity by 4-fold one hour after ADO application. Moreover, at any given ADO concentration, the duration of beating rate depression can be prolonged in a concentration-dependent manner by ENT1 inhibitors, with the prolongation-response curve nearly matching the ENT1 IC_{50}s for tested ENT1 inhibitors (Draflazine, NBTI, Dilazep, and Dipyridamole). These findings suggest that the pool of ADO in the test well is cleared by uptake via endogenous ENT1 and support the role of equilibrative ADO uptake in limiting the duration of action of ADO on A1 receptors in cardiomyocytes. We are extending our investigation to the downstream target of A1 receptor activity, and the putative role of $IK_{ACH/ADO}$ as the underlying mechanism of beat rate slowing and irregularity. In conclusion, the hIPSC-CM model provides a useful model for investigation of ADO pharmacology of the heart.

J.P. Imredy: None. **B. Balasubramanian:** None. **E.V. Lis:** None. **A. Lagrutta:** None. **F. Sannajust:** None.

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A Unique Jervell Lange-Nielsen Syndrome Mutation Modeled in Induced Pluripotent Stem Cell Derived Cardiomyocytes

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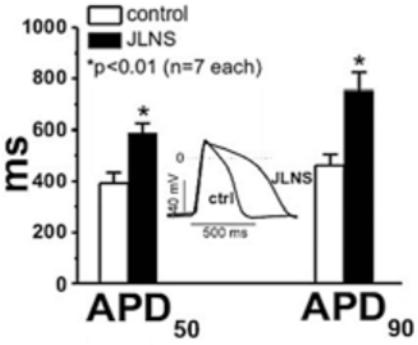
Introduction: Current screening for mutations in human disease is turning increasingly to next-generation methods that map short reads to a reference sequence. We report here an unusual variant that was undetected by next generation sequencing in a patient diagnosed with Jervell Lange-Nielsen syndrome (JLNS) and initial results in an induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) model.
Methods and Results: A diagnosis of JLNS was made in a middle-aged woman with congenital deafness and QT intervals as long as 800 msec. However, next-generation sequencing found only a heterozygous *KCNQ1* mutation, R518X. Convinced by the clinical

phenotype that a second causative variant was highly likely, we used Sanger sequencing of PCR KCNQ1 amplicons to identify a 36-basepair poly-adenine tract, encoding 12 lysines, inserted within the coding sequence at the 5' end of exon 15. Electrophysiological studies in patient-specific IPSC-CMs revealed marked prolongation of ventricular-like action potentials (Figure).
Conclusion: Long inserts of the type we identified here have not been previously reported in the long QT syndromes. We speculate that next generation-based short reads containing this variant could not be mapped to a reference sequence and thus this type of variant will be missed by next-generation analysis unless bioinformatics filters are specifically modified to include this possibility. Validation of this long QT syndrome iPSC-CM model provides a human cell based platform for drug discovery and mechanistic studies to further our understanding of disease pathogenesis.



K. Bersell: None. **T. Yang:** None. **D. Roden:** None.

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Epigenetic Signatures Contribute to the Superior Endothelial Cell Identity in Human Induced Pluripotent Stem Cells Derived From Endothelial Cells
Mingtao Zhao, Shijun Hu, Rajini Srinivasan, Fereshteh Jahaniani, Ning-Yi Shao, David Knowles, Won Hee Lee, Tomek Swigut, Joanna Wysocka, Michael Snyder, Joseph Wu, Stanford Cardiovascular Inst, Stanford, CA

Human induced pluripotent stem cells (iPSCs) can be derived from multiple types of somatic cells by transient overexpression of four Yamanaka factors. Epigenetic memory of the tissue of origin is seen in early passage iPSCs, which may interfere the directed differentiation towards target lineages in disease modeling and drug discovery. Here we derived human iPSC from three types of somatic cells of the same individuals: fibroblast (FB-iPSCs), endothelial cells (EC-iPSCs) and cardiac progenitor cells (CPC-iPSCs). We then differentiated them into endothelial cells by using sequential administration of Activin, BMP4, bFGF and vEGF. EC-iPSCs show higher EC differentiation propensity and EC-specific markers (PECAM1 and NOS3) gene expression in early passage iPSCs than FB-iPSCs and CPC-iPSCs. In vivo, EC-iPSC-ECs display significantly greater revascularization capacity than those of FB-iPSCs and CPC-iPSCs when transplanted to the hindlimb ischemic mice. In addition, transplanted EC-iPSC-ECs were recovered with a higher percentage of CD31+ population and higher EC-specific markers (PECAM1, KDR and ICAM) gene expression by using single cell qPCR. In vitro, EC-iPSC-ECs exhibit better endothelial cell character maintenance along with extensive culturing and passaging. Several chromatin signatures, including H3K27ac, H3K4me1 and p300 were found highly enriched in ECs and EC-iPSCs, but not in human embryonic stem cells (ESCs). Gene ontology analysis indicates that the differentially enriched regions are primarily associated with angiogenesis and vascular development, reflecting the residual epigenetic signatures in EC-iPSCs. Finally EC-specific enhancer markers undergo dynamic changes during the process of EC fate commitment and differentiation, though the majority of them sustain conserved pattern in EC-iPSCs, CPC-iPSCs and FB-iPSCs. In conclusion, these results highlight that the residual epigenetic signatures of tissue of origin may affect lineage differentiation propensity in early-passage human iPSCs.
M. Zhao: None. **S. Hu:** None. **R. Srinivasan:** None. **F. Jahaniani:** None. **N. Shao:** None. **D. Knowles:** None. **W. Lee:** None. **T. Swigut:** None. **J. Wysocka:** None. **M. Snyder:** None. **J. Wu:** None.

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Embryonic Extracellular Matrix and Cell Fate Determination
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Background- The determination of cell fate during development is governed by intrinsic factors, but also by interaction with the milieu in which they reside. The extracellular matrix (ECM) from rapidly developing tissue should form a rich signaling environment for cellular proliferation and differentiation.
Hypothesis- Murine embryonic ECM can be prepared by detergent decellularization that is morphologically preserved, biocompatible for cell culture, and at E13.5 substantial enough to permit vascular catheterization and recellularization by perfusion.
Methods and Results- To test the contribution of embryonic extracellular matrix (ECM) to the determination of cell fate, we undertook isolation of ECM from developing murine embryos. Triton X-100 and SDS detergent decellularization were used to isolate ECM from E10.5 and E13.5 embryos. Acellularity was confirmed by pico-green DNA assay (98.7 % ± 0.95 of DNA removed compared to control), as well as the lack of visible nuclei by H & E and DAPI histology. The matrix scaffolds were washed thoroughly with PBS and culture media to return them to a biocompatible state. Murine embryonic stem cells (mESC) modified to express EGFP were cultured on the exterior or the interior of the ECM scaffolds. mESCs seeded on the exterior of the E10.5 scaffolds or perfused through the E13.5 umbilical vasculature were highly adherent and proliferative during the 17 day culture period as evidenced by fluorescent microscopy. Perfused mESCs exhibited engrafted in the heart, liver, and vascular conduit E13.5 matrix 2 days post-infusion. Histology confirmed the attachment and morphologic alteration of the cultured cells on the exterior of the E10.5 ECM and presence of the perfused cells in the E13.5 embryo matrix interior.
Conclusion- Biocompatible, acellular morphologically preserved embryonic ECM can be extracted from E10.5 and E13.5 murine embryos. By E13.5 the structural integrity of the acellular matrix can sustain vascular perfusion for delivery of mESCs to internal organoid structures. These ECM preparations support the proliferation and maintenance of mESCs externally and internally.
S.M. Kren: None. **C.S. Chapman:** None. **A. Loza:** None. **D.J. Garry:** None. **M.G. Garry:** None.

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Simultaneous Analysis of the Contraction Kinetics, Force Generation, Calcium Handling, and Membrane Potential of Single Stem Cell Derived Cardiomyocytes
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Stem cell-derived cardiomyocytes are increasingly used for studying cardiac physiology and pathophysiology in vitro. However, current techniques for functional assessment of CMs are optimized for mature myocardial cells and are not well suited for the study of stem cell derived CMs that lack distinct cellular edges and well-developed sarcomeres.
We hypothesized that statistical analysis of movies of contracting CMs can be used to comprehensively assess changes in cellular morphology and compute myocyte contractile kinetics and force generation concurrently with calcium cycling and electrophysiology.
We have performed pairwise statistical similarity measures between all frames in a video of human stem cell-derived cardiomyocytes contracting on a flexible substrate. We then generated a similarity matrix to assess change in cell morphology over time and compute the contraction kinetics. In adult cardiomyocytes this approach produced contraction curves highly similar to those generated by traditional edge detection technology with a Pearson's correlation coefficient of 0.98. We further calculated the contractile force generated during myocyte contraction with a biomechanical model and validated this approach using conventional traction force microscopy. Both methods yielded highly similar results with a mean difference in peak force of 0.01μN (95% limits of agreement -0.05μN to 0.03μN). Addition of the calcium indicator Fluo-4 allowed for the detection of subtle changes in contractility and calcium cycling in response to isoproterenol and verapamil. Likewise, the addition of the membrane potential dye FluoVolt allowed for the assessment of the cardiotoxicity of dofetilide. We show that characterization of contractility and action potential together detected more variation in peak amplitude than action potential alone after application of dofetilide (29.4% vs. 1.3%; p=0.0084).
We have developed a highly versatile novel methodology for the simultaneous quantitative analysis of contraction kinetics, force generation, calcium cycling, and electrophysiology in human cardiomyocytes. This novel approach has the potential for broad application in the study of cardiac disease modeling, drug discovery and drug cardiotoxicity screening.
J.D. Kijlstra: None. **D. Hu:** None. **N. Mittal:** None. **P. van der Meer:** None. **A. Garakani:** 7. Ownership Interest; Modest; Reify Corp. **I. Domian:** None.

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A Novel Anti-cell Death Mechanism by Targeting the Biosynthesis of Oligosaccharide in ER
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The pathological expression of many of the deadly human diseases such as myocardial infarction, renal failure etc. is cell death. In cancer research, myriads of ways have been found to kill a cell. However, we are still craving for an applicable mechanism to prevent cell death. The majority of the current research is to directly target the apoptosis pathway. While some compounds have been developed, none of them are pharmaceutically important likely due to the inherent toxic nature of the target. Here, we report a novel mechanism to block the cell death pathway by activating the cells' own protective programs coded for stress. Components of the unfolded protein response (UPS) in ER have been implicated in the protection of ischemia/reperfusion heart in recent research. By targeting the biosynthesis of oligosaccharide, which activated UPS as indicated by the expression of the ER chaperones Grp94 and Grp78, we were able to completely block the Hsp90 inhibitor induced apoptotic cell death as well as the oxidative stress (as exemplified by sodium nitroprusside, SNP) induced necrotic cell death in multiple cell lines. Caspase-3 activation and PARP cleavage were nearly completely inhibited. While awaiting further exploration, the biosynthesis of the oligosaccharide could turn out to be an applicable target for acute conditions such as cardiac injury because of its relatively safe nature.
X. Luo: None. **W. Zhou:** None. **L. Wei:** None. **L. Wei:** None. **F. Zhang:** None.

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MiR-4260 Promotes the Proliferation, Migration, and Tube Formation of Human Umbilical Vein Endothelial Cells
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MicroRNAs (miRNAs, miRs), endogenous small non-coding RNA, have been shown to act as essential regulators in angiogenesis which plays important roles in improving blood flow and cardiac function following myocardial infarction. The current study investigated the potential of miR-4260 in endothelial cell function and angiogenesis using human umbilical vein endothelial cells (HUVEC). Our data demonstrated that overexpression of miR-4260 was associated with increased proliferation and migration of HUVEC using EdU incorporation assay (17.25%±1.31 vs 25.78%±1.24 in nc-mimics vs miR-4260 mimics, respectively) and wound healing assay, respectively. While downregulation of miR-4260 inhibited the proliferation (17.90%±1.37 vs 10.66%±1.41 in nc-inhibitor vs miR-4260 inhibitor, respectively) and migration of HUVEC. Furthermore, we found that miR-4260 mimics increased (129.75±3.68 vs 147±3.13 in nc-mimics vs miR-4260 mimics, respectively), while miR-4260 inhibitor decreased the tube formation of HUVECs in vitro (123.25±2.17 vs 92±4.45 in nc-inhibitor vs miR-4260 inhibitor expression, respectively). Our data indicate that miR-4260 contributes to the proliferation, migration and tube formation of endothelial cells, and might be essential regulators for angiogenesis. Further study is needed to investigate the underlying mechanism that mediates the role of miR-4260 in angiogenesis by identifying its putative downstream target genes.
Q. Sun: None. **D. Lv:** None. **Q. Zhou:** None. **Y. Bei:** None. **J. Xiao:** None.

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Cardiac-specific Overactivation of the Mechanistic Target of Rapamycin Complex 1 Induces Metabolic, Structural and Functional Remodeling
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The heart remodels metabolically and structurally before it fails. Metabolically, the heart increases its reliance on carbohydrates for energy provision. Structurally, the heart hypertrophies to sustain increased hemodynamic stress. There is evidence suggesting that the activation of the mechanistic Target Of Rapamycin Complex 1 (mTORC1) pathway is closely tied to glucose uptake by the heart to drive the metabolic and structural remodeling. We have previously shown that with insulin stimulation or increases in workload, the glycolytic intermediate glucose 6-phosphate (G6P) is required to activate mTORC1. Sustained mTORC1 activation leads, in turn, to ER stress and contractile dysfunction. Studies by others in the kidney have shown that mTORC1 activation upregulates glucose transporter 1 (Glut1) expression and glucose uptake. We therefore test the hypothesis that chronic mTORC1 overactivation results in G6P accumulation, and precedes structural and functional remodeling in the heart. We developed mice with inducible, cardiac-specific deficiency of the protein

tuberin (TSC2), a member of the tuberous sclerosis complex, the principal inhibitor of mTORC1. Intracellular G6P concentrations were measured enzymatically. Immunoblotting was performed on protein markers to confirm activation of mTORC1 downstream targets and of the unfolded protein response. Histologic analysis were performed to assess structural changes. Serial echocardiograms were performed to evaluate cardiac function. The results indicate that chronic mTORC1 activation through inducible, cardiac-specific deletion of TSC2 is accompanied by G6P accumulation and metabolic remodeling. Metabolic remodeling precedes structural and functional remodeling. We suggest that in the heart, sustained mTORC1 activation is a key driver of metabolic and structural remodeling.
G. Davogustto: None. **R. Salazar:** None. **H. Vasquez:** None. **H. Taegtmeyer:** None.

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MiR-4261 Controls Endothelial Cells Angiogenesis
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Myocardial infarction (MI) is among major causes of morbidity and mortality associated with coronary artery disease. Angiogenesis improves tissue perfusion and cardiac repair after MI. Therefore, angiogenesis is considered to be a novel therapeutic way for ischemic heart diseases. MicroRNAs (miRNAs, miRs) have been reported to play important roles in regulating post-ischemic neovascularization. The current study aims at investigating the role of miR-4261 in angiogenesis. We found that miR-4261 mimics increased, while miR-4261 inhibitors decreased the proliferation of human umbilical vein endothelial cells (HUVEC) using EdU incorporation assay (17.25%±1.31% vs 30.91%±0.92% in nc-mimics vs mir-4261-mimics, 17.91%±1.36% vs 8.51%±0.82% in nc-inhibitor vs mir-4261-inhibitor, respectively) and CCK-8 assays (0.84±0.04 vs 1.38±0.04 in nc-mimics vs mir-4261-mimics, 0.80±0.02 vs 0.72±0.01 in nc-inhibitor vs mir-4261-inhibitor, respectively). The wound healing assay showed that miR-4261 mimic transfection resulted in a significant increase in the migration of HUVEC compared to that of the negative controls while miR-4261 inhibition had the opposite effects. Tube formation assays showed that HUVEC transfected with miR-4261 mimics increased the number of tubes formed (57.25±2.56 vs 81.5±2.53 in nc-mimics vs mir-4261-mimics, respectively), while miR-4261 inhibitor-transfected cells had the opposite effect (56.55±0.45 vs 41.38±0.52 in nc-inhibitor vs mir-4261-inhibitor, respectively). These results indicate that miR-4261 play an important role in regulating angiogenesis. However, it remains unknown which target gene mediated the effects of miR-4261. Thus, it will be of great interest to further investigate the molecular mechanisms of miR-4261 in the proliferation, migration, and tube formation of HUVEC in vitro. MiR-4261 could be a potential therapeutic target to enhance angiogenesis.
Q. Zhou: None. **D. Lv:** None. **Q. Sun:** None. **P. Chen:** None. **Y. Bei:** None. **J. Xiao:** None.

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Vasonatrin Peptide Inhibits Endoplasmic Reticulum Stress and Attenuates Myocardial Ischemia/reperfusion Injury in Diabetic Rats
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Aims: Diabetes mellitus (DM) increases morbidity/mortality of ischemic heart disease. Although the ability of the natriuretic peptides to modulate cardiac function and cell proliferation has been recognized, their effects on myocardial ischemia/reperfusion (MI/R) injury is still unclear. This study was to investigate the effects of the artificial synthetic natriuretic peptide — vasonatrin peptide (VNP) on MI/R injury in diabetic rats, and underlying mechanisms.
Method: The high-fat diet-fed streptozotocin induced diabetic rats were subjected to MI/R (30 min/4 h) and VNP treatment (100 μg/kg, i.v., 10 min before R). *In vitro* study was performed using H9c2 cardiomyocytes subjected to hypoxia/reoxygenation (H/R, 3 h/6 h) and incubated with or without VNP (10^{-8} mol/L).
Result: The diabetic state aggravated MI/R injury and showed more severe myocardial functional impairment than normal state. VNP treatment (100 μg/kg, i.v., 10 min before R) significantly improved ± LV dP/dt_{max} and LVSP, and decreased infarct size, apoptosis index, caspase-3 activity, serum CK and LDH levels (n=8, $P<0.05$). Moreover, VNP inhibited endoplasmic reticulum (ER) stress by suppressing GRP78 and CHOP, and consequently increased Akt and ERK1/2 expression and phosphorylation levels (n=3, $P<0.05$). These effects were mimicked by 8-Br-cGMP (1 mg/kg, i.p., 20 min before R), a cGMP analogue, whereas inhibited by KT-5823 (0.5 mg/kg, i.p.), the selective inhibitor of PKG ($P<0.05$). Pretreated DM rats with TUDCA (50 mg/kg, i.p.), an inhibitor of ER stress, couldn't further promote the VNP's cardioprotective effect. Additionally, gene knockdown of PKG1α with siRNA blunted VNP's inhibition of ER stress and apoptosis, while overexpression of PKG1α resulted in significant decreased ER stress and apoptosis in H/R H9c2 cardiomyocytes (n=6, $P<0.05$).
Conclusion: We demonstrated that VNP protects diabetic heart against MI/R injury by inhibiting ER stress via cGMP-PKG signaling pathway.
H. Zhang: None. **W. Xing:** None. **F. Gao:** None.

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Increased Myocardial Sensitivity to Ischemic Injury in an Animal Model of Posttraumatic Stress Disorder
Boyd R Rorabaugh, Albert Bui, Sarah L Seeley, Anna Krivenko, Eric D Eisenmann, Megan E Fry, Joseph D Lawson, Lauren E Stoner, Phillip R Zoladz, Ohio Northern Univ, Ada, OH

Background: Posttraumatic stress disorder (PTSD) is a psychological disorder characterized by the formation of traumatic memories following exposure to a life threatening event. In addition to psychological manifestations, PTSD promotes atherosclerosis and increases the incidence of myocardial infarction. However, it is unknown whether the effects of PTSD are limited to increasing the incidence of myocardial infarction or if PTSD also increases infarct severity. Therefore, we used an animal model of PTSD to determine whether posttraumatic stress influences infarct size and postischemic recovery of cardiac contractile function.
Methods: Rats were subjected to a well-established animal model of PTSD that is based on predator exposure and psychosocial stress (Zoladz et al., Stress 11:259-281). Rats subjected to this model exhibit many PTSD-like characteristics including the formation of traumatic memories, increased anxiety, increased startle reflex, hypertension, and alterations in the hypothalamic-pituitary adrenal axis. Male rats (7 weeks of age) were either subjected to psychosocial stress (n = 9) or continuously housed in their home cages (n = 8) for 31 days. Hearts were subsequently isolated and subjected to 20 minutes of ischemia and 2 hours reperfusion on a Langendorff isolated heart system.
Results: Stressed rats exhibited significantly elevated corticosterone concentrations and anxiety-like behavior in the elevated plus maze. Infarct sizes were significantly larger in hearts from stressed rats (44.7 ± 1.7 % of area at risk) compared to nonstressed rats (31.0 ± 5.4 % of area at risk). Consistent with increased myocardial injury, postischemic recovery of rate pressure product (stressed = 16,922 ± 1,554

mmHg*bpm; nonstressed = 26,407 ± 2,977 mmHg*bpm) and +dP/dT (stressed = 1,901 ± 189 mmHg/sec; nonstressed =3,259 ± 498 mmHg/sec) were significantly attenuated in hearts from stressed rats. Furthermore, postischemic end diastolic pressure was significantly elevated in hearts from stressed (57 ± 6 mmHg) compared to nonstressed (32 ± 7 mmHg) rats.
Conclusion: This animal model suggests that PTSD may make the myocardium more sensitive to ischemic injury through a mechanism that is independent from its ability to promote atherosclerosis.
B.R. Rorabaugh: None. **A. Bui:** None. **S.L. Seeley:** None. **A. Krivenko:** None. **E.D. Eisenmann:** None. **M.E. Fry:** None. **J.D. Lawson:** None. **L.E. Stoner:** None. **P.R. Zoladz:** None.

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Pluripotent Stem Cell Microrna-294 Induces Cardiomyocyte Proliferation and Augments Cardiac Function after Myocardial Infarction
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Rationale: Embryonic heart is characteristic of rapidly dividing cardiomyocytes that give rise to sufficient numbers required to build a working myocardium. In contrast, cardiomyocytes retain some proliferative capacity in the neonates but lose most of it in adulthood. Embryonic stem cell cycle (ESCC) miRs are a class of microRNAs regulating the unique cell cycle of ESCs and their characteristic pluripotency. Nevertheless, expression of miR-294, a member of the ESCC miRs is lost during developmental transitions from the ESCs to mature cells. Effect of miR-294 to induce cardiac proliferation and heart function has not been previously studied.
Objective: To determine whether miR-294 drives cardiomyocyte cell cycle reentry leading to augmentation of cardiac function after myocardial infarction.
Methods and Results: miR expression analysis in the heart during development revealed elevated levels of miR-294 in the prenatal stages while the expression was lost in the neonates and adults as confirmed by qRT-PCR. Neonatal ventricular cardiomyocytes (NRVMs) were treated with miR-294 mimic to determine the effect on proliferation and cell cycle. Elevated mRNA levels of cyclins A2, E1, CDK2 together with c-myc, E2F1 and E2F3 was observed in NRVMs treated with 25nM mimic for miR-294. Additionally, miR-294 treated NRVMs showed in AKT phosphorylation along with enhanced protein levels of cyclin D1 and E2F1. Increased expression of p-histone 3, Ki67 and Aurora B kinase (G2/M) was confirmed by immunocytochemistry in NRVMs after miR-294 treatment compared to control cells. Administration of miR-294 in mice subjected to myocardial infarction demonstrated augmentation of cardiac function in mice receiving miR-294 8 weeks after injury. Increase myocyte proliferation was observed in the heart after miR-294 treatment as analyzed by BrdU uptake, p-Histone 3 and Aurora B expression by immunostaining. Concurrently, a decrease in infarct size along with decreased apoptosis was observed in the miR-294 hearts compared to the control.
Conclusion: Ectopic expression of miR-294 recapitulates embryonic signaling and enhances cardiomyocyte ability to proliferate and reenter the cell cycle leading to augmented cardiac function in mice after myocardial infarction.
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Mitochondrial Dysfunction Mediated Myocardial Stunning Following Resuscitation From Cardiac Arrest
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Rationale: Severe myocardial contractile dysfunction following resuscitation from cardiac arrest (CA) is a major contributor to CA mortality. The pathophysiology and etiology of this dysfunction is not known and there are no pharmacological therapies known to improve outcomes. Previously, we demonstrated that Dynamin related protein 1 (Drp1) is activated and recruited to the mitochondria during CA and that the Drp1 inhibitor Mdivi-1 improves post CA survival.
Objective: To determine the effects of CA length on myocardial and mitochondrial function. We also sought to determine the effects of Mdivi-1 on post CA outcomes.
Methods and Results: Asystolic cardiac arrest (CA) was induced in mice by IV injection of 0.08 mg/g KCL. CPR begun at 4, 8, 12, and 16 minutes post-cardiac arrest had rates of return of spontaneous circulation (ROSC) of 100%(12/12), 93%(14/15), 71%(10/14), and 44% (4/9) and 2-hour survival of 100%(12/12), 67%(10/15), 50%(7/14), and 11%(1/9). Transthoracic echocardiography 15 min post-resuscitation demonstrated percent fractional shortening of 36±4% (Sham,n=6), 30±4% (4 minCA,n=11), 24±5% (8minCA,n=10), 15±2% (12minCA,n=12). In surviving animals, myocardial dysfunction persisted for 2 hours post-resuscitation, but slowly recovered to baseline by 72 hours. No evidence of myocardial necrosis, inflammation, or apoptosis was noted following resuscitation. Progressive increases in mitochondrial derived reactive oxygen species (ROS) during CA was observed by MitoSOX red myocardial tissue staining. Mitochondria isolated from 12 min CA hearts demonstrated decreased substrate coupled and uncoupled respiration. Mdivi-1, a mitochondrial inhibitor of division (fission), improved survival and neurological scores in mice following an 8 min cardiac arrest compared to controls.
Conclusions: Severe, time dependent myocardial stunning (contractile dysfunction in the absence of irreversible injury) was observed following asystolic cardiac arrest. This myocardial stunning was associated with mitochondrial injury and improved by an inhibitor of Drp1. Strategies targeting ischemia/reperfusion-induced changes in mitochondrial dynamics hold promise for improving myocardial function and survival following cardiac arrest.
W.W. Sharp: 2. Research Grant; Significant; NIH K08 K08 HL103901-01A1, NIH R03 HL110826-01A1. **L. Piao:** None. **Y. Fang:** None. **D.G. Beiser:** None. **J.K. Liao:** None. **S.L. Archer:** None.

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C1q-TNF Related Protein-9, a Novel Cardioprotective Cardiokine, Requires Proteolytic Cleavage to Generate a Biologically Active Globular Domain Isoform

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The discovery of the cardiac secretomes, known as "cardiokines", significantly enhanced appreciation of the local microenvironment's influence upon disease development. We previously reported cardiomyocytes produce cardioprotective adiponectin (APN). However, cardiac APN levels are several orders of magnitude below adipocytes, thereby making APN an unlikely physiologically relevant cardiokine. C1qTNF-related proteins (CTRP1-15) is a newly descovered family of APN paralogs. However, whether any of the CTRP members may function as a "true" cardiokine remains unknown. Here we demonstrated that several CTRPs (CTRP1,4,7,9,13) were expressed in the heart at levels significantly greater than APN. Most notably, the mRNA level of CTRP9 exceeded APN by >100-fold in cardiac tissue. Adult cardiac cells were isolated and cultured in vitro. Conditioned medium was collected 1-8 hours after culture and CTRP9 protein levels were determined by ELISA. A significant increase of CTRP9 in conditioned medium was detected as early as 1 hour in culture, steadily accumulating thereafter. These results indicate CTRP9 is secreted by cardiac cell at levels that may function as a cardiokine. Addional experiemnt unexpectedly demonstarted that CTRP9 circulates in the plasma primarily in the globular domain isoform (gCTRP9), opposing from APN which circulates as full length multimers. Recombinant full length CTRP9 (fCTRP9) was cleaved when incubated with cardiac tissue extracts, generating gCTRP9, a process inhibited by protease inhibitor cocktail. To gain more insight into the biological significance of gCTRP9 production, isolated adult cardiomyocytes were treated with gCTRP9 or fCTRP9. gCTRP9 rapidly activates cardiac survival kinases, including AMPK, Akt, and eNOS. However, fCTRP9-mediated kinase activation is much less potent, and significantly delayed. Kinase activation by fCTRP9, but not gCTRP9, is inhibited by protease inhibitor cocktail. These results demonstrate for the first time CTRP9 is a novel cardiokine undergoing proteolytic cleavage to generate cardioprotective isoform, gCTRP9. Enhancing cardiac CTRP9 production and/or its proteolytic post-translational modification are of therapeutic potential, attenuating cardiac injury.

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Characterization of Secretome From C-kit+ Cells Derived From Neonate and Adult Heart Patients

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Background: Despite the regenerative benefit reported in recent human clinical trials (SCIPIO trial) using human cardiac stem cells (CSCs) (identified by ckit+CD45-/lin-), low retention and differentiation of transplanted hCSCs into cardiomyocytes are insufficient to explain the myocardial regeneration. This discrepancy has led to the paracrine hypothesis of stem cell action that indicates communication between newly transplanted stem cells and reparative native cells, essentially jumpstarting the repair process. Methods: We derived CSCs from the biopsies of right atrial appendage (RAA) of neonatal human subjects (nCSCs, age < 2 months) and from adult human subjects (aCSCs, age >50 years). CSCs were grown upto 90% confluency and incubated with basal medium (Ham's/F12) for 72 hours. Conditioned medium (CM) was collected and further analyzed. Results: The expression levels of various key cytokines were analyzed using ELISA and data showed nCSCs secrete higher levels of the angiogenic cytokines like SDF1, VEGF-A, ANG, SCF, PDGB, and FGF2 compared to over 20 other cytokines we examined as compared to aCSCs. CM derived from nCSCs showed more angiogeneic potential as compared to CM derived from aCSCs as analyzed by HuVEC tube assay formation. We also did a comparative analysis of CM from nCSCs and aCSCs using mass spectrometry. Molecular distribution of lead proteins revealed that the yhCSCs has more angiogenic, anti-inflammatory and proliferative proteins while ahCSCs have more of apoptotic proteins Conclusion: These data provide preliminary evidence that there is a difference in the cytokine secretion by the nCSCs as compared to aCSCs and implicates highly expressed cytokines as the key to myocardial recovery.

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Activation of the Homeostatic Intracellular Response Leads to Increased Myocardial Mitochondrial Turnover in Patients Undergoing Cardiac Surgery

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BACKGROUND: Previously we showed that the homeostatic intracellular repair response (HIR2) is activated in the hearts of patients undergoing cardiac surgery. Autophagy is a principal component of this beneficial response that clears fragile mitochondria and protein aggregates. Moreover, we have previously shown that mitochondrial elimination through autophagy (mitophagy) is a key element in ischemic preconditioning. Thus, an important mechanism of cardioprotection appears to involve the upregulation of autophagy which facilitates the clearance of vulnerable mitochondria to limit I/R injury. We hypothesized that this protective action leads to turnover of the existing mitochondrial population in the heart during the resolution of I/R injury. The purpose of this study was to examine if the mechanism of HIR2 extends to remodeling the existing mitochondrial population of the heart.

STUDY DESIGN: Autophagy and mitochondrial turnover were assessed in 10 patients undergoing coronary artery bypass or valve surgery requiring cardiopulmonary bypass. Biopsies of the right atrial appendage obtained before initiation and after weaning from cardiopulmonary bypass were processed to yield whole tissue lysates and mitochondria-enriched heavy membrane fractions. Samples were analyzed for autophagy by immunoblotting for LC3, Beclin-1, ATG5-12, and p62. Mitochondrial turnover was assessed by monitoring Tom70, Cox4, Drp1, p62 and Parkin in tissue lysates and heavy membrane fractions.

RESULTS: Heart surgery was associated with a robust increase in autophagy indicated by depletion of LC3, Beclin-1, ATG5-12 and p62, as well as the mitophagy and fission regulator Drp1. Parkin increased in the mitochondrial fraction after bypass. Surprisingly, post-bypass tissue lysates showed a marked increase in mitochondrial markers Tom70 and Cox4, suggesting mitochondrial biogenesis.

CONCLUSIONS: These findings provide evidence for the first time in humans that coordinated mitophagy and biogenesis are part of the homeostatic response to I/R, pointing to the importance of studying this aspect of HIR2. Strategies designed to amplify HIR2 during cardiac stress may represent an entirely new approach

to myocardial protection in patients undergoing heart surgery.

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NEDD8 Ultimate Buster-1 Long (NUB1L) Protein Regulates Atypical Neddylation and Protects Against Myocardial Ischemia-reperfusion Injury

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Neddylation is a ubiquitination-like pathway that covalently conjugates NEDD8 to target proteins and involves in diverse cellular processes. Under stress conditions, NEDD8 forms a chain or mixes with ubiquitin to modify protein substrates in NEDD8 conjugating enzymes-independent manner (atypical neddylation). The functional consequence of atypical neddylation remains unexplored in any cell types including cardiomyocytes. Here we report that increased neddylated proteins were observed in desmin-related cardiomyopathic (DRC) mouse hearts, mouse hearts subjected to myocardial ischemia-reperfusion (I/R) and human failing hearts. In cultured cardiomyocytes, multiple cellular stresses induced atypical neddylation, which was attenuated by NUB1L overexpression but exaggerated by loss of NUB1L, revealing NUB1L as a negative regulator of atypical neddylation. Activation of atypical neddylation by forced expression of NEDD8 accumulated a proteasome surrogate substrate GFPu, while suppression of atypical neddylation by NUB1L overexpression enhanced the degradation of GFPu and a DRC-linking misfolded protein. NUB1L is necessary and sufficient to protect cardiomyocytes against proteotoxic stress-induced cell injury. In vivo, cardiac-specific overexpression of NUB1L (NUB1L-O/E) in mice dose-dependently reduced neddylated proteins and facilitated the degradation of the proteasome surrogate substrate. NUB1L-O/E mice displayed no discernible cardiac structural and functional abnormality at baseline, but exihibted reduced apoptotic cardiomyocytes, limited infarct sizes and preserved cardiac function in response to I/R. We therefore conclude that NUB1L suppresses atypical neddylation, enhances proteasome proteolytic function and protects against myocardial I/R injury. Targeting atypical neddylation could be a novel therapeutic strategy to treat cardiac ischemic cardiomyopathy.

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An Exercise-induced MicroRNA Pathway That Protects Against Apoptosis and Pathological Cardiac Dysfunction

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Background: Exercise induces physiological cardiac growth and protects against adverse cardiac remodeling. microRNAs (miRNA) are important regulators in cardiovascular pathology and disease. Less is known about miRNA roles in the cardiac effects of exercise, and we investigated their roles in exercise-induced cardiac growth.

Methods and Results: Based on cardiac miRNA profiling in two exercise models (swimming, running) and functional screening in neonatal rat cardiomyocytes (NRCMs), miR-222 was selected for further study. miR-222 was upregulated ~2-fold in both exercise models, pathological hypertrophic and failing hearts ($p<0.05$). We found that ERK, JNK and p38 signal pathways are involved in FBS increasing miR-222 in NRCMs. miR-222 increased NRCM proliferation and size ($p<0.01$) and reduced NRCM apoptosis induced by serum starvation with or without doxorubicin. Four potential miR-222 targets (*p27*, *Hipk1*, *Hipk2*, and *Hmbox-1*) were identified and confirmed as direct targets. siRNA knockdown (KD) of p27 or Hipk2 inhibited NRCM apoptosis.

To investigate miR-222's cardiac role *in vivo*, we made transgenic mice with cardiac-specific, inducible (TgI-miR-222) or constitutive (TgC-miR-222) expression of miR-222. RT-PCR showed that miR-222 is increased ~6-fold in TgI-miR-222 hearts after induction and ~10-fold in TgC-miR-222 hearts. Surprisingly, both transgenic lines have normal heart size and function, and the 4 miR-222 targets are unaffected at baseline. After ischemia-reperfusion injury (IRI), TgI-miR-222 had no difference in initial infarct size or dysfunction but were protected against adverse remodeling over the next six weeks with better function ($p<0.01$), less cardiac fibrosis (68%, $p<0.05$), and reduced CM apoptosis (300%, $p<0.05$). Two weeks after TAC, TgC-miR-222 had less increase in cardiac mass (HW/TL, $p<0.05$), better cardiac function (Δ32%, $p<0.01$), reduced expression of markers of fibrosis (*Col1a1*, *Col3a1*, *Mmp2,* $p<0.05$) and apoptosis (*Caspase 1, 3, 8,* $p<0.05$). Four months after TAC, TgC-miR-222 had improved survival ($p<0.05$) compared to wide type controls.

Conclusion: Cardiac miR-222 is upregulated by exercise and protects against adverse remodeling and cardiac dysfunction after IRI or TAC.

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***Klf5* and *Ppara* Expression is Increased at the Early Stage and Reduced at the Late Stage of Myocardial Ischemia/Reperfusion in Mice**

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Inhibition of cardiac fatty acid oxidation (FAO) is considered beneficial after ischemia reperfusion (I/R). Krüppel-like factors (KLF) have an important role in metabolism. In a model of cardiac energetic deficiency (LPS-treated mice), *in silico* promoter analysis and whole genome array analysis indicated cardiac KLF5 as important regulator of *Ppara*. Gene expression analysis in human ventricular myocytes (AC16) treated with Ad-Klf5 followed by ChIP analysis confirmed that KLF5 is a positive transcriptional regulator of *Ppara*. Mice with cardiomyocyte-specific *Klf5* ablation *(αMHC-Klf5$^{-/-}$)* that we made had reduced cardiac *Ppara* expression and other FAO-related genes. The role of PPARα activation in I/R injury is unclear as both beneficial and detrimental effects have been reported. We aimed to gain insight in the effects of KLF5 and PPARα on acute I/R injury.

To mimic I/R *in vitro*, AC16 cells were subjected to hypoxia (9.5 h) followed by short (1h) or prolonged (14h) normoxia. *Ppara* expression was initially (1h) increased ($p<0.05$) and then (14 h) decreased ($p<0.05$). Klf5 expression pattern showed a trend of similar changes. Infarction was performed by 30 min of

left coronary artery occlusion followed by 2 or 24 hours of reperfusion in wild type mice. I/R resulted in a trend for an initial increase in *Klf5* (2-fold, p=0.05), *Ppara* (3-fold, p=0.08), and *Pdk4* (8-fold, p<0.01) mRNA at 2h post-surgery. *Klf5* (2-fold; p<0.01) and *Ppara* (9-fold; p<0.05) expression were decreased 24h post-surgery. Consistent with *Ppara* changes, there was a 2-fold decrease of *Cpt1* (p<0.01) and *Vlcad* (p<0.01) and a trend for decreased *Aox* (2-fold), *Lcad* (2-fold), and *Pdk4* (6-fold) compared to sham. Echocardiography indicated normal cardiac function after 24h post-surgery. Despite reduced FAO, *αMHC-Klf5*$^{-/-}$ mice subjected to I/R had a marked increase in mortality; 40% (4 of 10) of *αMHC-Klf5*$^{-/-}$ mice died within the first 24h of reperfusion while no mortality was observed in wild type mice that underwent I/R.
In conclusion, I/R is associated with an increase in *Klf5* and *Ppara* in the first hours of reperfusion followed by a decrease in *Klf5* and *Ppara*. Increased mortality for *αMHC-Klf5*$^{-/-}$ mice with I/R injury suggests that the initial increase may be an adaptive response that is critical for survival.
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AdipoRon, the First Orally Active Adiponectin Receptor Activator, Attenuates Post-ischemic Myocardial Apoptosis via AMPK Activation and Oxidative Stress Reduction
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Adiponectin (APN) is a cardioprotective molecule. Its reduction in diabetes exacerbates myocardial ischemia/reperfusion (MI/R) injury. Although APN administration in animals attenuates MI/R injury, multiple factors limit its clinical application. The current study investigated whether AdipoRon, the first orally active molecule that binds APN receptors, may protect the heart against MI/R injury, and if so, to delineate the involved mechanisms. Wild type (WT) or gene manipulated mice were treated with vehicle or AdipoRon (50 mg/kg, 10 minutes prior to MI) and subjected to MI/R (30 minutes/3-24 hours). Oral administration of AdipoRon to WT mice significantly improved cardiac function (P<0.01) and reduced infarct size (P<0.01). At cellular level, AdipoRon attenuated post-ischemic cardiomyocyte apoptosis determined by DNA ladder formation, TUNEL staining, and caspase-3 activation (P<0.01). MI/R-induced apoptotic cell death was significantly enhanced in APN deficient mice (APNKO), and AdipoRon attenuated MI/R injury to the same degree observed in WT mice, indicating the pathological exageration caused by APN deficience can be rescured by a small molecule APN receptor activator. Cardiomyocyte-specific inhibition of AMPK (AMPK-DN), the most significant molecule mediating APN's metabolic regulatory function, also increased post-ischemic apoptosis. Interestingly, AdipoRon's anti-apoptotic action was partially inhibited, but not lost in AMPK-DN mice, indicating the anti-apoptotic effect of AdipoRon cannot be completely atributed to AMPK activation. AdipoRon significantly attenuated post-ischemic oxidative stress in an AMPK-independent fashion, as evidenced by reduced NADPH oxidase expression and superoxide production in both WT and AMPK-DN mice. Collectively, these results demonstrate for the first time that AdipoRon, an orally active APN receptor activator, effectively attenuated post-ischemic cardiac injury, supporting APN receptor agonists as a promising novel therapeutic approach treating cardiovascular complications caused by obesity-related disorders such as type 2 diabetes.
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A Decrease in Mitochondrial, but Not Cytosolic, Iron Protects Against Cardiac Ischemia-reperfusion Damage Through a Reduction in Ros
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Introduction: Iron is essential for the activity of several cellular proteins, but excess free iron can cause cellular damage through production of reactive oxygen species (ROS). Iron accumulation in mitochondria, the major site of cellular iron homeostasis, leads to cardiomyopathy. However, it is not known whether a reduction in baseline mitochondrial iron (as opposed to iron in other cellular compartments) can protect against ischemia-reperfusion (I/R) injury in the heart. We hypothesized that since mitochondria are the major site of iron homeostasis and that mitochondrial iron can lead to oxidative damage, a reduction in mitochondrial iron at baseline would be sufficient to protect against I/R injury.
Results: Transgenic (TG) mice with cardiomyocyte-specific overexpression of the mitochondrial iron export protein ATP-binding cassette (ABC)-B8 had significantly lower mitochondrial iron in the heart than nontransgenic (NTG) littermates at baseline, but their cardiac function and the expression of key antioxidant systems were similar to NTG littermates. In response to I/R, TG mice displayed significantly less apoptosis and lipid peroxidation products and better preserved cardiac function than NTG littermates, suggesting that a reduction in mitochondrial iron protects against I/R injury. To confirm these results, we next took a pharmacological approach to assess the effects of a reduction in mitochondrial vs cytosolic iron on the response to I/R using 2,2'-bipyridyl (BPD, a mitochondria-accessible iron chelator) and deferoxamine (DFO, an iron chelator that can only reduce cytosolic iron). Mice pretreated with BPD but not DFO are protected against I/R injury. In addition, BPD but not DFO treatment in rat cardiomyoblast H9C2 cells significantly lowered chelatable mitochondrial iron and protected against H2O2 induced cell death. These results suggest that a reduction in baseline mitochondrial, but not cytosolic, iron is sufficient to protect against I/R injury.
Conclusions: Our findings demonstrate that selective reduction in mitochondrial iron is protective in I/R injury. Thus, targeting mitochondrial iron with selective iron chelators may provide a novel approach for treatment of ischemic heart disease.
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Heparin Derived Oligosaccharide Inhibits Vascular Intimal Hyperplasia in Balloon Injured Carotid Artery and the Mechanism Involved
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To determine the effects of heparin-derived oligosaccharides (HDOs) on vascular intimal hyperplasia (IH) in balloon-injured carotid artery and the mechanism involved. The animal model was established by rubbing the endothelia within the common carotid artery (CCA) of male rabbits along with a high-cholesterol diet. The arterial IH was testified by histopathological changes of the CCA. Serum lipids were detected using automated biochemical analysis; Expression of mRNAs corresponding to vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), vascular

cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), scavenger receptor class B type I (SR-BI) and ATP-binding cassette transporter A1 (ABCA-1) were analyzed using reverse transcription polymerase chain reaction assays. Expressions of VEGF, VCAM-1, MCP-1, SR-BI and ABCA-1 proteins were detected by western blotting. Enzyme-linked immunosorbent assays were used to quantify expression levels of VEGF and bFGF. The results implied that administration of HDO significantly inhibited common carotid artery histopathology and restenosis that was induced by balloon injury. Treatment with HDO also significantly decreased mRNA and protein expression levels of VEGF, bFGF, VCAM-1, MCP-1, and SR-BI in the arterial wall, however ABCA-1 expression levels were elevated. HDO treatment led to a reduction in various serum lipids (total cholesterol, triglycerides, high-density and low-density lipoproteins). We concluded that, in a rabbit model, HDO can ameliorate IH and the mechanisms might involved VEGF, bFGF, VCAM-1, MCP-1, SR-BI and ABCA-1.
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PP2A/HSP70 Dynamically Regulates HDAC2 Phosphorylation and its Activity in Cardiac Hypertrophy
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Rationale: Cardiac hypertrophy is an adaptation for increased hemodynamic demands by underlying diseases and histone deacetylase (HDAC) 2 phosphorylation and following its activation are closely associated with those of process. Recently, we have demonstrated that the acetylation of HDAC2 K75 could induce S394 phosphorylation; however, specific mechanism for inter-modifications regulation in the single protein largely remains unclear. Objective: We aimed to delineate the regulation mechanism how K75 acetylation modulates S394 phosphorylation and which phosphatase regulates HDAC2 phosphorylation in the cardiac hypertrophy. Methods and Results: We found that the catalytic subunit of protein phosphatase (PP) 2A bound to HDAC2 in the H9c2 cell. PP2A kept HDAC2 unphosphorylated in the absence of hypertrophic stresses. Hypertrophic stresses-induced activation of pCAF, however, induced HDAC2 K75 acetylation, which then allowed PP2A to dissociate from HDAC2. This dissociation leads CK2α1 to bind to and phosphorylate HDAC2. Hypertrophic stresses induced HSP70 which then preferentially bound to phosphorylated HDAC2 rather than to unphosphorylated one. Forced expression of PP2CA not only reduced enzyme activity of HDAC2 but inhibited hypertrophic response in the cardiomyocytes. On the other hand, HSP70 bound to phosphorylated HDAC2 in order to mask the phosphor-HDAC2 from PP2CA. HDAC2 phosphorylation and following activation of intrinsic activity were regulated by binding of PP2CA to HDAC2. PP2A functioned as a negative regulator for cardiac hypertrophy by targeting of HDAC2 S394 phosphorylation. Conclusion: Taken together, HDAC2 forms a complex with PP2A in the absence of hypertrophic stresses and remains inactivated. HDAC2 acetylation results in both detachment of PP2A and binding of CK2α1 for phosphorylation, which is maintained by the association with HSP70 during development of cardiac hypertrophy.
G. Eom: None. **S. Yoon:** None. **H. Min:** None. **Y. Nam:** None. **D. Kwon:** None. **H. Kook:** None.

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The Scaffolding Protein Enh1 is Essential for the Protein Kinase C or Protein Kinase D-dependent Activation of the Transcription Factor Camp-response Element Binding Protein in Cardiomyocytes
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PDZ-LIM proteins form a family of the scaffolding protein essential for both embryonic and post-natal development. ENH1 (PDLIM5) is a PDZ-LIM protein, composed of an N-terminal PDZ domain and 3 LIM domains at the C-terminal end. The enh gene encodes for several splice variants that have opposite functions. ENH1 promotes the cardiomyocytes hypertrophy whereas ENH splice variants lacking LIM domains prevents it. At the molecular level, ENH1 interacts with Protein kinase C (PKC) and Protein Kinase D1 (PKD1) both kinases playing a pivotal role in the pathological remodeling of the heart. In addition, the binding of ENH1's LIM domains to PKC is sufficient to activate the kinase without any stimulation. However, the downstream events of the ENH1-PKC/PKD1 complex remain unknown. PKC and PKD1 are known to phosphorylate the transcription factor cAMP-response element binding protein (CREB) in cardiomyocytes. We therefore hypothesized that ENH1 could be a play a role in the PKC/PKD1-dependent activation of CREB. We first found that ENH1 expression is necessary to induce the phosphorylation of CREB at Ser133 in neonatal rat ventricular cardiomyocytes. On the contrary, the overexpression of ENH3, a LIM-less cardiac-specific splice variant, inhibited the phosphorylation of CREB-Ser133. Concomitantly, both real-time qPCR and promoter assay showed that the overexpression of ENH1 enhanced but ENH3 prevented the transcriptional activation of a CREB-target gene, the immediately early gene c-fos. Finally, we found that ENH1 regulated the translocation of phosphorylated CREB to the nucleus. Taken all together, our results suggest that ENH1 plays an essential role in CREB's activation and dependent transcription in cardiomyocytes. At the opposite, ENH3 splice variant inhibited the CREB activity in cardiomyocytes. In conclusion, our work describes a new molecular mechanism involving ENH splice variants with opposing functions.
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Epidermal Growth Factor Receptor (EGFR) Family Dimeric Partners Switch During Pathological Stress in microRNA-7 Transgenic Mice
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miRNA-7 is known to target epidermal growth factors receptor 1(EGFR1) in cancer cells. EGFR family members (EGFR 1, 2, 3 and 4) are known to form homo- and/or hetero-dimers to mediate downstream signals . Our previous study in human heart failure (Naga Prasad etal., JBC, 2009) showed that EGFR2 (ERBB2) was targeted by miRNA-7. Based on this study, we developed transgenic (Tg) mice with cardiomyocyte-specific overexpression of miRNA-7. miRNA-7 Tg mice have age dependent deterioration in cardiac dysfunction and is associated with cardiac dilation as measured by echocardiography (3 months - 60% FS, 6 month -52% FS &12 months - 24%FS) and yet, they survive well for more than a year. To investigate whether pathological stress would accelerate the deterioration in cardiac function, miRNA-7 Tg mice were subjected to transverse aortic constriction (TAC) for two weeks. In contrast to the wild type littermates which undergo hypertrophic response following TAC, miRNA-7 Tg mice have accelerated cardiac dysfunction and dilation within two weeks. Biochemical analysis interestingly showed differential switching of dimeric EGFR partners in hearts of the miRNA-7 Tg mice compared to their sham controls. Our presentation will discuss the differential downstream signaling induced by changing of EGFR

dimeric partners induced by pathological stress like TAC in the wildtype and miRNA-7 Tg mice.
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Hyperglycemia-induced Prorenin Receptor Abundance on Plasma Membrane of Kidney Collecting Duct Cells: is a Novel Mechanism to Explain Diabetic Nephropathy?
Venkateswara R Gogulamudi, Danielle Y Arita, Camille RT Bourgeois, Ryosuke Satou, Minolfa C Prieto, Tulane Univ Health Sciences Ctr, Sch of Med, New Orleans, LA

Activation of the renin-angiotensin system (RAS) leads to nephropathy during Diabetes Mellitus (DM). Prorenin levels are increased in the circulation of DM patients and predict microvascular damage. In streptozotocin (STZ)-induced type 1 DM rats, the collecting duct is the major source of prorenin in the kidney. The prorenin receptor (PRR), a new RAS component, elicits intracellular signals linked to fibrosis upon binding of prorenin at the cell plasma membrane (PM). We propose that PRR may contribute to the development of tubulointerstitial fibrosis by being locally activated by prorenin. To support this concept, we tested the hypothesis that hyperglycemia increases membrane bound PRR to collecting duct cells. To address this hypothesis, we used T1DM rats (N=10) induced with a single STZ injection (ip; 200ng/kg x 7 d) as well as mouse collecting duct M-1 cells, treated at different time intervals 0, 5 min, 1, 12, and 24 h with normal glucose (NG; 5mM glucose+20mM mannitol) and high glucose (HG; 25mM) to assess PRR trafficking alterations induced by glucose. After 7-days induction, STZ-rats showed plasma glucose as 428±13 vs.138±9 gr/dl and plasma insulin as 0.07±0.02 vs 2.42 ng/ml; compared to controls. By immunofluorescence (IF) in rat kidney sections, PRR was mainly localized on the apical aspect of collecting duct cells in STZ-rats, while in controls most of PRR signal was observed intracellularly. Although PRR mRNA levels did not differ in the collecting ducts between groups; its protein levels were augmented in STZ-rats. Importantly, compared with NG-treated cells, PRR protein levels (membrane bound) were significantly higher in PM extracts from M-1 cells treated with HG, and a maximum peak was observed at 1 h. Interestingly, in de-convoluted IF images of M-1 cells, PRR was localized mainly in the perinuclear areas during NG conditions; however, in HG-treated cells, PRR was found toward the cell surface. These data suggest that hyperglycemia induces PRR trafficking alterations of PRR. The present results suggest that hyperglycemia-induced PRR abundance on the PM in the collecting duct might be a novel mechanism underlying the development of diabetic nephropathy, particularly tissue fibrosis in DM.
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V. Gogulamudi: None. **D. Arita:** None. **C. Bourgeois:** None. **R. Satou:** None. **M. Prieto:** None.

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Hyperinsulinmia Promotes Cardiac Dysfunction via Upregulation of Phosphodiesterase in Heart
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Accumulating evidence suggests that hyperinsulinemia contributes to heart dysfunction. Here we show that insulin signaling is responsible for high fat diet (HFD) feeding-induced expression of phosphodiesterase 4D (PDE4D) in the myocardium of mice. The increased expression of PDE4D, in concert with reduced phosphorylation of phospholamban (PLB), promotes systolic and diastolic heart dysfunction. We revealed that insulin-mediated induction of PDE4D was dependent on β2AR-mediaed β-arrestin2-ERK pathway, which is transactivated in a GRK2-dependent fashion. Deletion of β2AR gene significantly attenuated insulin-induced phosphorylation of extracellular signal-regulated kinase (ERK) and Akt, as well as the upregulation of PDE4D expression, which prevented the heart dysfunction. β-arrestin2 KO mice did not display increased PDE4D expression and did not develop systolic or diastolic dysfunction following HFD. In brief, these data indicate that chronic hyperinsulinimia leads to heart dysfunction by increasing PDE4D expression via β2AR-GRK2-β-arrestin2 -ERK pathway, which suggests that β2AR signaling could be an attractive therapeutic target for preserving or improving cardiac function in subjects with insulin resistance.
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Cardiac Beta-arrestin2 Promotes Contractility by Enhancing SERCA2a SUMOylation and Activity
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Heart failure (HF) is the number one killer disease in the western world and new and innovative treatments are needed. Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)-2a is a crucial, for contractile function, calcium-handling protein expressed in the mammalian myocardium and its downregulation is one of the molecular hallmarks of chronic HF. Its activation is part of the signaling mechanism by which the β_1-adrenergic receptors (ARs) increase cardiac contractility. Agonist-bound β_1ARs however, like most G protein-coupled receptors (GPCRs), undergo functional desensitization/internalization due to the actions of βarrestin1 or -2. These two arrestins are universal GPCR adapter proteins, mediating G protein-independent signaling via multi-protein scaffolding, and, among the cellular processes they can regulate, is protein SUMO (small ubiquitin-like modifier)-ylation, which generally increases protein stability/levels. In the heart, βarrestin1 appears detrimental, whereas βarrestin2 beneficial, for structure and function post-myocardial infarction (MI). Post-MI βarrestin1 knockout mice also display elevated SERCA2a activity and better contractility than post-MI wild type mice. In addition, reduced cardiac SERCA2a SUMOylation is known to underlie its downregulation in HF, decreasing cardiac contractility. Thus, in the present study, we sought to investigate a potential involvement of cardiac β_1AR-activated βarrestins in regulation of SERCA2a SUMOylation and activity. By studying individual βarrestin knockout heart extracts, we found that βarrestin2, but not βarrestin1, interacts with SERCA2a in the mouse heart in vivo, promoting the latter`s SUMOylation and activity. This interaction is direct, as indicated by pull-down and FRET experiments. Finally, via in vitro studies in the cardiomyocyte-like cell line H9c2, we found that this interaction is both β_1AR-, and beta-agonist-specific, and leads to increased Ubc9-dependent SERCA2a SUMOylation, which, in turn, acutely enhances SERCA2a activity in H9c2 cells. These results suggest that βarrestin2, presumed to also decrease cardiac function by desensitizing βARs, may actually (directly) enhance cardiac contractility, thereby opposing βarrestin1 in that regard.
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Sam68 Impedes the Recovery of Arterial Injury by Augmenting Inflammatory Response

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Background: The role of Src-associated in mitosis 68 kDa (Sam68) in cardiovascular biology has not been studied. A recent report suggests that Sam68 suppresses TNF-α-induced NF-κB activation. Since NF-κB plays a critical role in vascular inflammation and injury via generation of inflammatory cytokines and recruitment of inflammatory cells, we sought to dissect the mechanism by which Sam68 regulates NF-κB signaling and its functional significance during vascular injury.
Methods & Results: The endothelial denudation injury was induced in the carotid arteries of Sam68-/- and WT mice. Sam68-/- mice displayed an accelerated re-endothelialization and attenuated neointima hyperplasia, which was associated with a reduced number of macrophages and lowered expression of pro-inflammatory cytokines (i.e., TNF-α, IL-1β and IL-6) in the injured vessels. Importantly, the ameliorated vascular remodeling was recapitulated in WT mice after transplantation of bone marrow (BM) from Sam68-/- mice, suggesting beneficial role was attributed largely to BM-derived inflammatory cells. In cultured Raw264.7 macrophages, knockdown of Sam68 resulted in a significant reduction in the TNF-α-induced expression of TNF-α, IL-1β, and IL-6 and in the level of nuclear phospho-p65, indicating an attenuated NF-κB activation. These results were confirmed in peritoneal macrophages and macrophages differentiated from BM mononuclear cells of Sam68-/- and WT mice. To identify molecular mechanisms, Raw264.7 cells were treated with TNF-α and Vehicle, followed by Sam68 co-immunoprecipitation and mass-spec identification of Sam68-interacting proteins. Specifically, TNF-α treatment results in altered interactions of Sam68 with Filamin A (FLNA), a cytoskeleton protein known to be involved in NF-κB activation. Loss- and gain-of-function of Sam68 and FLNA suggest their mutual dependence in NF-κB activation and pro-inflammatory cytokine expression, and Sam68 is required for TRAF2-FLNA interaction.
Conclusions: Our results for the first time suggest that Sam68 promotes pro-inflammatory response in injured arteries and impedes recovery, and this effect is attributed, in part, to the exaggerated NF-κB activity via Sam68-FLNA interaction and consequent TRAF2 stabilization.

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The E2F1 Transcription Factor Suppresses Cardiac Fibrosis via Downregulating Syndecan-4 Expression and Smad2/3 Phosphorylation

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Rationale: The E2F1 transcription factor is best known as a cell-cycle regulator; recent reports suggest its important role in cardiovascular system.
Objective: To determine whether E2F1 regulates cardiac fibrosis.
Methods and Results: Following Angiotensin II (Ang II) administration, E2F1-null (E2F1-KO) mice displayed a more severe cardiac fibrosis and higher levels of cardiac phospho-Smad2 (pSmad2) and pSmad3 than WT mice. Consistently, levels of pSmad2 and pSmad3 were elevated in E2F1-KO fibroblasts, which was associated with a significantly increased expression of collagen I (Col I) and α-smooth muscle actin (α-SMA) and interestingly, cell surface proteoglycan syndecan-4 (Sdc4). Knockdown of Sdc4 significantly attenuated the elevation in Smad2/3 phosphorylation and Col I and α-SMA expression in E2F1-KO fibroblasts. Remarkably, either chemical inhibition of Smad2/3 signaling or morpholino-mediated knockdown of Sdc4 expression abrogated the difference in the degree of Ang II-induced cardiac fibrosis between WT and E2F1-KO animals.
Conclusions: E2F1 suppresses Ang II-induced cardiac fibrosis by downregulating Sdc4 expression and Sdc4-mediated Smad 2/3 activation.

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Structure and Function Relationship of Phospholipid Transfer Protein in Lipid Transfer Activity Revealed by Electron Microscopy

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Human phospholipid transfer protein (PLTP) mediates the transfer of lipids among atheroprotective high-density lipoproteins (HDL) and atherogenic low-density lipoproteins (LDL) by an unknown mechanism. Delineating this mechanism would be an important step toward the understanding and regulation of PLTP for treating cardiovascular diseases, hypoalphalipoproteinemia and hyperalphalipoproteinemia. Using electron microscopy, negative-staining, and single-particle image processing, we discovered that PLTP penetrates each class of HDL, LDL and liposome independently, and also bridges a ternary complex with one of its distal end-domains penetrating into HDL and another distal domain interacting with LDL. These new insights into PLTP interaction with lipoproteins and liposomes provide a molecular basis for analyzing PLTP-dependent lipid transfer between lipoprotein particles.

M. Zhang: None.

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Network-based Approaches to Identify Novel Regulators of Heart Failure

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Heart failure is a highly heterogeneous disorder characterized by the interactions of multiple environmental and genetic factors. While reductionistic approaches have made significant inroads into characterizing the pathophysiology of the syndrome, they are unable to properly dissect the complex interactions between sets of genes and pathways which result in the emergent phenotypes . Systems genetics offers a means by which these interactions may be identified and explored. We have developed a resource, the Hybrid Mouse Diversity Panel (HMDP) to perform systems-level analyses in mice. Nine week old female mice from 93 unique inbred lines of the HMDP were give 30 ug/g/day of isoproterenol through an abdominally implanted Alzet micropump. After three weeks, mice were sacrificed along with age-matched controls. A portion of the left ventricle was arrayed on an Illumina Mouse Ref 8.0 platform.
Maximal Information Component Analysis was used to construct gene networks, and a module of 41 genes was identified which shows strong correlation to a number of important phenotypic traits, including heart weight and cardiac fibrosis. This module contains a

number of genes of interest, including Lgals3, a diagnostic marker for heart failure. Through the use of structural equation modeling, we identified several key genes within the module for further analysis, the most important of which is the metalloprotease Adamts2. We have performed a series of in vitro analyses demonstrating the important role of Adamts2 in this module using neonatal rat ventricular myocytes. Knockout of Adamts2 results in an amelioration of the hypertrophic response to catecholamine stimulation as well as a reduction of hypertrophic markers such as Nppa and Nppb. Furthermore, we observe that other genes in the module no longer respond to catecholamine stimulation after knockdown of Adamts2.

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Inhibition of MEF2 by Atenolol Modifies the Transcriptome During Cardiac Hypertrophy to Improve Heart Function

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Cardiac hypertrophy is a growth response of the adult heart that often results from increased mechanical loading due to high blood pressure and myocardial damage caused by ischemic heart disease. The β1 selective β-adrenergic antagonist, atenolol, is historically one of the most frequently prescribed of all medicines used to preserve heart function following myocardial damage. Transcription factor, myocyte enhancer factor 2 (MEF2), is required for early cardiac development and has also been implicated in cardiac hypertrophy. The effect of β-blockade on the gene program that is activated following cardiac hypertrophy as heart failure progresses is not fully understood. Here we show that treatment with atenolol leads to in vivo changes within the murine heart to reverse cardiac hypertrophy, and this is accompanied by repression of MEF2 activity.

Cardiac hypertrophy was simulated using transverse aortic constriction (TAC) for four weeks in a transgenic MEF2-lacZ mouse model, followed by six weeks of vehicle (AT-) or atenolol treatment (AT+). Physiological responses in cardiac function, fibrosis, and cardiomyocyte size in TAC+AT+ mice were consistent with cardiac hypertrophy and demonstrated increased MEF2 activity compared to control (TAC-AT-). Atenolol treatment resulted in an overall improvement in cardiac function of TAC+ mice, showing a decrease in cardiomyocyte size, fibrosis and MEF2 activity. RNA was isolated from the left ventricle of the heart to determine changes in mRNA and lncRNA using RNA-seq. Atenolol reversed the expression of a specific subset of genes and lncRNA that become upregulated by TAC which corresponded to gene ontology terms related to metabolism and the immune system.

Together, these data demonstrate that atenolol treatment during cardiac hypertrophy inhibits MEF2 activity and concomitantly leads to improved cardiac function. Thus, inhibition of MEF2 may be necessary to improve or reverse the effects of long-term heart failure.

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Epicardial-to-Myocyte Hypertrophic Signaling is Mediated by Epicardial/Mesenchymal Status and Protein Arginine Methyltransferase-1

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The epithelial-to-mesenchymal transition (EMT) is an important cellular mechanism in a diverse range of biological processes such as development, wound healing, cancer metastasis, and organ fibrosis. Epicardial cells are mesothelial cells lining the outer surface of the heart that are an important progenitor population and a source of growth factors during development. Epicardial cells undergo EMT and invade the myocardium, differentiating into cardiac fibroblasts and coronary smooth muscle cells. Of note, resident fibroblasts of epicardial origin are a major cellular mediator of cardiac fibrosis. Our recent work has established an important function for the protein arginine methyltransferase PRMT1 in EMT. We hypothesized that PRMT1 is required for EMT in a mouse epicardial cell line (MEC-1). We found that PRMT1 is required for a subset of the EMT marker changes in epicardial EMT, such as upregulation of vimentin, fibronectin, and slug, as well as loss of E-cadherin. Furthermore, PRMT1 knockdown reduced MEC-1 migration and invasion, suggesting that PRMT1 is critical for epicardial progenitor function. Epicardial cells secrete a variety of signaling factors that affect cardiomyocyte proliferation and structure. This is a critical component of heart development, and may also affect heart diseases such as hypertophic heart failure. Therefore we assessed whether EMT and PRMT1 affect the paracrine functions of epicardial cells. Co-culture of MEC-1 cells with rat neonatal cardiomyocytes caused cardiomyocyte hypertrophy, and this was enhanced when MEC-1 cells were pre-treated with TGF-β to induce EMT. Interestingly, MEC-1 cells treated with PRMT1 siRNA also induced cardiomyocyte hypertrophy, but TGF-β pre-treatment of these cells did not enhance this effect. In conclusion, epicardial EMT is largely dependent on PRMT1. Epicardial cells also promote cardiomyocyte hypertrophy, which is enhanced in epicardial cells that have undergone PRMT1-mediated EMT towards a mesenchymal fate. These studies establish a role for protein methylation in the EMT process, and could lead to novel treatments for heart failure and other diseases affected by EMT.

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A microRNA Targets the Epigenetic Reader BRD4 to Suppress Transcription Elongation in the Heart

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BRD4 is a member of the BET family of proteins, which contain tandem reader domains (bromodomains) that bind to acetylated histone tails within chromatin. We recently demonstrated that JQ1, a small molecule that prevents BRD4-chromatin interaction, potently blocks pathological cardiac hypertrophy and improves cardiac function in pre-clinical models. BRD4 functions as a nodal regulator of the transcriptional program for cardiac hypertrophy by recruiting P-TEFb to gene regulatory elements, resulting in phosphorylation of RNA polymerase II (Pol II), and subsequent transcription elongation. Here, we describe a signal-dependent mechanism for regulation of BRD4 in the heart. BRD4 protein expression is dramatically upregulated in cardiomyocytes exposed to cues for pathological, but not physiological, hypertrophy. In unstimulated cardiomyocytes, BRD4 protein expression is maintained at low levels by a microRNA, which directly targets the

3' UTR of the BRD4 transcript, thereby preventing translation of this acetyl-lysine reader. In response to signals for hypertrophy, expression of the microRNA is down regulated through a histone deacetylase (HDAC)-dependent mechanism, resulting in robust induction of BRD4 protein synthesis. Whole genome analyses establish a crucial role for the microRNA in the control of Pol II elongation in the heart via BRD4 down regulation. These findings define a novel chromatin signaling axis that can be targeted at multiple levels (epigenetic reader, microRNA or HDAC) for the treatment of heart failure.

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The First Mouse Mutants of a Novel Epigenetic Modifier, Rearranged L-Myc Fusion, Display Defects in Heart Development

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Rearranged L-Myc Fusion, Rlf, was recently identified as a novel epigenetic modifier from a mouse N-ethyl-N-nitrosourea mutagenesis screen. The mice used in this study carry a multi-copy green fluorescent protein (GFP) transgene linked to an erythroid specific α-globin promoter that is sensitive to epigenetic silencing. Three independent mouse lines with mutations in Rlf were each found to have a decrease in GFP expression, suggesting Rlf acts an epigenetic modifier. Our study is the first to reveal a role for Rlf in epigenetics.
Preliminary phenotyping has found loss of Rlf results in perinatal lethality. Late gestation homozygous null Rlf mutants were found to weigh significantly less than their heterozygous or wild type littermates. Histological analysis of mid gestation embryos has identified a potential heart defect in homozygous mutants. Rlf mutants display a thin compact layer, an overabundance of trabeculae and a fenestrated interventricular septum. The Rlf mutant phenotype is reminiscent of ventricular noncompaction defects observed in humans, such as left ventricular noncompaction cardiomyopathy.
RNA-seq analysis of mid gestation Rlf wild type and null hearts, prior to the observation of a cardiac defect, was undertaken to determine which pathways may be regulated by Rlf in the heart. More genes were observed to be significantly down-regulated in Rlf mutant hearts compared to wild types. Pathway analysis of differentially expressed genes showed genes involved in cell-cell adhesion, cell signalling, glycosylation and the Notch pathway were dysregulated. These findings indicate that Rlf may play a critical role in cardiac development.
Whole genome bisulphite sequencing of different embryonic tissue/stages has shown loss of Rlf results in an increase in methylation at a large number of distinct loci across the genome. Many of which were found to overlap sites reported to be putative regulatory elements, and histone marks associated with active or poised regulatory elements in the heart.
Our data suggest Rlf plays a key role in regulating gene expression pathways during heart development. These are the first mouse mutants available to study how Rlf functions as an epigenetic modifier and the phenotypic consequences of Rlf inactivation.

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Impaired TNFα Signaling Deregulates Basal Nrf2-Antioxidant System in Myocardium

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Antagonizing TNF-α and its signaling is proven to be a successful therapeutic strategy in various disease processes. Although blocking TNF-α signaling attenuated the disease activity, the overarching clinical contraindications remains to be a greater concern and reasons for the adverse effects are largely unknown. TNF-α induces ROS and as ROS is pivotal for activation of antioxidant transcription factor, Nrf2, we posit that any impairment in TNF-α signaling could affect Nrf2-dependent basal redox homeostasis. HL-1 cardiomyocytes, TNFR1/2 double knockout mice (DKO) that shows hampered TNF-α signaling were used as experimental models. Electron Paramagnetic Resonance Spectroscopy (EPR) measurements revealed that as low as 2 ng TNF-α/ml, ROS was induced significantly (p10ng/ml in HL-1 cells. TNF-α (2-5 ng /ml, for 2 h) evoked robust ($p<0.05$, n=3) nuclear translocation of Nrf2 and increased nuclear binding (Trans AM DNA binding analysis) along with significant ($p<0.05$) induction in trans-activation of Nrf2 targets. Additionally, this was associated with 2-fold increase ($p<0.05$) in intracellular glutathione (GSH) that is associated with significant cell death only at higher (>10 - 50 ng/ml) and later time points (12 h and 24 h). These results suggest that TNF-α mediated ROS induction activates Nrf2 dependent antioxidant system in cardiomyocytes. In vivo experiments with TNFR1/2 DKO demonstrates that the expression of Nrf2-regulated genes were significantly downregulated indicating a weakened antioxidant system. Also, the acute exercise stress (AES) induced Nrf2 trans-activation observed in WT mice was significantly blunted in TNFR1/2 DKO suggesting a need for TNF signaling in stress induced Nrf2 activation. These results support the concept that complete and/or sustained blockade of TNF signaling may result in compromised Nrf2-dependent antioxidant defense in the myocardium. This further necessitates that during chronic anti-TNF-α treatment, a finely tuned and maintenance of specific threshold of TNF-α signaling is essential to avoid oxidant stress based complications in cardiovascular system.

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Reciprocal Cardiac Chromatin Regulation by Ctcf and Hmgb2

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Chromatin remodeling plays an essential role in cardiac gene reprogramming under pathological conditions. However, we currently lack a detailed understanding of how this process is structurally coordinated across the genome. Previous RNA and proteomic analyses of an in vivo model of heart failure performed by our group identified the chromatin structural proteins CTCF and high mobility group protein B2 (HMGB2) as regulators of heart disease. CTCF is an essential factor in the maintenance of global chromatin organization through the facilitation of long-range interactions and chromatin looping. HMGB2 is a non-nucleosomal structural protein that packages nucleosomes into higher order structures.

An unbiased genomic analysis of ~100 strains of mice treated with isoproterenol (ISO) followed by transcriptome and phenotype analyses revealed a general down-regulation of CTCF at the mRNA level across the different strains after ISO treatment, whereas the HMGB2 response was strongly influenced by common genetic variation (i.e. between strains). ChIP-seq data, ChIP-reChIP-PCR experiments (detection of concomitant presence of two proteins at a given locus) and immunofluorescence showed CTCF and HMGB2 bind the same regions of the genome, yet they do not physically co-localize in myocyte nuclei. Loss of HMGB2, which induces hypertrophy in myocytes, causes de-condensation of chromatin, as directly measured by micrococcal nuclease digestion; CTCF knockdown has no effect on global chromatin accessibility by this assay. Examination of other chromatin structural proteins like histone H1 suggests this interaction between CTCF and HMGB2 is specific. Lastly, we showed that CTCF and HMGB2 regulate each other's expression as well as the transcription of cardiac hypertrophy genes and ribosomal RNA. Our data show that basal HMGB2 (but not CTCF) expression correlates with cardiac mass. The response of HMGB2 levels to heart failure stimuli is highly influenced by genetics, whereas the decrease in CTCF following (ISO) is unaffected by genetic variation. These findings reveal molecular mechanisms of chromatin compaction in cardiac cells, linking them with genetic variability, and revealing a reciprocal relationship between HMGB2 and CTCF in cardiac hypertrophy.

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Association Between Serum Ghrelin Levels and Coronary Artery Disease: a Meta-analysis

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Background: Ghrelin (GH) is a gastrointestinal endocrine peptide regulating multiple biological processes including adipogenesis, glucose metabolism, cell differentiation and proliferation. Recent studies demonstrated that GH inhibits pro-atherogenic changes in vessel wall via inhibition of nuclear factor - B activity, a transcriptional factor mediating production pro-inflammatory cytokines and adhesion molecule expression in the endothelium.

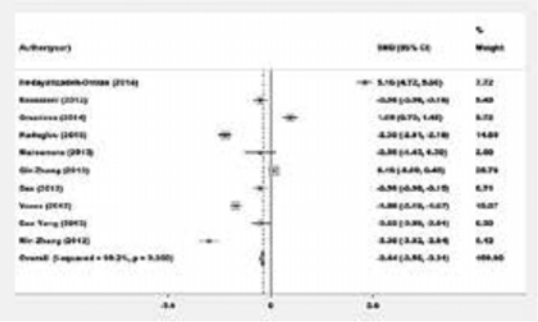
The aim of the current study is to conduct a meta-analysis to evaluate the relationship between serum GH levels and coronary artery disease (CAD).

Methods: We searched MEDLINE, CINHAL and COCHRANE databases for studies reporting serum GH levels in the CAD and non CAD study population. We included case controls, cohort and cross-sectional studies. We calculated the weighted standardized mean difference (SMD) in serum GH levels between the CAD and control groups.

Results: Our search strategy yielded 285 articles and we included 10 studies enrolling 1855 participants. The median age of the CAD group was 62 yrs. (IQR 60 - 63) compared to 61 yrs. (IQR 58 - 65) in the control group. The median body mass index in the CAD group was 28 kg/m2 (IQR 27.9 - 28) compared to 27 kg/m2 (IQR 26 - 27) in the control group. The unweighted median serum GH levels in the CAD group were 0.66 ng/ml (IQR 0.3 - 1.6) compared to 0.76 ng/ml (IQR 0.38 - 4.9) in the control group.

The SMD of GH level was -0.44 (95% CI -0.56,-0.31) p<0.001 comparing those in the CAD group and control group.

Conclusion: Serum GH levels are significantly and inversely associated with CAD. Current findings warrant the need to further investigate the role of GH in the pathogenesis of CAD.



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Cell-based Therapies for Heart Failure: Changes in Cytokine Expression From Mesenchymal Stem Cells and Induced Pluripotent Stem Cell Derived Cardiomyocytes

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Mesenchymal stem cells (MSCs) use paracrine signaling to modulate the cellular microenvironment via expression of cytokines, chemokines, and adhesion molecules to aid and promote endogenous repair. Induced pluripotent stem cell derived cardiomyocyte (iPSC-CMs) and mesenchymal stem cells (MSCs) together may play a synergistic role in changing the microenvironment milieu to allow for endogenous cellular repair through paracrine signaling. Cytokine expression is involved in the progression of heart failure (HF). Using a rat model of HF, cell based therapies with a fibroblast embedded patch only, iPSC-CM patch, and MSCs via tail vein (IV) or intracardiac injections (IC) to the left ventricle (LV) were administered, and RNA was subsequently isolated, and real-time polymerase chain reaction (PCR) was performed for analysis of gene expression.

Evaluation of gene expression revealed significant increases in the expression of connexin 43 with iPSC-CMs ($p<0.05$). Expression of MMP9 was decreased with MSCs alone ($p<0.05$) but with the use of the patch with both cell types, its levels were significantly increased ($p<0.05$). Myosin heavy chain was seen to

increase significantly with increasing cell numbers in iPSC-CM therapy ($p<0.05$). Markers of angiogenesis including vascular endothelial growth factor, angiopoietin, and insulin like growth factor were significantly increased with iPSC-CM patch therapy ($p<0.05$). Up-regulation of angiogenic cytokines and cardio-protective cytokines may help in slowing progression of HF. Interestingly, we also observed an increase in some markers, which were associated with HF. In conclusion, both iPSC-CM patch and MSCs altered signaling in the setting of HF, perhaps leading to improvement of at the cellular level. An iPSC-CM-based patch and MSCs as an adjuvant therapy may be able to play a role in the setting of HF as cellular therapeutic approach.

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In vitro Modeling of the Pacemaker/working Myocardial Fate Decision by the Shox2-nkx2-5 Antagonistic Mechanism

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Our recent studies have identified a Shox2-Nkx2-5 antagonistic mechanism that regulates pacemaker/working myocardial cell fate decision in the developing venous pole in mice, particularly in the junction domain of the sinoatrial node (SAN) and pulmonary vein myocardium where Shox2 and Nkx2-5 are co-expressed. In such regulatory mechanism, the stronger transcription output by Shox2 promotes pacemaker cell fate, while stronger Nkx2-5 output favors working myocardial fate. Interestingly, we also found that a majority of Hcn4-positive cells derived from mouse embryonic stem (mES) cells co-express Shox2 and Nkx2-5, suggesting that in vitro differentiated pacemaker-like cells also adopt such an antagonistic mechanism to regulate cell fate. To establish an in vitro modeling system to further dissecting the functional importance of the Shox2-Nkx2-5 antagonism and for future in vitro differentiation of pacemaker cells, we established induced pluripotent stem (iPS) cell lines from mice carrying allelic series of Shox2 and Nkx2-5, as well as lineage specific Cre and RosamTmG reporter alleles. These iPS cell lines are being tested to prove the principle that the Shox2-Nkx2-5 antagonistic mechanism also functions in iPS cell-derived cardiomycytes to regulate cell fate, and are being used for lineage tracing of differentiated cardiomycytes. Furthermore, these cell lines could also be applied to modeling human diseases with Nkx2-5 mutations in vitro.

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Using the Embryonic Heart as an Instructive Template for Cardiac Tissue Engineering

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The engineering of highly aligned cardiomyocytes into functional heart muscle remains a primary challenge in cardiac tissue engineering. Researchers have shown that micropatterned topography and chemistry as well as mechanical and electrical gradients are all effective at inducing some degree of alignment. However, which approach works best in terms of electromechanical function of the engineered cardiac muscle is still an active area of research. Because formation of new heart muscle in mammals primarily occurs during cardiogenesis, we asked whether the embryonic heart could be used as an instructive template for the design of more effective cardiac tissue engineering scaffolds. Specifically, we hypothesized that micropatterns of fibronectin based on fibronectin fibril size and architecture in embryonic myocardium could improve cardiomyocyte alignment relative to 20 μm wide, 20 μm spaced fibronectin lines, a control pattern used widely in the literature. To test this, we first imaged the fibronectin matrix in the ventricles of day-5 embryonic chick hearts and imaged this in 3D using a multiphoton microscope. This fibronectin structure was then converted into a photomask for photolithography and subsequent patterning of fibronectin onto cover slips using microcontact printing. Samples with the biomimetic patterns or control patterns were seeded with embryonic chick cardiomyocytes, cultured for 3 days and then stained and imaged to visualize the myofibrils. Image analysis to quantify alignment showed that the ability of the biomimetic pattern to induce cardiomyocyte alignment increased with cell density, suggesting that cell-cell interactions play an important role in the formation of aligned embryonic myocardium. Disruption of the cadherins junctions using blocking antibodies confirmed this conclusion. In the future we will use human induced pluripotent stem cell-derived cardiomyocytes to engineer more clinically-relevant human heart muscle and analyze electromechanical function of the tissues including contractile force and action potential propagation.

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Allogeneic Cardiosphere-derived Cells From an Aged Donor Elicit Long-term Improvements in Left Ventricular Function and Myocyte Proliferation in a Mini-swine Model of Chronic Myocardial Ischemia

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Objective: Virtually all large animal studies of cell-based therapy have employed young donor-recipient pairs. Since aging may impact the reparative ability of allogeneic cardiosphere-derived cells (CDCs), we tested whether CDCs from an aged donor promote functional repair of ischemic myocardium using a mini-swine model that enabled serial assessment of left ventricular (LV) function over an extended follow-up period.
Methods: Immunosuppressed mini-swine (cyclosporine 100 mg/day) with a chronic (4-months) LAD stenosis were untreated (n=8) or received 20 million allogeneic aged CDCs (n=10). Cells were cultivated from a 9-year old mini-pig and infused into the 3 major coronary arteries under continuous flow (1 million cells/min). LV function was assessed by echocardiography at baseline and over a 3-month follow-up period, at which time histological assessment of myocyte morphometry, proliferation (Ki67), and cell retention (Y-FISH) was performed.
Results: Wall thickening (WT) of the ischemic LAD region was impaired at baseline (LAD: 42.7 ± 2.1% vs. Remote: 81.4 ± 4.8%, $p<0.01$) and not different between treatment groups. One month later, LAD WT improved in aged CDC-treated animals (from 41.0 ± 3.1 to 53.0 ± 1.7%, $p=0.01$) but remained depressed in untreated animals (from 45.1 ± 2.8% to 44.9 ± 2.6%, $p=0.97$). Extended follow-up revealed that LAD WT continued to improve, reaching 60.2 ± 1.6% ($p=0.01$) 3-months after CDC treatment. Histological analyses demonstrated morphometric changes consistent with myocyte regeneration, including an increase in myocyte nuclear density (1231 ± 34 vs. 1094 ± 34 nuclei/mm^2, $p=0.02$) and reduction in myocyte diameter (13.9 ± 0.2 vs. 14.5 ± 0.3 μm, $p=0.05$). Increases in $Ki67^+$ myocytes (468 ± 92 vs. 199 ± 32 nuclei/10^6 myocyte nuclei, $p=0.02$) and rare

Y^+ cells in sex-mismatched recipients (0.5 ± 0.2 cells/cm^2) supported endogenous myocyte proliferation as the primary source of new myocytes. **Conclusion:** CDCs from an aged donor retain their reparative capacity to promote functional improvement and increase myocyte number following global intracoronary infusion. The progressive improvement in LV function 3-months after treatment with ongoing myocyte proliferation supports a long-lasting benefit after a single infusion.

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C-kit+ Cardiac Progenitor Cells Contain a Subpopulation of Slowly Adherent Cells With Higher Cardiomyogenic and Pro-angiogenic Profile

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Background: Isolation of a pure population of cardiomyogenic cardiac stem cells (CPCs) has been the Holy-Grail in cardiac regeneration. C-kit, a cell surface receptor, has been proposed as a marker of CPCs and the result of multiple preclinical studies and a phase I clinical trial has been encouraging. Nonetheless, optimization of isolation methods is desirable given the heterogeneity of c-kit positive CPCs. We hypothesize that isolation of c-kit positive CPCs based on differential adhesion identifies a superior population of cells.
Methods: Non-adherent cells from digested murine hearts were transferred to successive flasks at 2, 4, 24, 48 and 72 hours. Hence, 5 fractions of cells were isolated based on their adhesion profile and labeled rapidly-adherent (1 and 2) and slowly-adherent (3 to 5). Cells were then expanded and sorted based on c-kit expression and expanded to seven passages. Cells were analyzed for surface markers and cardiomyogenic and pluripotent expression profile by flow-cytometry and real-time polymerase chain reaction. Differentiation potential of the two fractions was examined and their paracrine profile compared using human umbilical vein endothelial cells and neonatal rat cardiomyocytes.
Results: C-kit expression was maintained in the slowly-adherent fraction while precipitously dropping in the rapidly-adherent one. In addition, the former expressed lower levels of mesenchymal/fibroblast (CD90.2) and macrophage (CD11b/CD45) markers and higher levels of pluripotent (Oct-4, Nanog, Dppa-3, Rif) and cardiac markers (Nkx2.5, Gata4, Mybpc3 and cTnI) comparing to the latter. Finally, slowly-adherent CPCs had superior paracrine profile compared to rapidly-adherent one.
Conclusion: C-kit positive CPCs cells isolated from murine myocardium are heterogeneous and better isolation methods are required to enrich a purer population of cardiomyogenic stem cells. In that vein, isolation of c-kit positive CPCs based on their adhesion profile identifies a subpopulation of cells capable of maintaining c-kit positivity and superior in terms of cariomyogenic and pro-angiogenic potential.

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Hydrogel Mattress, an in vitro Platform to Enhance Maturation and Evaluate Contractile Function of Individual hiPSC-CMs

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Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) have great potential as tools for human heart disease modeling and drug discovery. However, their contractile properties have not been routinely evaluated; as current methods are not accessible for most laboratories. We sought to develop a more efficient method to evaluate hiPSC-CM mechanical properties, at the single cell level. Individual hiPSC-CMs were cultured on a hydrogel based platform, termed the "hydrogel mattress," and their cellular contractile properties evaluated using video-based edge detection. We found that hiPSC-CMs maintained on the mattress reproducibly exhibited robust cell shortening, in dramatic contrast to hiPSC-CMs maintained in a standard manner. We further found that contraction and peak cell shortening amplitude of hiPSC-CMs on mattress was comparable to that of freshly isolated adult ventricular mouse CM. Importantly, hiPSC-CMs maintained on the mattress exhibited several characteristics of a native CM, in terms of myocyte elongation, calcium handling and pharmacological response. Finally, using this platform, we could calculate the traction force generated by individual CMs. In summary, the Hydrogel mattress platform is a simple and reliable *in vitro* platform that not only enables the quantification of contractile performance of isolated hiPSC-CMs, but also enhances CM maturation. This flexible platform can be extended to *in vitro* disease modeling, drug discovery and cardiotoxicity testing.

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Unique Features of Cortical Bone Stem Cells Associated With Enhanced Cardiac Repair

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Rationale: Adoptive transfer of bone marrow and cardiac derived stem cells (CDCs) into failing human hearts has been shown to be safe, yet these cells have only induced modest improvements after myocardial infarction (MI). Recently we have shown in a mouse model that cortical bone derived stem cells (CBSCs) induced a greater enhancement of cardiac function after MI through enhanced paracrine signaling and transdifferentiation of CBSCs into new cardiac tissue. However, the reparative potential of CBSCs relative to other stem cell types including bone marrow derived mesenchymal stem cells (MSCs) and CDCs is not known.
Objective: To characterize surface marker expression, proliferation, survival, migration and differentiation capacity of swine CBSCs relative to MSCs and CDCs.
Methods and Results: CBSCs, MSCs and CDCs were isolated from Gottingen miniswine. CBSCs were morphologically distinct from MSCs and CDCs, with differences in length to width ratio and overall cell surface area. Cell surface marker profiling using RT-PCR analysis revealed that CBSCs express some of the classical MSC markers such as CD106, CD271, CD105, CD90 and CD29 and are negative for CD45 and CD11-b. CBSCs had an enhanced proliferation capacity versus CDCs and MSCs, measured by CyQuant assay. Concurrently CBSCs had significantly decreased population-doubling time (3.57 and 1.26 fold decrease) as compared to MSC and CDCs. CBSCs exhibit enhanced survival after exposure to apoptotic stimuli as compared to MSCs measured by Annexin-V staining. A significantly greater % of CBSCs expressed markers of

cardiac lineage commitment when exposed to dexamethasone than did CDCs or MSCs. Markers of cardiac lineages including GATA-4, α SMA, Troponin T, sm22 were measured with RT-PCR and immunocytochemistry.
Conclusion: CBSCs have enhanced proliferative, survival capacity and cardiac lineage commitment versus CDCs and MSCs that could account for their enhanced effects on cardiac regeneration after myocardial infarction.
S. Mohsin: None. **C.D. Troupes:** None. **T.E. Sharp:** None. **T. Starosta:** None. **E.J. Agra:** None. **S. Smith:** None. **H. Kubo:** None. **R.M. Berretta:** None. **S.R. Houser:** None.

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Assessing the Paracrine Hypothesis in Cardiac Regeneration Through Endogenous c-Kit+ Cells
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Multiple studies have shown an innate capacity of the adult mammalian heart to partially regenerate following stress or injuries such as myocardial infarction (MI). However, this modest capacity is insufficient to resolve the significant damage that occurs post-MI, thus efforts have centered on bolstering this regenerative potential by delivery of various stem or progenitor cell types. To-date, over fifty clinical trials have evaluated cell-based therapeutics to induce regeneration in the setting of ischemic heart disease - mostly using bone marrow mononuclear cells (BM-MNCs) although c-Kit$^+$ cardiac progenitor cells (CPCs) and mesenchymal stromal cells (MSCs) from bone were also evaluated. Collectively, cell therapy has demonstrated clinical safety; yet overall effectiveness has been questioned, as evidence that transplanted cells differentiate into cardiomyocytes is lacking. Hence, studies showing some functional benefit have ascribed it to enhanced endogenous regeneration via paracrine factors secreted by the implanted cells. One hypothesis is that these factors stimulate resident c-Kit$^+$ progenitors to generate new myocardium. We recently developed an inducible genetic system to trace endogenous c-Kit$^+$ cells in the murine heart. We now use this system to evaluate whether delivery of different progenitor-like cell types can enhance c-Kit$^+$-derived myocyte or vessel formation in the uninjured heart; using a dual-reporter strategy to differentially fate map transplanted cells versus endogenous c-Kit$^+$ cells. We observed that BM-MNCs induced a modest increase in c-Kit$^+$-derived, CD31$^+$ vessels proximal to the site of injection, although no new myocytes were observed. There was no evidence for fusion between BM-MNCs and host myocardium, or for prolonged engraftment, as by 6 weeks virtually all injected cells were cleared. Taken together, this suggests that BM-MNCs are capable of inducing an acute c-Kit$^+$-mediated vascular response in the uninjured heart. We are also currently evaluating CPCs, bone marrow or adipose-derived MSCs, and bone stromal cells for their capacity to stimulate c-Kit$^+$ cells, either without injury or after MI. Our goal is to better define the paracrine hypothesis, improving cell-based therapies for application in humans.
R.J. Vagnozzi: None. **M. Maillet:** None. **M.A. Sargent:** None. **J.D. Molkentin:** None.

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Empowering Cardiac Progenitor Cell-mediated Repair of Injured Myocardium by Overexpressing P2Y2 Nucleotide Receptor
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Heart failure is a leading cause of death in the US due to the limited capability of adult mammalian heart to regenerate following injury. Autologous stem cell therapy holds promise for regeneration of injured myocardium after myocardial infarction. However, stem cells derived from diseased organs exhibit impaired proliferative and migratory capabilities and increased susceptibility to cell death. Empowering stem cells from diverse origins, including cardiac progenitor cells (CPCs), with pro-survival genes has been attempted. Despite the well-established roles of purinergic signaling mediated by extracellular nucleotides in regulating diverse cellular responses in cardiovascular diseases, it has not been well-defined in CPCs. Our preliminary data show, for the first time, that the majority of P2 purinergic receptors are expressed and exhibit functional responses to ATP and UTP in mouse and human CPCs. Since previous findings have shown that the G protein-coupled $P2Y_2$ receptor ($P2Y_2R$) induces cardioprotective responses in animal models as well as human cardiomyocytes and regulates a wide range of signaling pathways that are crucial to tissue repair in various experimental models and in stem cells from diverse origins, we aim to determine whether $P2Y_2R$ plays similar roles in CPCs. Our preliminary data show that the $P2Y_2R$ agonists ATP and UTP enhance human CPC (hCPC) proliferation, migration and survival. Interestingly, hCPCs that exhibit relatively slower growth kinetics and higher levels of senescence markers show a dramatic decrease in $P2Y_2R$ expression as compared to fast-growing hCPCs consistent with our hypothesis that overexpressing $P2Y_2R$ participates in rejuvenating hCPCs and improving their growth capabilities. This hypothesis will be tested *in vivo* by determining whether $P2Y_2R$ overexpression in hCPCs improves their reparative potential for injured mouse myocardium. We also introduce the novel hypothesis that $P2Y_2R$-induced regenerative responses in hCPCs involve the activation of Hippo signaling that is known to be regulated by different GPCRs, which links the extracellular nucleotides released during cardiac ischemia to extracellular matrix sensing and Hippo signaling that have been recently implicated in cardiac regeneration.
F. El-Sayed: None. **M. Sussman:** 7. Ownership Interest; Significant; CardioCreate Inc..

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Paired-like Homeodomain-2 Promotes Mouse Myocardial Regeneration
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The lack of self-renewal capacity in mature hearts is a major reason for heart failure after myocardial infarction (MI). To repopulate a damaged heart with de novo cardiomyocytes, one promising strategy is to reintroduce mature cardiomyocytes into mitotic cycle. We have previously reported that the mouse Hippo signaling is a major heart-size control pathway during development. Knocking-down of Hippo (Hippo-deficient) activates its downstream effectors (Yap/Taz) and promotes juvenile and adult myocardial regeneration after MI. Here we further dissected the Hippo pathway and identified the paired-like homeodomain transcription factor 2 (pitx2) as a potential cofactor of Yap, the major target of Hippo-kinase-cascade. Co-IP assays indicated a direct interaction between Pitx2 and Yap; knocking-down of pitx2 in Hippo-deficient heart resulted in severe scarring after MI, implicating a requirement of pitx2 for the regeneration of Hippo-deficient heart. Pitx2 expression is induced in injured myocardium, and is required in neonatal cardiac regeneration. Immuno-staining, ChIP-seq and RNA-seq studies showed that

pitx2 positively regulates cell proliferation and the expression of antioxidant scavenger genes. In addition, increased reactive oxygen species (ROS) in pitx2 knockdown heart is partially responsible for impeding neonatal myocardial regeneration. Further studies revealed that over-expression of pitx2 in adult cardiomyocytes is sufficient to promote the restoration of myocardial structure and function after MI. Together, these evidences revealed a new role of pitx2 in ventricular myocardium and showed the potential of pitx2 as a therapeutic target in future cardiac regenerative medicine.
G. Tao: None. **P.C. Kahr:** None. **Y. Morikawa:** None. **M. Zhang:** None. **L. Li:** None. **Z. Sun:** None. **B.A. Amendt:** None. **J.F. Martin:** None.

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Induced Pluripotent Stem Cell-based Model of Cardiac Arrhythmia: New Platform for Drug Screen
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Human induced pluripotent stem cell (iPSC)-based model of cardiac diseases has been proved to be useful and valuable for identifying new therapeutics. However, the use of human iPSC-based model of cardiac diseases for drug screen is hampered by the high-cost and complexity of methods used for reprogramming, in vitro differentiation, and phenotyping. To address the limitations, we first optimized a protocol for reprogramming of human fibroblasts and keratinocytes into pluripotency using single lipofection and the episomal vectors in a 24-well plate format. This method allowed us to generate multiple lines of integration-free and feeder-free iPSCs from seven patients with cardiac diseases and three controls. Second, we differentiated human iPSCs derived from Timothy syndrome patients into cardiomyocytes using a monolayer differentiation method. We found that Timothy syndrome cardiomyocytes showed slower, irregular contractions and abnormal calcium handling compared to controls, which were consistent with previous reports using a retroviral method for reprogramming and using an embryoid body-based method for cardiac differentiation. Third, we developed an efficient approach for recording action potentials and calcium transients simultaneously in control and patient cardiomyocytes using genetically encoded fluorescent indicators, ArcLight and R-GECO1. The dual optical recordings enabled us to observe prolonged action potentials and abnormal calcium handling in Timothy syndrome cardiomyocytes. We confirmed that roscovitine rescued the phenotypes in Timothy syndrome cardiomyocytes and these findings were consistent with previous studies using conventional electrophysiological recordings and calcium imaging with dyes. The approaches using our optimized methods and dual optical recordings will improve iPSC applicability for disease modeling to test potential therapeutics. With those new approaches in hand, next we plan to use the iPSC-based model of Timothy syndrome to investigate novel molecules involved in the pathogenesis of Timothy syndrome and to screen and identify new therapeutic compounds for Timothy syndrome patients.
L. Song: None. **M. Yazawa:** None.

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Influence of Age and Ischemia on Cardiac Subsarcolemmal and Interfibrillar Mitochondria in a Novel Model of Estrogen Deficiency
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Altered mitochondrial respiration (MR) and calcium retention capacity (CRC) are proposed cardiac cell death mechanisms exacerbated by aging in males. The present study aimed, for the first time, to determine changes in mitochondrial subpopulation function with age and ischemia/reperfusion (I/R) injury in the female heart. A novel model to recapitulate human menopause/age interactions was used in F344 female rats ovariectomized (OVX) at 15mo and studied at 24mo (MO OVX; n=15), vs adult (6mo; n=18). MR and CRC were assessed in isolated subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria following in vivo coronary artery ligation (CAL; 31 min I and 10 min R) or sham. State 3 MR energized by either complex I (CI) or complex II (CII) substrates was selectively reduced by age in SSM ($p<.02$), and by I/R in IFM ($p<.05$). The I/R-dependent decrease in CRC was 64% (18 vs 29.5) greater in MO OVX vs. adult IFM, suggesting earlier mitochondrial permeability transition pore (MPTP) opening. At CI, but not CII, cyclosporine A (CsA) enhanced CRC 20% (103 vs 86) more in SSM and 75% (98 vs 56) more in IFM from adult compared to MO OVX, suggesting reduced protective efficacy with age and MPTP involvement. Additionally, mitochondrial cyclophilin D increases with age, while cytoplasmic RIP1 is increased with age and I/R further implicating the MPTP mechanism and link with programmed necrosis in the aged female heart. In contrast to males, our data suggest a sex-specific phenotype whereby reductions in both SSM and IFM dynamics may play an additive role in the enhanced susceptibility to I/R injury and myocardial infarction in the aged female heart, which remains the leading cause of death in older women.
A.M. Garvin: None. **N.C. Aurigemma:** None. **D.H. Korzick:** None.

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Regulation of Adult Cardiomyocyte Proliferation and Repair by Tbx20
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Background: In the adult heart, there is increasing evidence that cardiomyocytes can proliferate, but these rates are exceedingly low and are not sufficient for myocardial repair after injury. In the embryonic heart, the T-box transcription factor Tbx20 is required for cardiomyocyte proliferation, and Tbx20 overexpression (Tbx20OE) promotes fetal characteristics in adult cardiomyocytes when initiated before birth in mice. We hypothesize that Tbx20OE, when induced in adult cardiomyocytes, promotes proliferation and improves cardiac repair after injury. Methods and Results: Mice were generated with tamoxifen-inducible Tbx20OE specifically in differentiated cardiomyocytes driven by αMHCMerCreMer. Tbx20OE initiated in adult cardiomyocytes leads to increased numbers of mononucleated proliferating cardiomyocytes, increased expression of cyclins, and increased total numbers of cardiomyocytes. No changes in cardiac function or heart weight/body weight ratios were observed, although the average size of cardiomyocytes with Tbx20OE is smaller than controls. In addition cardiac proliferative pathways, including AKT, Yap1, and IGF1, are activated, while cell cycle inhibitors, including Meis1 and p21, are decreased. In order to determine if Tbx20 promotes cardiac repair after injury, Tbx20OE was induced 3 days post-myocardial infarction (MI). In Tbx20OE mice subjected to MI, cardiac function was significantly improved, the infarct scar size was smaller, and increased pHH3+ cardiomyocytes were observed. Likewise, capillary density and expression of VegfA and bFGF are increased in the injured myocardium with Tbx20OE. Ongoing studies will determine direct regulatory interactions of Tbx20 with genes and

regulatory pathways that promote cardiomyocyte proliferation and repair in adults.
Conclusions: Tbx20OE in adult CM activates cell proliferation also improves cardiac function and repair in mice when induced post-MI.

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Production of De Novo Cardiomyocytes in Adult Asxl2-/- Hearts

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The chromatin-associated protein ASXL2 is highly expressed in the heart, where it critically regulates the levels of two repressive histone marks, trimethylated H3 lysine 27 and ubiquitinated H2A. We have previously observed that Asxl2-/- hearts displayed abnormal growth during adulthood, resulting in disproportionally larger heart from 4 months on. This abnormal growth is not due to cardiomyocyte hypertrophy, as demonstrated by measurement of comprehensive dimensions of isolated cardiomyocytes. BrdU labeling showed an elevation in proliferation index in Asxl2-/- hearts at 3 months. At the time of labeling, with rare exceptions, labeled cells are interstitial non-cardiomyocytes in both wildtype control and Asxl2-/- hearts. The vast majority of labeled cells stained positive for vimentin, a mesenchymal marker, and negative for Nkx2.5, Gata4 or c-kit. EdU pulse-chase experiments were performed to determine the fate of labeled cells. After a 4-week chase, a significant number of EdU+/cTnT+ cardiomyocytes were present in Asxl2-/- hearts but not in wildtype control hearts. Expression of the gap junction protein Cx43 was detected between EdU+ cardiomyocytes and neighboring EdU- cardiomyocytes, suggesting electrical coupling. Cardiomyocytes were isolated from pulsed-chased Asxl2-/- hearts for additional morphological analysis. Isolated EdU+/cTnT+ cardiomyocytes vary greatly in cell size, shape and nucleation status, ranging from small, spindle-shaped and mononuclear to large, rod-shaped and binuclear. Taken together, our data suggest that new cardiomyocytes are produced in adult Asxl2-/- hearts from a population of proliferative interstitial cells that are distinct from c-kit+ cardiac stem cells. This raises intriguing possibilities for cardiac repair and regeneration.

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The 18 kDa FGF-2 Prevents the Doxorubicin-induced Cardiac Damage and Amp-activated Kinase Activation, in vitro and in vivo

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Introduction: Protection of the heart from chemotherapeutic (Doxorubicin, DOX) drug-induced toxicity is a desirable goal, to limit side effects of cancer treatments. DOX toxicity has been linked to the activation (phosphorylation) of the AMP-activated kinase, AMPK. The 18 kDa low molecular weight isoform of fibroblast growth factor 2 (Lo-FGF-2) is a known cardioprotective and cytoprotective agent. In this study we have tested the ability of Lo-FGF-2 to protect from DOX-induced damage in rat cardiomyocytes in vitro, and in transgenic mouse models in vivo, in relation to AMPK activation.
Methods: Rat neonatal cardiomyocytes in culture were exposed to DOX (0.5 μM) in the presence or absence of pre-treatment Lo-FGF-2 (10 ng/ml). Compound C was used to block phosphorylation (activity) of AMPK. Levels of cell viability/death (using Calcein-AM/Propidium iodide assay), phospho -and total AMPK, and apoptotic markers such as active caspase 3 were analyzed. In addition, transgenic mice expressing only Lo-FGF2, and wild type mice, expressing both high molecular weight (Hi-FGF2) as well as Lo-FGF2 were subjected to DOX injection (20 mg/kg, intraperitoneal); echocardiography was used to examine cardiac function at baseline and at 10 days post-DOX.
Results: DOX-induced cell death of cardiomyocytes in culture was maximal at 24 hours post-DOX coinciding with significantly increased in activated (phosphorylated) AMPK. Compound C attenuated DOX-induced cardiomyocyte loss. Pre-incubation with Lo-FGF-2 decreased DOX induced cell death, and also attenuated the phosphorylation of AMPK post-DOX. Relative levels of phospho-AMPK were lower in the hearts of Lo-FGF2-expressing male mice compared to wild type. DOX-induced loss of contractile function (left ventricular ejection fraction and endocardial velocity) was negligible in Lo-FGF2-expressing mice but significant in wild type mice.
Conclusion: Lo-FGF-2 protects the heart from DOX-induced damage in vitro and in vivo, by a mechanism likely involving an attenuation of AMPK activity.

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Qiliqiangxin Attenuates Doxorubicin-induced Cardiomyopathy via Regulating Autophagy

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Aims: Doxorubicin-induced cardiomyopathy, as a common complication of cancer chemotherapy, greatly limits the clinical implication of doxorubicin in the treatment of cancer. Our previous clinical trial and animal studies have shown that Qiliqiangxin, a traditional Chinese medication, was protective for adverse cardiac remodeling. This study aimed at determine the therapeutic effect of Qiliqiangxin in mouse doxorubicin-induced cardiomyopathy.
Methods and results: Three groups of mice (control, n=6; doxorubicin+saline, n=7; doxorubicin+Qiliqiangxin, n=7) were followed up for 4 weeks. Echocardiography was taken at the end of 4 weeks to evaluate cardiac function. Mice from the doxorubicin+saline group showed reduced ejection fraction (EF) and fractional shortening (FS) as compared with the mice from the control group. Qiliqiangxin could partly restore the reduction of EF and FS in doxorubicin-induced cardiomyopathy mice. Genes related with autophagosome formation (becline1, ATG4, ATG7, LC3) and autophagosome-lysosome fusion (Cathepsin B, Cathepsin D, Cathepsin L) were determined by quantitative reverse transcription polymerase chain reactions (RT-PCRs) in heart samples from mice of the three groups (control, doxorubicin+saline, doxorubicin+Qiliqiangxin) at the end of 4 weeks. All these 7 genes were found to be increased in doxorubicin-induced cardiomyopathy mice, implying doxorubicin may disturb the autophagy flux of mice hearts through promoting autophagosome formation and inhibiting autophagosome-lysosome fusion. Interestingly, the expression of these 7 genes were

decreased in the doxorubicin+Qiliqiangxin group, implying that Qiliqiangxin may protect the cardiac function in doxorubicin-treated mice through regulating the autophagy flux of mice hearts. Moreover, autophagy-related proteins LC3 and P62 were determined by western blotting. Qiliqiangxin reversed the increase of LC3II level and the decrease of P62 level in the doxorubicin-treated mice hearts.
Conclusion: Qiliqiangxin may protect the cardiac function of doxorubicin-induced heart failure mice through regulating autophagy in the heart. Qiliqiangxin might be a novel drug for doxorubicin-induced cardiomyopathy.
S. Shen: None. **L. Tao:** None. **X. Wu:** None. **J. Xiao:** None. **X. Li:** None.

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Mir-195-3p/-5p Decrease Cardiac Fibroblast Proliferation and the Transdifferentiation into Myofibroblasts

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Aims: MicroRNAs (miRNAs, miRs) contribute to many essential physiological and pathological processes including fibrosis. This study aims at investigating the role of miR-195-3p/-5p in cardiac fibroblast proliferation and the transdifferentiation into myofibroblasts.
Methods and results: In isolated primary neonatal cardiac fibrobasts (NRCFs), forced expression of miR-195-3p/-5p with agomiRs could attenuate fibrobast proliferation as determined by EdU and Ki67 staining while inhibition of miR-195-3p/-5p with antagomiRs could increase fibrobast proliferation. By quantitative reverse transcription polymerase chain reactions (RT-PCRs) and western blotting (WB), α-SMA (a marker of myofibroblast transdifferentiation) was found to be suppressed in the miR-195-3p/-5p agomiR-treated NRCFs at both mRNA and protein levels, while was increased in the miR-195-3p/-5p antagomiR-treated NRCFs. Moreover, Chek-1 was identified as a target gene of miR-195-3p/-5p responsible for cardiac fibroblast proliferation and the transdifferentiation into myofibroblasts by RT-PCR and WB and immunofluorescent staining. Silencing of Chek-1 attenuates cardiac fibroblast proliferation and the transdifferentiation into myofibroblasts as detected by α-SMA/EDU staining. In addition, Chek-1 mediated the effects of miR-195-3p/-5p in cardiac fibroblast proliferation and the transdifferentiation into myofibroblasts.
Conclusion: . Therefore, miR-195-3p/-5p might be promising therapeutic targets for cardiac fibrosis.
S. Shen: None. **X. Wang:** None. **D. Xu:** None. **L. Tao:** None. **X. Wu:** None. **J. Xiao:** None. **X. Li:** None.

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Drp1 Accumulates in Mitochondria and Plays a Protective Role in the Heart in Response to Pressure Overload

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Dynamin-related protein 1 (Drp1) plays an essential role in maintaining the quality control of mitochondria through mitochondrial (Mt) fission and mitophagy. We investigated how Mt function, autophagy and Drp1 are regulated in the heart during pressure overload (PO) and whether endogenous Drp1 plays an important role in regulating cardiac function. Mice were subjected to transverse aortic constriction (TAC) at multiple time points between 6 hours and 30 days. Left ventricular (LV) weight/tibial length (LVW/TL) was significantly elevated at Day 5 (TAC vs Baseline; 6.21 ± 0.28 vs 4.59 ± 0.36, $p<0.05$). Ejection fraction (EF) was maintained at Day 5 (79±5 vs 82±7%), but gradually decreased thereafter (30 days; 51±12%, $p<0.05$). LC3-II was decreased (-40.0%, $p<0.05$) while p62 accumulated (1.84 fold, $p<0.05$) significantly at Day 5. Both Mt ATP content (-65.6%, $p<0.05$) and production (-90.3%, $p<0.05$) were reduced significantly at Days 7 and 14, respectively, and thereafter. Mt mass, evaluated by electron microscopy, was also reduced (-19.9%, $p<0.05$) at Day 7. Drp1 accumulated in mitochondria at Day 7, and S616 phosphorylation of Drp1, associated with increased activity, was increased at Day 7. Thus, PO suppresses autophagy and induces Mt dysfunction by Day 7, at which time Drp1 accumulates in mitochondria and Mt mass is decreased. To examine the functional significance of endogenous Drp1 during PO, cardiac-specific heterozygous Drp1 knock out (Drp-hetCKO) mice were subjected to TAC. At Day 7, decreases in EF (57± 11 vs 80 ± 7%, $p<0.05$) and increases in LVW/TL (7.22 ± 0.26 vs 5.86 ± 0.65, $p<0.05$) and lung weight/TL (13.03 ± 1.09 vs 7.00 ± 1.31, $p<0.05$) were exacerbated in Drp-hetCKO compared to in control mice. LV end diastolic pressure was significantly higher (20.0 ± 5.7 vs 7.4 ± 3.1 mmHg, $p<0.05$) and myocardial fibrosis (14.1 ± 2.5 vs 6.2 ± 4.3 %, $p<0.05$) was greater, and Mt mass was also significantly greater in Drp-hetCKO than in control mice (relative Mt mass, 1.21 ± 0.46 vs 1.00 ± 0.02, $p<0.05$). These results suggest that PO inhibits autophagy and induces mitochondrial dysfunction by Day7, which coincides with Mt accumulation of Drp1. Drp1 plays an adaptive role in this condition, mediating decreases in Mt mass and protecting the heart from dysfunction.
A. Shirakabe: None. **Y. Ikeda:** None. **T. Saito:** None. **P. Zhai:** None. **J. Sadoshima:** None.

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c-fos and Caspase 8 Mediate Palmitate-induced Cardiomyocyte Apoptosis

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BACKGROUND: We previously demonstrated that p38α activation mediates palmitate (PA)-induced cardiomyocyte apoptosis, and that specific p38α siRNA markedly and dose-dependently attenuates apoptosis rates in the presence of PA. We tested the hypothesis that c-fos and caspase 8 would be up-regulated concurrently with p38a.
METHODS AND RESULTS: Immortalized human adult ventricular cardiomyocytes (**AC16** cells) were exposed to high physiological levels of PA. The p38α-dependent pathway was evaluated using p38α siRNA knockdown. PA dose-dependently increased transcription of an AP-1 factor, c-fos, in AC16 cardiomyocytes (control:0.34±0.11, 150 μM PA:0.83±0.19, 300 μM PA:1.58±0.45, n=5, $p<0.05$). c-fos protein expression increased dose-dependently with PA treatment (control:0±0%, 150 μM PA:26%±28%, 300 μM PA:258%±103%, n=4, $p<0.05$). Phospho-c-fos levels increased dose-dependently as well (control:0±0%, 150 μM PA:73%±41%, 300 μM PA:302%±62%, n=4, $p<0.05$). Transcription of a putative downstream effector, caspase 8, also increased (control:0.94±0.10, 150 μM PA:1.40±0.10, 300 μM PA:1.42±0.11, n=3, $p<0.05$) but protein levels did not.
p38α knockdown dose-dependently blocked the PA-induced increase in phospho-c-fos protein level (control siRNA:0±0%, 300 μM PA:206±47%, 300 μM PA+30 pmol siRNA:98±31%, 300 μM PA+60 pmol

ABSTRACTS

siRNA:58±40%, 300 μM PA+120 pmol siRNA:-21±19%, n=4, p<0.05), without a clear reduction in total c-fos. Interestingly, caspase 8 level was also dose-dependently reduced by p38α knockdown (control siRNA:0±0%, 300 μM PA:-25±3%, 300 μM PA+30 pmol p38α siRNA:-38±13%, 300 μM PA+60 pmol p38α siRNA:-54±7%, 300 μM PA+120 pmol p38α siRNA:-46±11%, n=3, p<0.05).
CONCLUSIONS: This study in cardiomyocytes demonstrated that 1) PA dose-dependently increases gene expression of c-fos and protein levels of total c-fos and phospho-c-fos, 2) PA increases transcription of caspase 8, and 3) reduced p38α expression dose-dependently attenuates PA-induced increases in c-fos and phospho-c-fos protein levels, and decreases caspase 8. Our results suggest that p38α activated by PA up-regulates the downstream transcription factor, c-fos, and maintains caspase 8 level. These molecules may underlie PA-induced cardiomyocyte apoptosis.
C.C. Oh: None. **M. Nguy:** None. **D. Karen:** None. **R. Migrino:** None. **D. Schwenke:** None. **K. Thornburg:** None. **P. Reaven:** None.

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Mechanism of Cardiotoxicity of Tyrosine Kinase Inhibitors
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Aim: It is becoming evident that many of the signaling elements driving cancer cell division, for which kinase inhibitors (KIs) are targeted, are the same as those necessary for cardiomyocyte viability. Adverse cardiac events have been reported for a number of KIs. Aim of this study was to identify KIs that induce toxicity to cardiomyocytes and to elucidate the central molecular mechanisms mediating the toxicity.
Methods: 288 anti-cancer agents (123 are KIs) were screened for their ability to induce cardiotoxicity in cultured primary cardiomyocytes. Cell viability assay was done by measuring the ATP levels in cardiomyocytes after exposure of cells to a 3-log concentration range of each compound for 24 hours. Toxicity data was combined with kinase profiling data to identify protein kinases mediating the toxicity of KIs. In parallel, the molecular mechanism mediating the cardiomyocyte toxicity of dasatinib, a second generation Bcr-Abl and Src family tyrosine kinase inhibitor, was investigated. The role of Src kinase in regulation of cardiomyocyte viability was analyzed by siRNA-mediated Src knockdown. Overexpression of wild type Src and dasatinib- resistant Src in cardiomyocytes was performed to investigate the role of Src in regulating the cardiomyocyte toxicity of dasatinib. Western blotting was used to investigate for downstream signaling targets of Src.
Results: Of the KIs, 70 compounds decreased the cardiomyocyte viability by 10-80 %. The kinase profiling data showed that IGF1R, MEK/ERK pathway, PI3K and Src kinases are the key kinases regulating cardiomyocyte viability. Dasatinib treatment dose-dependently increased cardiomyocyte death. Depletion of Src by siRNA also reduced cardiomyocyte viability. Dasatinib treatment attenuated FGF induced ERK phosphorylation. Overexpression of dasatinib-resistant mutant of Src, but not wild type Src, protected the cardiomyocyte from dasatinib toxicity and rescued the FGF-induced ERK phosphorylation.
Conclusions: Cardiomyocyte toxicity of KIs can be attributed to inhibition of IGF1R, MEK/ERK pathway, PI3Ks and Src family kinases. Cardiomyocyte toxicity of dasatinib is mediated by Src kinase inhibition which in turn attenuates MEK/ERK pathway signalling.
M.F. Elmadani: None. **J. Ulvila:** None. **S. Khan:** None. **T. Alakoski:** None. **J. Magga:** None. **T. Aitokallio:** None. **K. Wennerberg:** None. **R. Kerkelä:** None.

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Role of Phosphodiesterase 1C Mediated Intra- and Extracellular cAMP Signaling in Pathological Cardiac Remodeling
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By acting as regulators of the cyclic nucleotides, the phosphodiesterases (PDEs) play important roles in diverse signaling pathways in the myocardium. Comparative studies revealed increased expression of the phosphodiesterase PDE1C in both experimental and human heart failure. Relative to WT littermates, mice with global PDE1C deletion experienced significantly reduced TAC-induced cardiac hypertrophy, fibrosis, and dysfunction, as well as reduced cardiomyocyte apoptosis, indicating a potential role for PDE1C in regulating cell survival. Isolated PDE1C KO cardiomyocytes were resistant to Ang II or Iso-induced cell death, and PDE1 inhibition in WT myocytes attenuated cell death similarly, indicating this was a cardiomyocyte-specific effect. As PDE1C can hydrolyze either cAMP or cGMP, this effect could have been mediated by either PKA or PKG; further in vitro studies revealed that while PKG inhibition did not alter the protective effects of PDE1 inhibition, PKA inhibition blocked it. To further characterize this signaling, we tested whether modulation of known cardioprotective cAMP/PKA-mediated pathways altered these effects. Remarkably, antagonism of the adenosine A2A/A2B receptors blocked the protective effects of PDE1C inhibition or depletion. Furthermore, stimulation with adenosine appeared to be synergistic with PDE1 inhibition: very low doses of adenosine and PDE1 inhibitor in combination induced a robust protective effect in cardiomyocytes. These results indicated that PDE1C may be involved in regulation of adenosine-produced cAMP; adenosine A2A receptors also appear to colocalize with PDE1C in isolated cardiomyocytes. Finally, we hypothesized that by increasing cAMP efflux, PDE1C depletion could potentiate the extracellular cAMP-adenosine pathway, further stimulating adenosine-mediated cAMP production in a feed-forward loop. Antagonism of this pathway blocked PDE1C inhibition-mediated cardioprotection, while addition of extracellular cAMP to isolated cardiomyocytes was protective in an adenosine receptor-dependent fashion, indicating the potential involvement of this pathway. Therefore, PDE1C appears to be a crucial regulator of protective extra- and intracellular signaling in cardiomyocytes.
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Interleukin-10-mediated Activation of AKT and Bcl2 Inhibits Chronic Angiotensin II-induced Pathological Autophagy
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Rationale: Although, autophagy is an essential cellular salvage process to maintain cellular homeostasis, pathological (stress-induced exaggerated/defective) autophagy can lead to cardiac abnormalities and ultimately heart failure. Therefore, a tight regulation of autophagic process would be important to treat chronic heart failure. Previously, we have shown that IL-10 strongly inhibited pressure overload-induced hypertrophy and heart failure, but role of IL-10 in regulation of pathological autophagy is not known. Hypothesis: We tested the hypothesis that IL-10 inhibits angiotensin II-induced pathological autophagy and this process, in part, led to improved cardiac function. Methods and Results: Pathological autophagy was induced in wild type (WT) and IL10-knockout (IL-10 KO) mice by angiotensin II (Ang II for 28 days) infusion. Ang II-induced left ventricular (LV) dysfunction and hypertrophic remodeling were accentuated in IL-10 KO mice compared to WT mice. IL-10 KO mice showed exaggerated autophagy as observed by Electron Microscopy and Western blotting (beclin 1, LC3 II/I and CHOP) with reduced AKT phosphorylation at serine-473. In neonatal rat ventricular cardiomyocytes (NRCM), Ang II treatment enhanced beclin1, LC3 and CHOP protein levels and inhibited AKT and 4EBP1 phosphorylation and Bcl2 levels. Interestingly, IL-10 inhibited Ang II-induced autophagic marker proteins. Additionally, IL-10 restored Ang II-induced suppression of AKT and 4EBP1 phosphrylation and restoration of Bcl2 protein level. Pharmacological inhibition of AKT via PI3K inhibitor (LY290002), reversed IL-10 responses on the Ang II-induced pathological autophagy, confirming that IL-10 mediated inhibition of autophagy is AKT dependent. Finally, as physical interaction of Bcl2 with beclin 1 is important to inhibit autophagy, we performed immunoprecipitation pull-down experiments, which showed Ang II disrupts the physical interaction of beclin 1 with Bcl2 and IL-10 reestablished this physical interaction to reduce autophagy. Conclusion: Our data provides a novel role of IL-10 in regulation of pathological autophagy and thus can act as a potential therapeutic molecule in treatment of chronic heart disease.

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The Role of Bax and Bak in Autophagic Cell Death

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In times of energy depletion, a cell will attempt to maintain metabolic homeostasis and viability by degrading and recycling organelles and intracellular components and proteins in a process referred to as autophagy. However, if the energy depletion persists, the cell will be overwhelmed by the autophagic process and will succumb to autophagic cell death. This form of cell death has been implicated in cardiac remodeling during heart failure and damage during ischemic injury. Two proteins that have been previously shown to play a role in virtually every form of regulated cell death, including autophagy, are Bax and Bak. These effectors are responsible for cytochrome-c release during apoptosis and effect mitochondrial permeability transition pore opening during regulated necrosis. Although the expression of either Bax or Bak is required for autophagic cell death to occur, the role of Bax/Bak in this type of cell death is poorly understood, although the lysosome appears to be centrally involved. Here we show that Bax/Bak DKO MEFs subjected to several days of serum starvation contain intact lysosomes compared to WT MEFs. Furthermore, the acidity of the lysosomes in starved DKO MEFs is preserved compared with starved WT MEFs. Bax and Bak are both found in isolated lysosomal preparations and Bax targeted to the lysosome can completely restore autophagic cell death in DKO MEFs. Finally, although Bax oligomerization is required for apoptosis, it is not necessary for autophagic cell death, as DKO MEFs expressing an oligomerization defective mutant of Bax are still susceptible to this form of death, as monomeric Bax can still increase membrane permeability. In conclusion our results suggest that lysosomal membrane permeability through Bax or Bak is required for autophagic cell death to occur and without Bax or Bak the lysosomes remain intact where they can function as an energy source during times of nutrient deprivation.

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174

The BH3-Only Protein BNIP3 Induces Mitochondrial Clearance via Multiple Pathways

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Autophagy plays an important role in cellular quality control and is responsible for removing protein aggregates and dysfunctional organelles. BNIP3 is an atypical BH3-only protein which is known to cause mitochondrial dysfunction and cell death in the myocardium. Interestingly, BNIP3 can also protect against cell death by promoting removal of dysfunctional mitochondria via autophagy (mitophagy). We have previously reported that BNIP3 is a potent inducer of mitophagy in cardiac myocytes and that BNIP3 contains an LC3 Interacting Region (LIR) that binds to LC3 on the autophagosome, tethering the mitochondrion to the autophagosome for engulfment. However, the molecular mechanism(s) underlying BNIP3-mediated mitophagy are still unclear. In this study, we discovered that BNIP3 can mediate mitochondrial clearance in cells even in the absence of a functional autophagy pathway. We found that overexpression of BNIP3 led to significant clearance of mitochondria in both wild type (WT) and autophagy deficient Atg5-/- MEFs. BNIP3 caused an increase in LC3II levels in WT MEFs, indicating increased formation of autophagosomes. In contrast, LC3II was undetectable in Atg5-/- MEFs. Furthermore, we found that BNIP3-mediated clearance in WT and Atg5-/- MEFs did not require the presence of Parkin, an E3 ubiquitin ligase which plays a critical role in clearing dysfunctional mitochondria in cells. Also, overexpression of Parkin did not enhance BNIP3-mediated mitochondrial clearance. When investigating activation of alternative cellular degradation pathways, we found that BNIP3 induced activation of the endosomal-lysosomal pathway in both WT and Atg5-/- MEFs. Mutating the LC3 binding site in BNIP3 did not interfere with the activation of the endosomal pathway and clearance of mitochondria in Atg5-/- MEFs. Thus, these findings suggest that BNIP3 can promote clearance of mitochondria via multiple pathways in cells. The role of autophagy in removing mitochondria is already well established and we are currently exploring the roles of the endosomal and alternative autophagy pathways in BNIP3-mediated mitochondrial clearance in myocytes.

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Pro-survival Function of Mef2 in Cardiomyocytes is Enhanced by β-blockers
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β1-adrenergic receptor (β1-AR) stimulation increases apoptosis in cardiomyocytes via activation of cAMP/ protein kinase A (PKA) signaling. β-adrenergic receptor antagonists, or β-blockers, oppose the action of PKA signaling by blocking the β1-receptor and effectively inhibit apoptosis and heart failure. The Myocyte Enhancer Factor 2 (MEF2) proteins have been implicated as nuclear targets for signaling cascades involved in muscle-gene expression and have important roles in proliferation, apoptosis and survival in multiple cell types. We previously reported that PKA signaling represses MEF2 activity. Here, we assessed whether β-blockers can inhibit neonatal cardiac myocyte apoptosis by interfering with PKA dependent MEF2 repression. We show that siRNA mediated MEF2 loss of function induced cardiomyocyte apoptosis. β1AR activation by isoproterenol treatment represses MEF2 transcriptional activity and promotes apoptosis in neonatal cardiomyocytes and, importantly, this effect was reversed in cells expressing a PKA resistant form of MEF2D (S121/190A), as indicated by FACS analysis. We also report that a β-blocker, Atenolol, antagonizes -adrenergic stimulation modulated MEF2 cellular localization in neonatal cardiomyocytes and this was reversed by atenolol treatment. In addition, we also document that Krüppel-like factor 6 (KLF6) is an important MEF2 target gene and loss of function analysis using siRNA-mediated knockdown of KLF6 expression resulted in cardiomyocyte apoptosis. Collectively, these observations, establish that MEF2 plays an important pro-survival role in cardiomyocytes which can be modulated by β-adrenergic signaling. These observations have important clinical implications and may contribute to novel strategies for preventing cardiomyocyte apoptosis associated with heart pathology.
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Interleukin 17 A, Hepatosplenic Mansonic Schistosomiasis and Atherosclerosis
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Introduction: The immune system plays an important role in the pathophysiology of schistosomiasis and atherosclerosis due to its activation of various immune cells and release of different inflammatory mediators. However, the pathologies of these diseases are not fully understood. The main aim of this study was to detect atherosclerotic disease in patients with mansonic schistosomiasis, while the secondary objectives were evaluate the serum concentrations of interleukin 17A (IL 17 A) and 22 (IL 22).
Hypothesis: Atherosclerosis is not common in patients with mansonic schistosomiasis.
Methods: This study included 30 patients (14 men, 16 women; mean age = 59.87 ± 7.50 years) with hepatosplenic mansonic schistosomiasis that agreed to participate in the study by signing the Free and Informed Consent Form. Ten healthy volunteers (four men, six women) were selected as controls for interleukin concentrations. Atherosclerosis was evaluated by measuring the carotid artery intima-media thickness (IMT ≥1.5 mm are indicative of atherosclerotic plaques). Shapiro-Wilk test was necessary to determine whether the variables followed a normal distribution. The continuous variables were evaluated using student's t test. P value ≤ 0.05 was considered statistically significant.
Results: The main clinical characteristics of the patients were as follows: benign arrhythmias, 19 patients (63%); hypertension, 17 (56%); smoking, 12 (40%); hepatitis B or C, nine (30%); peripheral vascular insufficiency, nine (30%); family history of CAD, nine (30%); diabetes mellitus, one (3.3%); leukemia, one (3.3%); and non-cardiac surgery, 24 (80%). There were no differences in the IL 17 A concentrations (15.63 ± 0.00 pg/ml vs 15.63 ± 0.00 pg/ml, p = 1) and in the IL 22 concentrations (7.81 ± 0.00 pg/ml vs 7. 81 ± 0.00 pg/ml, p = 1) between patients and controls. The overall mean of the intimal medial thickness was 0.7 ± 0.2 mm
Conclusions: None of patients had atheroma. The serum concentrations of IL 17A and IL 22 were equal between patients and controls (concentrations undetectable or low). It is possible that the immune response of patients with hepatosplenic mansonic schistosomiasis may attenuate the development of atherosclerosis.
D.C. Oliveira: None. **D. Santos:** None. **T. Alcantara:** None. **A.L. Coutinho:** None. **C. Brandt:** None.

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Interleukin 17 and Coronary Stenosis in Patients With Stable Coronary Artery Disease
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Introduction: The role of the immune and inflammatory pathways in patients with coronary artery disease (CAD) is important but not complete understood. The aim of this study was to evaluate concentrations of the interleukins 17 (IL 17) according to severity of coronary stenosis in patients with stable CAD
Hypothesis: There is no association between severity of coronary stenosis and IL 17 in patients with stable CAD.
Methods: This is a cross-sectional, prospective, analytical study, conducted from january to september, 2013. We included 40 patients (P) with stable CAD, CCS III or IV, ischemic myocardial scintigraphy, who had not been subjected to any kind of myocardial revascularization and with coronary stenosis ≥ 50% according to current coronary angiography. There were 20 healthy volunteers (C), to take up comparison of concentrations of IL 17. Interleukins were evaluated in serum of patients and after 48 hours of cells in culture with and without stimulus. IL 17 A concentrations were expressed in pg / ml. Coronary stenosis were classified as severe (> 70%) [SS] and intermediate (50 - 69%) [MS] according to coronary angiography.
Results: Stenosis ≥ 50% were found in the anterior descending artery in 31 patients, in the left circumflex artery in 19 patients, and in the right coronary artery in 24 patients. No cases of stenosis were observed in the left main. Eighteen patients (45%) had single-artery disease, 8 patients (20%) had two-artery disease, and 14 patients (35%) had multiarterial disease. The comparison between the groups showed: IL 17: Serum: P with SS = 3.91 (3.91 -- 72.27) vs P with MS = 3.91 (3.91 -- 3.91) vs C = 3.91 (3.91 -- 28.8), p = 0.53; culture 48 hours without stimulus: P with SS = 3.91 (3.91 -- 3.91) vs P with MS = 3.91 (3.91 -- 86.8) vs C = 3.91 (3.91 -- 53.3), p = 0.55; culture 48 hours with stimulus: P with SS = 241.8 (3.91 -- 2200) vs P with MS = 217.5 (3.91 -- 1346) vs C = 154.3 (3.91 -- 1353), p = 0.7.
Conclusions: There were no differences in concentrations of IL 17 according to severity of coronary stenosis, does not matter in serum or cell in culture. In conclusion, there was no association between severity of coronary stenosis and IL 17 in patients with stable CAD
D. Oliveira: None. **E. Heide:** None. **M. Pita:** None. **D.A.G. Oliveira:** None. **R. Pontes:** None. **I. Pitta:** None.

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Blocking the Acetylcholine Activated Inward Rectifier Potassium Current but not the Rapid Delayed Rectifier Potassium Current Restores Sinus Rhythm in the Chronically Fibrillating Sheep Heart

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Background: The acetylcholine activated inward rectifier potassium current (IKACh) was shown to be constitutively active in chronic atrial fibrillation. Its blockade has been proposed to be a possible antifibrillatory pharmacotherapy. We therefore tested the hypothesis that blocking IKACh with the bee venom peptide tertiapin, or the small molecule chloroquine, terminates chronic AF in the sheep heart.
Methods and Results: We tested our hypothesis using biochemistry, electrophysiology and molecular modeling. In patch clamp, the IC50 of IKACh block by tertiapin, and chloroquine was 60 nM, and 710 nM respectively, while dofetilde, a currently used class III antiarrhythmic, did not block the current. On the other hand, dofetilide and chloroquine blocked the rapid delayed rectifier potassium current (IKr) with an IC50 of 50nM, and 2.3 microM respectively, while tertiapine had no effects. Furthermore, molecular modeling indicated that 1 chloroquine molecule blocks the intracellular ion permeation vestibule of the tetrameric Kir3.1, a molecular correlate of IKACh, by interacting with amino acids important for the channel's rectification. Dofetilide did not interact with Kir3.1. In vitro fluorescence measurements of chloroquine titration into the purified Kir3.1 intracellular domain protein confirmed the computational results and indicated that chloroquine directly binds Kir3.1 with a stoichiometry of 1 chloroquine molecule per Kir3.1 tetramer. Finally, optical mapping of the chronically fibrillating sheep atria showed that while tertiapine, chloroquine, and dofetilide slowed down chronic AF and prolonged the action potential, only tertiapine and chloroquine (the IKACh blockers) restored normal sinus rhythm.
Conclusions: IKACh could be a more powerful anti atrial fibrillation target compared to IKr. Pharmacological blockade of IKACh with a peptide, or a small molecule similar to chloroquine could terminate chronic AF and restore normal sinus rhythm.

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Targeted Inhibition of Plasminogen Activator Inhibitor-1 Attenuates Weight Gain and Prevents Vascular Dysfunction Following a High Fat Diet

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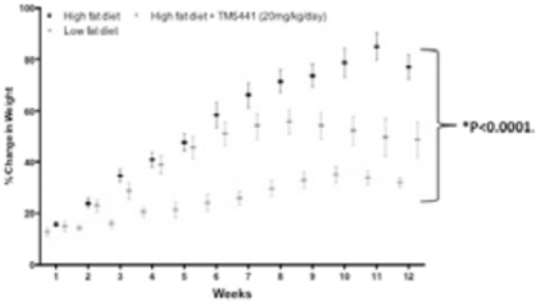
Introduction: Elevated plasminogen activator inhibitor-1 (PAI-1) is associated with obesity, but there is controversy whether PAI-1 causes or is a consequence of obesity. We sought to determine whether targeted PAI-1 inhibition with a novel small molecule antagonist (TM5441) alters the development of obesity and/or obesity-induced vascular dysfunction in a diet-induced obesity model.
Methods and Results: C57BL/6J mice were fed control, high fat diet (HFD), or high fat diet with TM5441 (HFD+TM5441) for 12 weeks. The HFD had marked weight gain (77±5%) as compared with control (32±2%). TM5441 significantly attenuated weight gain (49±8%, p=0.0075, Figure). HFD-induced hepatic triglyceride accumulation was attenuated by TM5441 (116±31 vs. 76±35 mg trig/g liver, p=0.03). Energy expenditure was reduced in the HFD compared to control (11.1±0.4 vs. 12.9±0.4 kcal/h/kg, p=0.005). However, HFD+TM5441 maintained a level of energy expenditure that was similar to control (13.2±0.6 kcal/h/kg, p=NS). The HFD group demonstrated higher systolic and diastolic blood pressure (141±3; 112±3 mm Hg) compared with control (122±7, 94±8; P<0.05 for both), while administration of TM5441 prevented diet-associated increase (120±6; 93±7 mm Hg, p=NS compared to control) at week 12. Pressure myography of mesenteric arteries in the HFD showed a significant rightward shift in the constrictor response to phenylephrine as compared to control (EC50: 14.5uM vs. 25.1uM, p=0.002). The HFD+TM5441 was similar to control (p=NS).
Conclusions: Inhibition of PAI-1 with TM5441 attenuates weight gain, enhances energy expenditure, and prevents obesity-related vascular dysfunction in a murine model of obesity.



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Cd248+cd8+ T Lymphocytes Suppress Pathological Vascular Remodeling in Human Thoracic Aortic Aneurysms

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Aortic aneurysms are characterized by vascular inflammation, neovascularization, and extracellular matrix destruction of the aortic wall. Although experimental studies indicate a potential role of CD248 in microvessel remodeling the functions of CD248 in human vascular pathologies remain unexplored. Here we aimed to study how CD248 interferes with pathological vascular remodeling of human aortic aneurysms. Immunofluorescent staining showed that CD248 expression was mainly localized in the CD8+ T cells infiltrating in the adventitia and media of aortic walls of patients with ascending thoracic aortic aneurysms (TAA). qPCR and immunofluorescent staining analyses revealed increased aortic CD248 expression and infiltrating CD248+CD8+ T cells in aortic aneurysms than in nonaneurysmal aortas. Flow cytometry analysis of human peripheral blood further identified a fraction of circulating CD248+ cells which was confined in the CD8+ T-cell compartment. The increased infiltrating of CD248+CD8+ T cells was coincident with reduced circulating CD248+CD8+ T

cells in patients with ascending TAA when compared with patients with coronary artery diseases (CAD) and healthy donors. The CD248+CD8+ T cells were characterized by upregulated IL-10 and downregulated IL-1β/INF-γ expression when compared with CD248-CD8+ T cells. Moreover, when co-cultured with human aortic endothelial cells, the CD248+CD8+ T cells not only downregulated endothelial expression of ICAM1/VCAM1 and MMP2/3 but also suppressed endothelial migration. Thus, this study shows that CD248 reduces pathological vascular remodeling via anti-inflammatory CD248+CD8+ T cells, revealing a CD248-mediated cellular mechanism against human aortic aneurysms.

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Human Heart Valve-derived Scaffold Improves Cardiac Repair in a Murine Model of Myocardial Infarction

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Cardiac tissue engineering using biomaterials with or without a combination of cardiac stem cell therapy offers a new therapeutic option for repairing infarcted heart. So far, cardiac tissue scaffold is designed mainly based on natural and synthetic biomaterials, which do not mimic human myocardial extracellular matrix. This raises a fundamental issue about the biocompatibility of currently used biomaterials with myocardial tissue. Here we hypothesized that human heart valve-derived scaffold (hHVS) may provide a clinically relevant novel biomaterial for cardiac repair. In this study, human heart valve tissue was sliced into 100 µm tissue sheet by frozen-sectioning and then decellularized to form the hHVS. Upon anchoring onto the hHVS, post-infarct murine BM c-kit+ cells exhibited an increased capacity for proliferation and cardiomyogenic differentiation in vitro. When used to patch infarcted heart in a murine model of myocardial infarction, either implantation of the hHVS alone or c-kit+ cell-seeded hHVS significantly improved cardiac function, as shown by transthoracic echocardiography as well as by hemodynamic measurements via a Millar catheter, and by reduced infarct size; while c-kit+ cell-seeded hHVS was even superior to the hHVS alone. Thus, we have successfully developed a hHVS for cardiac repair. Our in vitro and in vivo observations provide the first evidence for translating the hHVS-based cardiac tissue engineering into clinical strategies to treat myocardial infarction.

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Characterization of Electrophysiological and Conduction Parameters of the Heart in Trpm4-deficient Mice

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TRPM4 is a Ca2+-activated non-selective cation channel that belongs to the family of the Transient Receptor Potential (TRP) ion channels. Importantly, TRPM4 is impermeable for Ca2+ and is involved in different Ca2+-dependent cell functions, such as exocytosis, contraction and cell death. Trpm4 is known to be expressed in atrial and ventricular cardiomyocytes. The interest in the functional role of TRPM4 in the heart has risen further by the discovery of Trpm4 mutations that are linked to cardiac conduction disorders, including Progressive Familial Heart Block type I (PFHBI) and Brugada Syndrome. Both gain-of-function and loss-of-function mutation were described in patients with cardiac conduction diseases. Recently, our group showed that TRPM4 plays a role during the late repolarization phase of the action potential in murine ventricular cardiomyocytes and that deletion of the Trpm4 gene leads to shorter ventricular action potentials. To characterize if deletion of Trpm4 has an effect on the conduction properties of the heart, an in depth electrophysiological study was performed in living mice. An octapolar catheter was inserted into the right atrium and ventricle of the heart to measure intracardial electrograms. The atrial-His (AH) and His-ventricular (HV) intervals were calculated and no differences were found between WT and Trpm4-deficient mice. Additionally, more detailed conduction parameters of the heart were determined by use of programmed electrical stimulation (PES) protocols. Sinus node recovery time (SNRT) was not different between WT and Trpm4-deficient mice. Effective refractory period of atrium (AERP), AV node (AVNERP) and ventricle (VERP) were in the same range in WT and Trpm4-deficient mice. Wenckebach periodicity, the parameter for AV nodal conduction, was also not different between WT and Trpm4-deficient mice. These results suggest that deletion of Trpm4 has no effects on the conduction properties of the murine heart.

G. Jacobs: None. **M. Kecskes:** None. **R. Vennekens:** None.

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Improved Ca-cycling by Dexamethasone Preserves Contractility After Ischemia/Reperfusion in Diabetic Rat Cardiomyocytes

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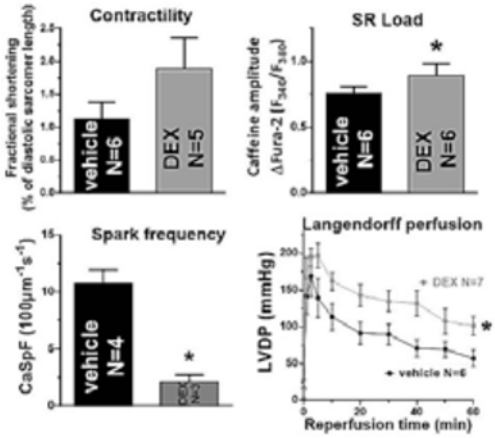
Glucocorticoid receptor (GR) stimulation is essential for normal heart function and dexamethasone (DEX) treatment has been shown to reduce ischemia reperfusion injury (IRI). Prevalence of diabetes is increasing and myocardial infarction in such patients is more severe and risk of complications is augmented. We tested the hypothesis, that GR stimulation with DEX protects diabetic myocardium from IRI by improving Ca cycling.

Cardiomyocytes from type 2 diabetic Zucker diabetic fatty rats were isolated and cultured in DEX (10 μg/mL) vs. vehicle for 24h. $[Ca]_i$ and fractional shortening (FS) was measured in electrical field-stimulated cardiomyocytes loaded with Fura-2 AM (10 μM). Compared to control, DEX-treated cells display increased Ca transient amplitude (0.33±0.03 vs. 0.46±0.04, P = 0.01, n=6 animals) and FS (fig.) DEX also significantly increased sarcoplasmic reticulum (SR) Ca content (Caffeine, 10 mM) and reduced SR Ca leak measured as Ca spark frequency (SpF), fluo-4 loaded myocytes, confocal microscopy (fig.).

To test, whether DEX reduced IRI, rats were treated with DEX (2 mg/kg i.p.) 24h before measurement (vehicle as control). Left ventricular developed pressure (LVDP) was monitored in Langendorff-perfused-hearts during global ischemia for 30 min and reperfusion. During reperfusion, DEX-treated hearts showed significantly greater LVDP ($P < 0.05$, 2way ANOVA, fig.).

In diabetic rats, glucocorticoid receptor stimulation with DEX leads to a more efficient Ca cycling with reduced SR Ca leakage, which translates to an increased contractility on the cellular level and protection against ischemia reperfusion injury in the whole organ.



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Histone Deacetylases Target the Sarcomere to Control Cardiac Relaxation in Rat Model of Heart Failure with Preserved Ejection Fraction

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Objective: Determine the role of histone deacetylase (HDAC) enzymes in the control of myocardial relaxation.
Methods: Dahl Salt Sensitive rats (DSSRs) were fed a 4% NaCl diet (high salt) or a 0.4% NaCl diet (low salt diet). High salt fed animals were further divided to receive ITF2357, a pan-HDAC inhibitor (3 and 30 mg/kg), or vehicle control for 10 weeks. Echocardiography was performed at 4, 6, 8 and 10 weeks. Pressure-volume analysis of left ventricular function was assessed prior to animal sacrifice. Hearts were preserved by flash freezing in liquid nitrogen. Myofibril mechanical studies were performed using frozen heart sections. Western blot and 2-dimensional gel analyses were performed.
Results: DSSRs fed high salt diet develop salt dependent hypertension, cardiac hypertrophy, and diastolic dysfunction as determined by E/A ratio, isovolumic relaxation time, ventricular wall thickness, and left ventricular end diastolic pressure. High salt diet did not cause significant cardiac fibrosis from baseline. ITF2357 treatment led to dose-dependent prevention of diastolic dysfunction. ITF2357 did not change the degree of high salt diet-induced hypertension, cardiac hypertrophy or fibrosis. To test the hypothesis that HDACs regulate cardiac relaxation at the level of the sarcomere, myofibril mechanical studies were performed. Myofibrils from DSSRs fed a high salt diet exhibited significant prolongation of linear and exponential phases of relaxation. In contrast, tension generation and the kinetics of activation were not significantly altered by high salt diet. HDAC inhibition with ITF2357 completely prevented prolongation of both phases of myofibril relaxation. In myofibril enriched fraction, HDAC 2 and HDAC 6 co-purified with the cardiac myofibril.
Conclusions: Diastolic dysfunction in DSSRs is due, at least in part, to relaxation abnormalities at the level of the sarcomere. HDAC inhibition targets myofibrils to improve diastolic cardiac function independently of hypertrophy, fibrosis and blood pressure. And HDAC 2 and HDAC 6 co-purify with the cardiac sarcomere.
Clinical Implication: HDAC inhibition may provide a novel therapeutic strategy for improving cardiac relaxation in heart failure with preserved ejection fraction.

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Chromogranin A is a Potent Prognostic Biomarker in Acute Heart Failure, and its Fragment Catestatin Regulates Cardiomyocyte Calcium Homeostasis by Camkiiδ Inhibition

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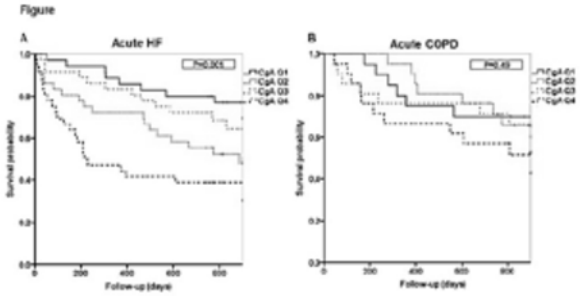
Background: Circulating chromogranin A (CgA) levels have been found associated with clinical outcomes in cardiovascular disease, but whether the CgA fragment catestatin (CST) may directly modulate cardiomyocyte Ca^{2+} handling is not known.
Methods: The prognostic utility of circulating CgA levels were compared between patients with acute HF (n=143) and chronic acute obstructive pulmonary disease (COPD, n=84). Functional effects of CST were assessed in isolated cardiomyocyte and explanted hearts.
Results: CgA levels were associated with mortality in acute HF patients, but not in acute COPD. (Figure; patients stratified according to CgA quartiles on hospital admission). Admission CgA levels were also associated with mortality after adjusting for other risk factors in multivariate analysis. We found CST to interact with Ca^{2+}/calmodulin (CaM)-dependent protein kinase II δ (CaMKIIδ) and to inhibit CaMKIIδ activity. CST also reduced CaMKIIδ-dependent phosphorylation of the ryanodine receptor 2 and phospholamban. In line with CaMKIIδ inhibition, CST reduced Ca^{2+} spark and wave frequency, attenuated Ca^{2+} sparkdimensions, increased sarcoplasmic reticulum Ca^{2+} content, andaugmented magnitude and kinetics of cardiomyocyte Ca^{2+} transients and contractions. Frequency dependent acceleration of relaxation was most pronounced in the Control group, an indication of CaMKIIδ activation, and this was attenuated by CST.**Conclusions:** CgA regulates cardiomyocyte Ca^{2+} handling via CaMKIIδ inhibition, which makes CgA an interesting CV biomarker and potential compensatory mechanism in situations of enhanced CaMKIIδ activity.



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Increased STIM1 Expression Results in Altered Calcium Handling and Heart Failure

ABSTRACTS

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Stromal interaction molecule 1 (STIM1) is a Ca2+ sensor that partners with Orai1, resulting in store-operated Ca2+ entry (SOCE) that is important for maintaining endoplasmic reticulum (ER) Ca2+ homeostasis. STIM1 is expressed in the heart and upregulated during disease, but its role in disease progression is unclear. In this study we used transgenic mice with STIM1 overexpression in the heart to model the known increase of this protein in response to cardiac disease. We found that STIM1 transgenic myocytes showed elevated Ca2+ entry following store depletion and STIM1 co-localized with the type 2 ryanodine receptor (RyR2) in the sarcoplasmic reticulum (SR). In addition, STIM1 transgenic mice exhibited sudden cardiac death as early as 6 weeks of age, while mice that survived past 12 weeks developed cardiac hypertrophy that progressed to heart failure, pulmonary edema, activation of the fetal gene program, alterations in mitochondrial structure, and reduced ventricular functional performance. When pre-symptomatic STIM1 transgenic mice were subjected to disease stimuli including pressure overload stimulation or neurohumoral agonist infusion, they showed greater pathology compared to control mice. STIM1 elevation also disrupted normal Ca2+ handling in cardiac myocytes, which showed spontaneous Ca2+ transients that could be inhibited by the SOCE blocker SKF-96265, as well as increased diastolic Ca2+ levels and elevated Ca2+ spark frequency. In keeping with this increase in Ca2+ cycling we also found that STIM1 elevation resulted in an increased baseline activity of cardiac nuclear factor of activated T-cells (NFAT) and Ca2+/calmodulin-dependent protein kinase II (CaMKII). This increased CaMKII activity did not, however, translate into additional RyR2 phosphorylation, suggesting that the augmented Ca2+ spark frequency observed was likely due to an elevation in SR Ca2+ load. Our results suggest that increased STIM1 expression elicits augmented Ca2+ entry, SR Ca2+ load and Ca2+ spark frequency, that leads to mitochondrial pathology and the induction of Ca2+ sensitive hypertrophic signaling pathways that contribute to cardiac disease.

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Metformin Increases Degradation of Ubiquitinylated Phospholamban via Autophagy in Cardiomyocytes

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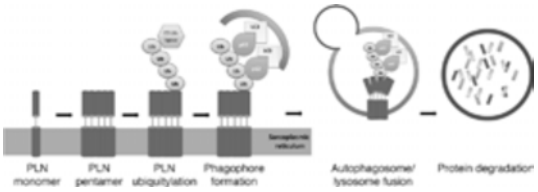
Phospholamban (PLN) is an effective inhibitor of the sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA) in striated muscles. Here, we examined PLN stability and degradation in primary cultured mouse neonatal cardiomyocytes (CMNCs) and mouse hearts using immunoblotting, molecular imaging, and [35S]-methionine pulse-chase experiments along with lysosome (chloroquine and bafilomycin A1) and autophagic (3-methyladenine and Atg5 siRNA) antagonists. Inhibiting lysosomal and autophagic activities promoted endogenous PLN accumulation, whereas accelerating autophagy with metformin enhanced PLN degradation in CMNCs. Metabolic labeling reaffirmed that metformin promoted wild-type and R9C PLN degradation. Immunofluorescence showed that PLN and the autophagy marker, microtubule light chain 3 (LC3), became increasingly co-localized in response to chloroquine and bafilomycin treatments. Mechanistically, pentameric PLN was polyubiquitinylated at K3 residue and this modification was required for p62-mediated selective autophagy trafficking. Consistently, attenuated autophagic flux in Hace1-null mouse hearts was associated with increased PLN levels determined by immunoblot and immunofluorescence. Our study identifies a biological mechanism that traffics PLN to the lysosomes for degradation in mouse hearts.



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Contractile Function and Myofilament Proteins of the Naked Mole-rat Heart Show Resistance to Oxidative Stress

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The naked mole-rat (NMR) is the longest-lived rodent, with a maximum lifespan of >31 years. Unlike every other mammal studied to date, this species withstands cardiovascular structural and functional changes for at least 75% of its lifespan. Due to the intersection of oxidative stress, aging, and cardiovascular disease, we questioned if NMRs were more resistant to oxidative stress-induced cardiac dysfunction compared to short-lived mice. Echocardiography showed that 7 days after a 20 mg/kg dose of doxorubicin (DOX), mice had a 25% decline in fractional shortening (36 ± 1% to 27 ± 2%). In contrast, the fractional shortening of NMRs was unchanged with DOX treatment (27 ± 1%). Previously we observed that while basal cardiac function is low, NMRs have a robust cardiac reserve, displaying a 1.7 fold increase in fractional shortening under exercise-like conditions. DOX-treated NMRs had a significant reduction in their dobutamine response, signifying a diminished cardiac reserve. Intriguingly, we found no changes in phosphorylation or expression of myofilament proteins in the NMR heart with DOX treatment. Mice on the other hand, increased the phosphorylation of cardiac myosin binding protein-C and switched expression from predominantly α-myosin heavy chain to the β-isoform. Electron microscopy showed that DOX caused marked mitochondrial swelling and loss of cristae as well as massive cardiac myofibrillar disarray in mice. Conversely, DOX-treated NMRs had only slight alterations to myofilament structure. NMRs additionally had twofold higher levels of glutathione in their hearts, indicating a high antioxidant capacity. These findings reveal that long-lived NMRs are less susceptible to oxidative stress-induced cardiac dysfunction than mice. The NMR's low

basal cardiac function, unique regulation of myofilament proteins, and high glutathione levels may all be integral in the species' ability to withstand oxidative damage and preserve cardiac function well into its third decade of life.
K.M. Grimes: None. **D. Barefield:** None. **D.A. Kramer:** None. **S. Sadayappan:** None. **R. Buffenstein:** 2. Research Grant; Modest; American Heart Association (12GRNT12030299).

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DWORF: Discovery and Characterization of a Cardiac Micropeptide Encoded in a Putative Long Noncoding RNA
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Background: Mammalian transcriptomes contain thousands of potential open reading frames (ORFs) with fewer than fifty codons. Most of these ORFs are presumed to be random and therefore noncoding, but recently several genes annotated as long noncoding RNAs (lncRNAs) were shown to encode functional micropeptides. **Objective:** Identify and characterize novel micropeptides encoded in cardiac-expressed lncRNAs using comparative genomics. **Methods and Results:** Using codon substitution frequency, a comparative genomics strategy, we identified several previously unknown small proteins, including a 34-amino acid micropeptide that we have named DWORF (Dwarf Open Reading Frame). DWORF is a tail-anchored transmembrane protein that localizes to the sarcoplasmic reticulum and is expressed only in cardiac ventricle and slow-twitch skeletal muscle by quantitative PCR. We subsequently derived a custom antibody targeting the N-terminal residues of DWORF. Western blots using this antibody revealed a 4 kDa protein that is expressed in cardiac ventricle and soleus muscle, but not other tissues. We further validated specificity of this signal by disrupting the reading frame of DWORF in mice using CRISPR/Cas9 mutagenesis, conclusively demonstrating that the mRNA encodes a small protein. Transgenic overexpression of DWORF in cardiac myocytes resulted in increased peak amplitude of Ca^{2+} transients and improved Ca^{2+} resequestration kinetics with no apparent adverse effects. These findings suggest that DWORF specifically enhances activity of the sarco/endoplasmic reticulum calcium ATPase (SERCA). **Conclusions:** Some putative lncRNAs encode micropeptides that can be identified using bioinformatics strategies. Micropeptides, though likely too small to have enzymatic activity of their own, may have modulatory functions in important cellular processes such as calcium handling, as we have demonstrated in the case of the muscle-restricted micropeptide DWORF.
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Serum HDL-C Decreases in MicroRNA-33b Knock-in Mice for an Intron of Sterol Regulatory Element-binding Factor 1 (Srebf1)
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Background: MicroRNAs (miRs) are small non-protein-coding RNAs that bind to specific mRNAs and inhibit translation or promote mRNA degradation. Recent reports, including ours, indicated that miR-33 (miR-33a) located within the intron of sterol regulatory element-binding factor (SREBF) 2 controls cholesterol homeostasis. Primates, but not rodents, express a second miR-33 gene (miR-33b) from an intron of SREBF1. To address miR-33b function in vivo, we developed humanized mice, in which a miR-33b transgene is inserted within a Srebf1 intron. Methods: The human miR-33b sequence was introduced into intron 16 of mouse Srebf1 because miR-33b is located in intron 16 of human SREBF1 and there are high homologies in exons 16 and 17 between human and mouse. The expression of serum miRNA was normalized with cel-miR-39 as a spike-in control. Results: We successfully established miR-33b knock-in (KI) mice with C57BL/6 background and this miR-33b KI strategy did not alter Srebf1 intron 16 splicing, which was confirmed by RT-PCR and sequencing. An LXR agonist T0901317, which induces Srebf1 expression, enhanced miR-33b expression in primary hepatocytes and the liver of miR-33 KI mice. The protein levels of known miR-33a target genes, such as ABCA1, ABCG1, and SREBP-1, were reduced compared with those in wild-type mice. Peritoneal macrophages from the miR-33b KI mice had a reduced cholesterol efflux capacity via apoA-I and HDL-C. Serum HDL-C levels were reduced by almost 35% in miR-33b KI mice. HPLC elution analysis showed that the decreased HDL-C levels were mainly composed of very large-, large-, medium sized HDL-C, which was compatible with the previous results of miR-33a deficient mice. Next, we measured miR-33b levels in serum of human. The expressions of miR-33b-3p were inversely correlated with serum HDL-C levels (P=0.025, R=0.336). Conclusions: miR-33b KI mice for an intron of Srebf1 showed reduced HDL-C level and serum miR-33b-3p levels were inversely correlated with HDL-C levels in human. These results indicate that miR-33b can be a potential target for raising HDL-C and may account for lower HDL-C levels in humans than those in mice. These mice will aid in elucidating the roles of miR-33s and screening of the drugs that can alter miR-33s levels and activities.
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Deletion or Knockdown of Myotonic Dystrophy Protein Kinase Does Not Affect Cardiac Conduction or Ejection Fraction in Mice
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Myotonic dystrophy type 1 (DM1), the most common form of muscular dystrophy in adults, causes dominantly-inherited muscle weakness, defects of cardiac conduction, variable LV dysfunction, and risk of sudden death. The genetic basis is an expanded CTG repeat in the 3' untranslated region of DMPK. DM1 patients are functionally hemizygous for DMPK protein, due to nuclear retention of mRNA having expanded repeats. The cardiac aspects are attributed to DMPK loss, toxicity of RNA with expanded repeats, or both. Dmpk heterozygous (+/-) and homozygous knockout (-/-) mice were reported to show AV conduction abnormalities resembling DM1 (Berul et al, JCI, 1999). In an effort to reduce RNA toxicity, antisense oligonucleotides (ASOs) targeting DMPK mRNA have recently entered clinical trials. DM1 phenotypes in skeletal muscle were corrected by ASO knockdown of toxic RNA in mice (Wheeler et al, Nature, 2012). While ASOs may have similar potential to mitigate RNA toxicity in the heart, there is risk of aggravated DMPK deficiency. To reexamine the role of DMPK in the conduction system we studied mice with Dmpk gene deletion or ASO knockdown. We obtained ECGs and echocardiograms on Dmpk -/- and +/- mice, compared

to WT littermates. The +/- mice were treated with Dmpk-targeting ASOs or saline. Subcutaneous injection of 50 mg/kg/wk ASO was started at age 2 months, then shifted to biweekly injections after 6 weeks. Dmpk expression in hearts of +/- mice was ~50% of WT, and was further reduced by ASOs (84 ± 3% decrease of mRNA, 93 ± 2% decrease of protein, relative to WT). Surface ECGs and echocardiography at 6 and 10 months showed no differences of heart rate, cardiac conduction, or ejection fraction in WT, saline-treated +/-, ASO-treated +/-, or -/- mice. Conscious, unrestrained ECGs obtained at 11-12 months by radiotelemetry showed no differences among WT, saline-treated +/-, ASO-treated +/-, or -/- mice. We conclude that ASOs can induce posttranscriptional silencing of Dmpk in murine hearts. Constitutive absence of DMPK did not impact cardiac conduction or contractility, and the same was true for ASO knockdown to levels <15% of WT. Our data support the idea that cardiac dysfunction in DM1 results mainly from RNA toxicity, which potentially could be prevented or alleviated by ASOs.

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High Fat Diet Promotes Haplotype Dependent Differential Transcriptional Regulation of the Human Angiotensinogen Gene

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Angiotensinogen (AGT) is the only known precursor to angiotensin II. Systemic renin angiotensin aldosterone system (RAAS) is activated in human and experimental models of obesity. RAAS activation in obesity is linked to the development of cardiovascular pathophysiologies. We have identified polymorphisms in 2.5 Kb promoter of human angiotensinogen gene (hAGT) that forms two haplotype (Hap) blocks: -6A/G (-1670A/G, -1562C/T, -1561T/C) and -217A/G (-532T/C, -793A/G, -1074T/C,& -1178G/A). Hap -6A/-217A (Hap -6A) is associated with human hypertension whereas, Hap -6G/-217G (Hap -6G) reduces cardiovascular risk. Here, we examine high fat diet-mediated allele-specific transcriptional regulation of the hAGT gene in adipose tissue, in vivo, in transgenic (TG) mice engineered with either haplotype of the hAGT gene. Twelve-week-old male TG mice with Hap –6A or –6G were fed normal diet (10% kcal as fat) and high fat diet (60% kcal as fat) for 10 weeks. Using Q - RT PCR and western blot we show increased hAGT expression in adipose tissue of the Hap -6A-TG mice after high fat diet than control diet (Hap -6A- 0.68±0.04 vs. Hap -6G- 0.33±0.03 A.U., $p<0.05$). ChIP assay shows greater chromatin binding of GR, MR, CEBPβ and STAT3 transcriptional factors (Hap -6A- 0.80±0.04 vs. Hap -6G- 0.26±0.06 A.U., $p<0.05$) to the hAGT transgenes in Hap -6A TG mice after high fat diet. No significant change was observed in the endogenous mouse AGT gene. In addition, after high fat diet, change (Δ) in inflammatory and redox markers was significantly ($p<0.05$) greater in TG mice with Hap I including, IL1 (4.6±0.8 vs. 2.1±0.49 fold), IL6 (4.0±0.69 vs. 2.1±0.2 fold) and NOX1 (8.3±0.4 vs. 2.5±0.6 fold). This is accompanied by reduction in levels of antioxidant defenses (SOD1: 0.97±0.0 vs. 1.4±0.1 fold; HO1: 0.77±0.1 vs. 1.3±0.2 fold) & activation of MAPK14 and ERK1/2 signaling. Taken together, our results show that SNPs in the hAGT Hap -6A favor high fat diet induced binding of transcriptional factors GR, MR, CEBP-β and STAT3 that lead to elevated expression of the hAGT in expanded mass of the adipose tissue. This also activates the RAAS with pathophysiological implications including, phosphorylation of kinases such as MAPK14 and ERK2, increase in tissue pro-inflammatory and oxidative stress molecules.

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RNA-sequencing of Disease-specific iPSC as a New Filter to Identify Genes Associated With Hypoplastic Left Heart Syndrome

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Hypoplastic Left Heart Syndrome (HLHS) is a complex multifactorial disease for which no definitive genetic causes have been found. Current genetic filtering strategies render lists of genes with unknown relevance in terms of pathogenesis. A complementary filter based on biological evidence would create a new approach to prioritize relevant candidate genes and mutations.
In our study, 5 members of a nuclear family including a child with HLHS were evaluated using echocardiography and their genetic information was obtained through whole genome sequencing (WGS). Data filtering including rarity, functional impact and mode of inheritance was implemented, resulting in identification of 34 genes with recessive or *de novo* variants potentially involved in the pathogenesis of HLHS. Additionally, iPSC were derived from proband and parents and subjected to RNA-sequencing at the undifferentiated state and following spontaneous differentiation. Comparative transcriptional analyses identified genes differentially expressed in proband samples at each stage. These gene sets were used as an additional filter for the previously generated WGS data. This strategy revealed that out of 34 mutated genes originally identified, 10 displayed transcriptional differences in undifferentiated iPSC from the HLHS-affected individual while 16 out of 34 mutated genes showed significantly different expression levels in differentiated cells from proband. Furthermore, expression dynamics were studied during guided cardiac differentiation for the 9 genes fulfilling all applied criteria. Two genes not previously linked to HLHS, ELF4 and HSPG2 were found to behave significantly different in HLHS-iPSC when compared to control counterparts.
In summary, filtering WGS data according to a new layer of transcriptional information that leverages iPSC plasticity allows prioritization of genes associated with HLHS in an in vitro model of disease.

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Characterization of Arrhythmogenic Dilated Cardiomyopathy Caused by Novel Filamin C Splice Variant in a Zebrafish Model

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Although dilated cardiomyopathy (DCM) is a serious and frequent genetic cause of heart failure, only 30-40% of cases can be attributed to a known DCM gene mutation. To identify and confirm additional disease genes involved in DCM, we performed whole exome sequencing in two multigenerational families with DCM, both from the same geographic region of Italy, and found a novel splice variant in the gene encoding filamin-C (FLNC). Previously characterized mutations in FLNC had been primarily linked to skeletal muscle disease, although none of the affected family members displayed skeletal myopathy. To confirm and further characterize the arrhythmogenic DCM phenotype observed in family members, we performed embryonic knockdown experiments using morpholino (MO) treatment in zebrafish (Danio rerio) targeting the FLNC ortholog, filamin Cb (flncb). Following MO injection into 1-2 cell stage zebrafish embryos, 63.4% (78 of 123) of viable flncb MO-injected embryos displayed a cardiac phenotype at 72 hours post fertilization (hpf) (vs. 17.0% [30 of 177] of control MO-injected embryos; p≤0.001). Increases in mortality were observed, with 20.8% (54 of 260) of flncb MO-injected embryos surviving at 7 days post fertilization (vs. 65% [162 of 249] of control embryos; p≤0.001). The flncb MO-injected embryos demonstrated pericardial edema, dysmorphic or dilated cardiac chambers, and abnormal looping of the heart tube suggestive of systolic dysfunction. The flncb MO-injected embryos additionally demonstrated a lower mean stroke volume than controls (0.076 vs. 0.181 nl; p=0.015), a reduced mean cardiac output (10.8 vs. 25 nl/min; p=0.02), and an increase in the fraction of retrograde blood flow over the cardiac cycle (0.42 vs. 0.03; p=0.027). Overall, this flncb MO treatment recapitulated a DCM phenotype similar to the state caused by the human splicing variant, supporting haploinsufficiency as the mechanism leading to DCM in these families. Our findings suggest that approaches to augment endogenous filamin C protein levels may represent a viable treatment strategy that warrants exploration in future studies.

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Microrna-132/212 Family Enhances Arteriogenesis After Hindlimb Ischemia Through Modulation of the Ras-MAPK Pathway

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Arteriogenesis is a complicated process induced by local shear stress caused by local occlusion and is enhanced by growth factors such as VEGF, which is secreted by inflammatory cells and endothelial cells in response to hypoxia. To search for microRNAs involved in arteriogenesis, we performed microarray analysis on hindlimb ischemia mice tissue. We observed a dynamic temporal regulation of microRNAs expression , among which miR-132 /212 were significantly upregulated 4 days after occlusion of femoral artery. The aim of our study was to unravel the role of miR-132/212 in arteriogenesis. In a pericyte-endothelial cell in vitro co-culture assay, overexpression of miR-132/212 in endothelial cells resulted in enhanced neovascularization capacity of the endothelial cells, including more tubular structures and junctions and longer total tubule length. Inhibition of miR-132/212 decreased total tubule length, and the number of junctions and tubule structures. Ex vivo aorta ring assay demonstrated more branches in wild-type than of miR-132/212 knockout mice. In line with this, microRNA132/212 knockout mice displayed slower perfusion recovery after hind-limb ischemia than wild type mice. Immunohistochemistry studies further demonstrated that knockout mice have similar number of collateral arteries but they are smaller than wild type. Based on microRNA targets prediction and luciferase assay we found that miR-132/212 enhanced Ras-MAPK signaling by directly inhibiting Rasa1, Spred1, and Spry1 expression which are inhibitors of the Ras-MAPK signaling pathway. In summary, our results suggests that miR-132/212 may promote arteriogenesis by modulation of Ras-MAPK signaling upon ischemia.

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Cardiotrophin-1 Promotes Physiologic Cardiac Hypertrophy

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The post-natal heart retains a remarkable adaptive capacity, which matches growth of the myocardium to increased physical demands. The beneficial form of myocardial growth is referred to as physiologic hypertrophy, and derives from a compensatory response to conditions that require increased blood volumes such as pregnancy and chronic exercise training. Despite the clear benefits associated with this form of adaptation, the molecular mechanism(s) that initiate or sustain such cardiac remodeling remain obscure. As such, identification of factors that promote physiologic hypertrophy will be of considerable interest to both cardiac biologists and clinicians. Here, we have begun to explore the role of the cytokine, cardiotrophin 1 (CT-1) as a central mediator of physiologic hypertrophy. We demonstrate that primary cardiomyocytes treated with recombinant human CT-1 protein engage a physiologic growth response, adding sarcomeres in series, a cellular/molecular change that is fully reversible with the removal of CT-1. This physiologic hypertrophy response to CT-1 is dominant, as CT-1 administration overrides the pathologic hypertrophy associated with agonists such as phenylephrine. In vivo delivery of human CT-1 protein leads to a rapid (14 days) alteration in the structure of the rat heart, with volume and wall dimension increases characteristic of exercise adaptation, changes that are fully reversible upon cessation of CT-1 treatment. In addition, CT-1 administration mitigates right heart failure in a model of pulmonary arterial hypertension, and sustains cardiac function in the post infarct rat heart. Finally, gene expression analysis revealed that CT-1 treatment leads to up-regulation of angiogenic factors within cardiomyocytes, suggesting that this cytokine engages a paracrine signaling mechanism to ensure that myocardial growth is matched by a robust angiogenesis.

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Selective Destruction of Adrenergic Cells in Mice Leads to Severe Left-ventricular Dysfunction at Rest With Apparent Stress-induced Recovery

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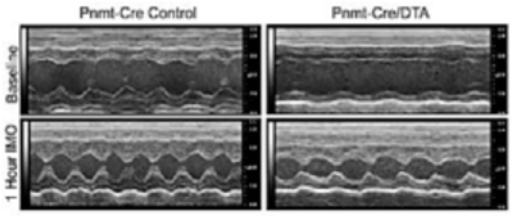
The catecholamines epinephrine and norepinephrine play critical roles in the maintenance of cardiovascular function. Phenylethanolamine-N-methyltransferase (Pnmt) catalyzes the conversion of norepinephrine to epinephrine and serves as a marker for adrenergic cells. We have previously shown that the selective destruction of Pnmt+ cells in the mouse produces severe left-ventricular dysfunction under anesthesia and that epinephrine deficiency alone does not recapitulate the phenotype. Here, we test the hypothesis that Pnmt+ cells are key modulators of the stress response to immobilization. Using a suicide reporter mouse model to ablate Pnmt+ cells (Pnmt-Cre/DTA), we achieve greater than 50% Pnmt+ cell reduction in the adrenal medulla and 97% reduction in Pnmt transcript. Remarkably, Pnmt+ cell destruction does not markedly diminish the cardiovascular response to restraint stress. At one hour of immobilization, heart rate and ejection fraction showed a similar increase in response to restraint in both groups, despite apparent heart failure in Pnmt-Cre/DTA mice at rest (Figure 1). Here, we show that, while Pnmt+ cells are required for the maintenance of basal cardiovascular function, loss of these cells does not diminish the cardiovascular response to restraint stress. These results suggest that left ventricular cardiac muscle has the capacity to respond to stress despite hypokinetic and poor ventricular function at baseline.



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Aberrant Endothelial-myocardial Crosstalk Causes Hypertrophy in Noonan Syndrome with Multiple Lentigines

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Congenital heart disease (CHD) is the most common birth defect worldwide; however, underlying mechanisms remain unknown. Loss-of-function mutations in PTPN11, the gene encoding the protein tyrosine phosphatase SHP2, are implicated in CHD and cause Noonan Syndrome with Multiple Lentigines (NSML). NSML presents with multiple cardiac defects, including hypertrophy. Here, we found that the NSML-associated adult-onset cardiac hypertrophy stems from aberrant signaling originating from developing endocardium. Embryonic NSML hearts showed diminished trabeculation and valvular hyperplasia, defects recapitulated in endocardial-, but not myocardial- or neural crest-, specific NSML mice. NSML hearts also developed ventricular septal defects, a phenotype reproduced only in myocardial-specific NSML hearts, suggesting NSML mutations have both cell autonomous and non-autonomous functions in cardiac development. Importantly, endocardial-specific expression of NSML was sufficient to induce adult-onset cardiac hypertrophy. Mechanistically, we observed aberrant AKT activity in NSML embryos, with decreased downstream FOXP1/FGF and NOTCH1/EPHB2 signaling, two pathways necessary for reciprocal crosstalk between developing endocardium and myocardium. Taken together, our data provide the first functional and disease-based evidence to suggest that critical mechanisms exist to control endocardial-myocardial crosstalk, the aberrant regulation of which may lead to CHD and adult-onset cardiac disease.

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Dissecting the Shox2-nkx2-5 Antagonistic Mechanism in the Pulmonary Vein Myocardium and Sinoatrial Node

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Atrial fibrillation is often triggered by ectopic pacemaking activity in the myocardium sleeves of the pulmonary vein (PV) and systemic venous return. However, the genetic programs that abnormally reinforce pacemaker properties at these sites and how this relates to normal sinoatrial node (SAN) development remain uncharacterized. We have identified a Shox2-Nkx2-5 antagonistic mechanism that primes the pacemaking cell fate in the PV myocardium and SAN in the embryonic stage. Specifically, Shox2 deletion in the Nkx2-5+ domain of the SAN caused sick sinus syndrome, associated with the loss of pacemaker program. Nkx2-5 hypomorphism rescued the requirement for Shox2 for the expression of genes essential for SAN development in Shox2 mutants. Similarly, the pacemaker-like phenotype induced in PV myocardium in Nkx2-5 hypomorphs reverted back to a working myocardial phenotype when Shox2 was simultaneously deleted. Shox2 interacts with Nkx2-5 directly, and a substantial genome wide co-occupancy of Shox2, Nkx2-5, and Tbx5 suggest a balanced transcription output is essential for the pacemaker cell fate determination. Further characterization of mice carrying allelic series of Shox2 and Nkx2-5 revealed an essential role of both Shox2 and Nkx2-5 in maintain the normal development of pulmonary vein myocardium and the structures in the venous pole, suggesting the importance of transcriptional precision of Shox2 and Nkx2-5 and a role of Shox2-Nkx2-5 antagonistic mechanism in integrating the transcriptional accuracy of Shox2 or Nkx2-5.

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Novel Cardiac Dyssynchrony Metrics Based on Ultrasound Images and Fibrosis

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Ventricular stiffening is a component of heart failure with preserved ejection fraction. This study focuses on building a dyssynchrony metric based on ultrasound images obtained from normal and fibrotic mouse hearts. The objective is to evaluate the association between regional fibrosis and ventricular wall dynamics as an indicator for the gradient of cardiac dyssynchrony.

Ultrasound images were obtained from a previous study in which mice were given thoracic aortic constriction to induce heart failure that included ventricular stiffening and fibrosis. Ultrasound video (frame rate 4.3ms) along the short axis of the mouse heart was captured using commercial software. On each short axis frame, six points were selected and traced on the endocardial wall. Each radial displacement pair is plotted in a loop diagram against one another in 15 unique combinations.

A loop whose points are close to the *ideal line u = v* corresponds to synchronous wall movement, while the area enclosed by the loop is proportional to time dyssynchrony. The inferior free wall and the section nearest the interventricular septum had 35% (26,48) interstitial fibrosis compared to the IS itself which had 9% (7, 13) in hearts with 25% fibrosis, and the contrast had the most variance (8.7%, P<0.0001). Strong predictors of the contrasting fibrosis included the trace perimeter and the maximal normalized displacement, but more pragmatically, the differences between the two conditions are apparent and consistent.

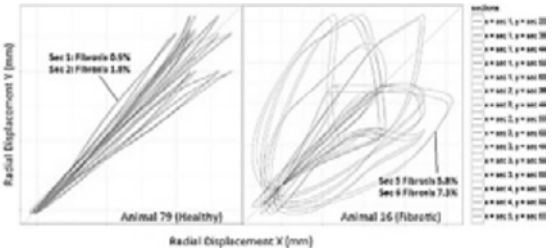

Figure 1 is the set of (u,v) pairs measured over the course of a heartbeat where u is the radial displacement of heart segment x measured when segment y had radial displacement v.

G. Shearer: None. **T. Hanson:** None. **J. Eclov:** None. **S. Courtier:** None. **S. Ortmeier:** None. **D. Ewert:** None.

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Enhancing Pgc-1α Activity Improves Heart Function Through Activating Mitochondrial Biogenesis in Chagas Disease

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Chronic chagasic cardiomyopathy (CCM) is presented with ventricular hypertrophy and contractile dysfunction that can lead to heart failure. I have found that a substantial decline in mitochondrial biogenesis and SIRT1/PGC-1α activity ensue in chronic chagasic mice. It was evidenced by the decline in mitochondrial DNA content as well as mRNA levels of mitochondrial encoded genes and mtDNA replication machinery. Further, the activity of SIRT1 (required for PGC-1α activation) was decreased and associated with decreased nuclear levels of PGC-1-regulated NRF1 transcription factor in chagasic hearts. The mitochondrial size and number were also reduced in chagasic heart, determined by electron microscopy. Therefore, we hypothesized that enhancing the SIRT1/PGC-1α activity by SIRT1 agonist would improve heart function through activating mitochondrial biogenesis in Chagasic disease. Mice were infected with T. cruzi, and beginning at day 90 post-infection (pi), treated with resveratrol (SIRT1 agonist) or metformin (AMPK agonist, can enhance SIRT1 activity) for 21 days; and then heart function was monitored at 150 days pi. We found that treatment with resveratrol partially attenuated the heart dysfunction (stroke volume, cardiac output, ejection fraction, heart rate) and cardiac hypertrophy in chagasic mice. These benefits were associated with improved expression of the mitochondrial DNA encoded genes and mtDNA content though the expression of genes involved in mtDNA replication was not improved. Treatment with metformin was not significantly beneficial in improving the CCM outcomes. The partial beneficial effects of resveratrol could be due to inefficient activation of SIRT1 or delayed start of the treatment. We plan to treat mice with SIRT1 agonist SIRT1720 (10 fold more active than resveratrol) during the indeterminate phase of T. cruzi infection in next set of experiments. This study will improve our understanding of the molecular and immune mechanisms of chagasic heart disease and will provide a novel treatment for chronically-infected chagasic patients.

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Genetic Ablation of Interleukin-18 Does Not Attenuate Hypobaric Hypoxia-Induced Right Ventricular Dysfunction

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Interleukin-18 (IL-18), a pro-inflammatory cytokine, has been implicated in pathogenic left ventricular hypertrophy and is elevated in plasma of heart failure patients. However, IL-18 blockade strategies in animal models of heart disease have been conflicting. Accordingly, the purpose of these experiments was to determine whether genetic ablation of IL-18 would protect male and female mice against hypobaric hypoxia-induced right ventricular (RV) dysfunction. We hypothesized that IL-18 knockout mice (KO) would be protected while wild type (WT) mice would show significant right ventricular dysfunction in response to exposure to hypobaric hypoxia (HH). KO and WT mice were exposed to HH for 7 weeks, and control (CO) mice were exposed to normoxic, ambient air. Following echocardiography, the RV was dissected and flash frozen for biochemical analyses. 7 week HH exposure trended toward an increase in IL-18 mRNA (p=0.08) in RV from WT mice. However, contrary to our hypothesis, IL-18 KO mice were not protected against HH-induced RV dysfunction, as evidenced by higher RV weights, elevated RV systolic pressure, and increased RV anterior wall thickness compared to normoxic KO mice. Importantly, these measurements were not significantly different from WT HH mice. Biochemical analyses suggest HH RV underwent early remodeling, as no changes were observed at the molecular level in the RV of HH mice compared to CO in either KO or WT animals. Compensatory upregulation of the other pro-inflammatory cytokines IL-2 and SDF-1 may have contributed to the lack of protection in IL-18 KO animals. These data suggest IL-18 signaling is not necessary for hypobaric hypoxia induced RV dysfunction, and blockade of IL-18 is not a viable therapeutic strategy in this model.

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Cardiomyocyte T-tubule Membrane Turns Over, Releasing BIN1 Containing Microparticles into Blood

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Bridging integrator 1 (BIN1) is a cardiac muscle protein that folds cardiomyocyte T-tubule membrane. BIN1 is intrinsic to cardiac health, and is reduced in acquired heart failure. Interestingly, we have found that BIN1 is also blood available, and that plasma BIN1 correlates with cardiac function, suggesting cardiac origin of plasma BIN1. We found that low plasma BIN1 correlates with failing muscle and predicts ventricular arrhythmia. However, the paradigm does not exist for an intracellular membrane associate cardiomyocyte protein to be homeostatically turned over into blood. In this study, using a mouse model with cardiac specific deletion of *Bin1* gene, we identified with biochemical techniques that plasma BIN1 levels directly correlate with cardiac tissue BIN1 levels, indicating cardiac origin. Furthermore, investigations using both super-resolution fluorescent imaging and flow cytometry analysis revealed that adult ventricular cardiomyocytes constantly release BIN1 into blood via membrane microparticle production. Microparticles are small membrane vesicles shed from plasma membrane of a variety of cell types including platelets, leukocytes, and endothelial cells. Using super-resolution three-dimensional stochastic optical reconstruction microscopy (3D-STORM), we found similar to the blood cells, isolated adult mouse cardiomyocytes release Annexin V positive microparticles with diameters ranging between 0.1 to 1.0 µm. These microparticles also carry BIN1 protein. Flow cytometry was also used to detect and quantify microparticles <1.0 µm in size from medium bathing a pure population of adult mouse cardiomyocytes. BIN1 microparticle release is proportional to actin stability and amount of T-tubule membrane folds. Compared to wild type cardiomyocytes, microparticle release is significantly reduced from myocytes with heterozygous deletion of *Bin1* gene. These data indicate that cardiomyocyte membrane undergoes dynamic turnover, releasing T-tubule folds into blood as microparticles. Furthermore, plasma BIN1 can be used as a direct measure of cardiomyocyte health and reserve.

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Detection and Determination of Protein Network Associated With Atrial Fibrillation Subtypes

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Atrial fibrillation (AF) is associated with increased risks of stroke, cardiac failure, and mortality. The underlying mechanisms and pathology of AF remain elusive. The aim of this study is to proteomically analyze the left atrial appendage tissue obtained from patients suffering from subtypes (paroxysmal, persistent, and long-standing persistent) AF.
MALDI Imaging mass spectrometry (MALDI-IMS) was applied to differentiate in classification of pathophysiological AF subtypes, through the direct (in situ) analysis of formalin-fixed paraffin embedded (FFPE) left atrial appendage (LAA) tissue. FFPE LAA tissue were collected from patients with predisposed paroxysmal (n = 9, mean age 69.0±3.1 years), persistent (n = 18, mean age 67.0±2.7 years), and long-standing persistent AF (n = 19, mean age 71.0±2.0 years). Sections were dewaxed and thereupon soused by trypsin solutions using an automated spraying device. Spectra were acquired at a mass range of m/z 800-3500Da and lateral resolution of 80 μm. Two hundred laser shots were acquired per pixel and random walk of 50/position. Data analyses were performed using SCiLS Lab software.
Component analysis of MALDI Imaging data through probabilistic latent semantic analysis results in a clear discrimination in the first 3 components of atrial fibrillation. Employing receiver operating characteristic analysis (AUC > 0.7), characteristic intensity distribution in given m/z values, which are discriminative for the considered cluster, was determined to distinguish between paroxysmal vs. persistent AF, and persistent vs. long-persistent AF, m/z values were determined between persistent vs long-persistent AF (1.59±0.12 vs 6.85±3.02, p = 0.02). Follow-up of neurological events in case-controlled assessment presented 13±12% in paroxysmal, 56±12% in persistent and 42±12% long-persistent AF.
The tissue-based proteomic approach provides clinically relevant beneficial information in improving risk stratification for AF patients. In the future, this obtained information might be considered new biomarker to support the diagnosis of the severity of AF status. They also suggest a new criterion to determine the most appropriate procedure for each AF subtype to improve postoperative outcomes.

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Mlip is a New Integrator of Physiological Stress Required for Proper Cardiac Adaptation

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Aging and diseases are generally a result of the inability of tissues to properly adapt to stress. The heart is particularly vulnerable to disequilibrium in homeostasis as its regenerative capacities are limited. Many molecular players have been identified as cardiac gate-keepers and integrators of stress, all essential to preserve cardiac function. However, the molecular basis of the relationship between aging and the pathogenesis of cardiac dysfunction remains poorly understood. Muscle enriched A-type lamin interacting protein (MLIP) is a unique mammalian protein of unknown function that was recently identified through its interaction with A-type lamins in the heart and as a modulator of cardiac hypertrophy in vitro. Here we report that young Mlip deficient mice develop dilated cardiomyopathy, manifested by an increase in heart mass with reduced cardiac function. Through global gene expression profiling of the Mlip deficient hearts, we identified a deregulation in mTOR signalling, a key stress sensing canonical pathway. Analysis of mTOR regulatory proteins (AMPK and AKT) revealed hyperactivation of the mTOR pathway and a deregulated integration of these two stress/nutrient sensors. These data support the notion that Mlip deficient hearts have impaired cardiac adaptation due to deregulated mTOR activity resulting in maladaptive remodeling, and the development of dilated cardiomyopathy. These results are further supported by a genetic association between

Mlip and early response to pro-hypertrophic stimulus. Collectively, these results demonstrate that Mlip is required for normal integration of physiological stress (postnatal cardiac growth, isoproterenol-induced hypertrophy) through the regulation of the AMPK/Akt/mTOR pathway to maintain cardiac homeostasis in the adult heart.

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microRNA-29b Contributes to Multiple Types of Muscle Atrophy

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Objective: Skeletal muscle atrophy has been observed in a significant proportion of heart failure patients while little is known about the mechanisms underlying muscle atrophy, especially in multiple types of muscle atrophy. MicroRNAs (miRNAs, miRs) are among key participants in gene regulatory networks and their dysregulation contribute to lots of diseases. However, miRNAs responsible for multiple types of muscle atrophy are unclear.
Methods and Result: miRNAs were screened by miRNA arrays and were further verified by real-time reverse transcription polymerase chain reaction with the gastrocnemius sample from denervation -induce muscle atrophy. miR-130b, miR-212, miR-21, miR-221/222, miR-223 and miR-29b were upregulated >3 fold in gastrocnemius samples from denervation mice than that of sham group. However, only miR-29b presented a common change (upregulated >3) in other types of muscle atrophy models including dexamethasone-induced and fasting-induced muscle atrophy. Over-expression of miR-29b in gastrocnemius by intramuscular injection with agomiR-29b significantly promoted muscle atrophy, which is evidenced by the change in morphology (the diameter of myofiber was decrease by 60%) and increased atrophy-related genes including Atrogin-1 and MuRF-1 (upregulated for 1.5 folds and 1.4 folds, respectively). This in vivo data suggest that miR-29b is sufficient to induce muscle atrophy. In multiple types of muscle atrophy C2C12 cell models (dexamethasone, TNF-alpha and H2O2 induced), miR-29b was increased while inhibition of miR-29b could prevent that effects. Using TargetScan software, we found that IGF1 and PI3Ka were potential target genes for miR-29b, which was further validated by luciferase assays and western blotting. Moreover, over-expression of IGF1 or PI3Ka ameliorated muscle atrophy caused by miR-29b overexpression, indicating that IGF1 and PI3Ka are two target genes responsible for miR-29b's pro-atrophy effects in muscle.
Conclusions: Our data indicated that via targeting IGF1 and PI3Ka. Inhibition of miR-29b might represent a novel therapy for multiple types of muscle atrophy including in heart failure.

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Left Ventricular Assist Device (LVAD) Reverses Inhibition on Beta-adrenergic Receptor Resensitization

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Beta-adrenergic receptor (βAR) down-regulation and desensitization are hallmarks of heart failure. Agonist occupied βAR undergo desensitization through phosphorylation by G-protein coupled receptor kinases leading to βAR internalization. Phosphorylated βAR becomes resensitized following dephosphorylation by protein phosphatase 2A (PP2A) in the endosomes and we have shown previously shown that PP2A activity is regulated by phosphoinositide3-kinaseγ (PI3Kγ). Traditionally, it has been considered that increased desensitization mechanisms underlie βAR dysfunction in heart failure but it is not known whether resensitization of βARs is altered and an integral contributor to heart failure. To test whether resensitization mechanisms are altered in heart failure and could play a role in recovery of βAR function post-LVAD, we used paired pre- and post-LVAD human heart samples along with non-failing hearts. Since resensitization occurs in endosomes, plasma membranes and endosomes were isolated from these human heart samples and assessed for PI3K activity, PP2A activity, β2AR phosphorylation and adenylyl cyclase (AC) activity as a measure of recovery in βAR function by G-protein coupling. Significant PI3Kγ activity was observed in the endosomes of pre-LVAD compared post-LVAD and non-failing human heart samples while plasma membrane PI3K activity remained similar across all samples. Similarly, AC activity was markedly reduced in plasma membranes and endosomes of pre-LVAD samples compared to post-LVAD and non-failing showing reduced ability of receptors to couple to G-proteins indicating reduced recovery of receptor function in post-LVAD heart samples. Consistent with the reduced recovery in βAR function in pre-LVAD samples, significant β2AR phosphorylation was observed in the endosomes and plasma membranes of pre-LVAD compared to post-LVAD and non-failing. Correspondingly, we observed significant reduction in endosomal PP2A activity in the pre-LVAD samples which was remarkably reversed in post-LVAD samples similar to the activity in non-failing. These studies suggest that resensitization is inhibited in end-stage human heart failure and may critically contribute to cardiac remodeling and hypertrophic response upon cardiac stress.

E.E. Martelli: None. **A. Chatterjee:** None. **R. Starling:** None. **C. Moravec:** None. **S.V. Naga Prasad:** None.

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Parkin Contributes to the Development of Cardiac Hypertrophy in Response to Cardiac Pressure Overload

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Parkin is an E3 ubiquitin ligase known to mediate mitochondrial clearance by marking damaged mitochondria for autophagy. Our lab has previously shown that Parkin is important for stress adaptation following myocardial infarction, and that loss of Parkin leads to accumulation of dysfunctional mitochondria. However, whether Parkin plays a role in cardiac adaptation to pressure overload is currently unknown. Here we investigated the functional importance of Parkin in cardiac hypertrophy and development of heart failure in response to hemodynamic stress. Wild type (WT), Parkin knock out ($Parkin^{-/-}$), and cardiac-specific Parkin transgenic (Parkin-TG) mice were subjected to trans-aortic constriction (TAC). Cardiac anatomy and function was evaluated by histology and echocardiography. Inflammation and hypertrophy gene expression profiles were assessed using qPCR and immunohistochemistry. We discovered that after 2

weeks of TAC, cardiac hypertrophy markers were not increased in hearts from Parkin$^{-/-}$ mice, and there was no increase in the heart weight to body weight ratio (HW/BW). However, after 8 weeks of TAC, Parkin$^{-/-}$ mice showed similar cardiac hypertrophy and loss of function as WT hearts. Parkin deficient hearts also displayed increased interstitial and perivascular fibrosis compared to WT hearts after 8 weeks of TAC. This suggests that there is a delay in activating the hypertrophy program in the absence of Parkin, and that lack of Parkin leads to excessive fibrosis. In contrast, Parkin-TG mice showed a rapid development of hypertrophy and progression to heart failure compared to WT mice. Interestingly, we observed no differences in either mitochondrial content or LC3 levels after two weeks of TAC in Parkin-TG hearts, suggesting that the rapid development of hypertrophy and early progression to heart failure was not due to excessive mitophagy. These data suggest that Parkin plays an important role in the activation of the cardiac hypertrophy program and that this function may be independent of its role in regulating mitophagy. Thus, this study provides novel insight into the functional importance of Parkin in the heart. Additional studies are needed to determine the mechanism of how Parkin regulates cardiac hypertrophy.

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Titin Phosphorylation by Protein Kinase G as a Novel Mechanism of Diastolic Adaptation to Acute Hemodynamic Overload

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Titin is the main determinant of myocardial passive tension and its distensibility is increased via phosphorylation by protein kinase G (PKG), activated by nitric oxide (NO) and natriuretic peptides (NP) upon acute overload. We hypothesized whether myocardial stretch led to decreased stiffness, optimizing diastolic filling along with the usual increase in contractility. Intact rat Langendorff hearts, strips dissected from the LV or right atrium of cardiac surgery patients and rabbit papillary muscles were acutely stretched for 15min. Passive tension (PT) was measured in skinned cardiomyocytes extracted from the LV of control and stretched rat hearts for sarcomere lengths (SL) 1.8-2.3μm before and after incubation with PKG. Rabbit muscles were incubated with a PKG inhibitor or, simultaneously, a NO synthase inhibitor, a NO scavenger, a NP receptor A antagonist. All-total titin phosphorylation was stained with Pro-Q Diamond and indexed to total-protein signals using SYPRO Ruby. Values are given as mean±SEM and statistical significance was set to $p<0.05$.

After acute stretch there was a progressive decrease in passive tension/diastolic pressure over 15min: 27±8% and 27±6% in human atrium and ventricular muscles, respectively, and 43±2% in rabbit papillary muscles and isolated hearts. This decrease in myocardial stiffness was significantly blunted by PKG inhibition (40%) and NO/NP pathway inhibition (29%). PT of cardiomyocytes was significantly lower (≈60%) in the previously stretched group for all SL. A similar effect, only significant in the control group, was observed after incubation with PKG. Titin phosphorylation increased markedly 15 minutes after acute myocardial stretch in human (atrial: 11±1% vs 41±8%; LV: 27±8% vs 71±21%) and rabbit (13±2% vs 23±3%) myocardium. The progressive decrease in myocardial stiffness after acute hemodynamic overload is preserved at the myofilamental level and seems to depend on PKG activity, representing a potential therapeutic target in patients with pathologically rigid myocardium. Moreover, blocking PKG activation seems to attenuate this adaptive diastolic response. Therefore, titin phosphorylation by this kinase is probably involved in this new myocardial response to stretch in both animals and humans.

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Intercellular Adhesion Molecule 1 Regulates Cardiac Remodeling and Function and T Cell Recruitment to the Heart in Pressure Overload Induced Heart Failure

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Background: Left ventricular (LV) dysfunction and Heart Failure (HF) are associated in humans with systemic inflammation, including increased circulating levels of pro-inflammatory cytokines and soluble intercellular cell adhesion molecule-1 (ICAM-1). Endothelial ICAM-1 regulates leukocyte recruitment into tissues, which in the heart can result in altered cardiac function. We **hypothesize** that ICAM-1 regulates cardiac remodeling by mediating leukocyte recruitment to the LV and thus contributing to worsening of cardiac function during pressure overload induced HF.

Methods and results: We used the mouse model of Thoracic Aortic Constriction (TAC) to induce LV remodeling and HF in WT and ICAM-1 deficient mice (ICAM-1$^{-/-}$). Immunohistochemistry, flow cytometry, qPCR, echocardiography and hemodynamics were used to investigate leukocyte infiltration into the LV, cardiac function, hypertrophy and fibrosis mechanisms taking place in response to TAC. Endothelial ICAM-1 was upregulated in WT mice in response to TAC as compared to Sham, correlating with LV T cell infiltration. In contrast, CD3+ and CD4+ T cell recruitment into the LV was significantly reduced in response to TAC in ICAM-1$^{-/-}$ mice as compared to WT mice. Further, indices of sistolic and diastolic function were preserved in ICAM-1$^{-/-}$ mice (dP/dt_{max} = WT TAC 5,627±549 vs. ICAM-1$^{-/-}$ TAC 8,396±1,495 ; dP/dt_{min} = WT TAC -5,614±1,195 vs. ICAM-$^{1-/-}$ TAC -8,832±2,274) and the End Diastolic Pressure was significantly lower than in WT TAC mice (31.0±7.0mmHg in WT TAC vs 8.1±7.8mmHg in ICAM-1$^{-/-}$TAC). Despite increased LV weight, ICAM-1$^{-/-}$ did not develop fibrosis in response to TAC, with blunted collagen deposition and lack of mRNA upregulation of fibrotic markers Collagen-I, TGFβ and SMAα four and ten weeks after TAC when dilated cardiomiopathy is established in WT mice.

Conclusion: Our data indicate that ICAM-1 regulates LV T cell infiltration, cardiac function and fibrosis in HF induced by TAC. Further studies will determine whether ICAM-1 contributes to HF pathogenesis exclusively by regulating T cell interactions with the LV endothelium or participating in novel mechanisms regulating cardiac cell function, which could represent new targets for the treatment of this deadly syndrome.

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Inhibition of Protein Phosphatase 2A (PP2A) Leads to Beta-adrenergic Receptor Dysfunction With Cardiac Stress Following Pressure-overload Hypertrophy

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Beta-Adrenergic receptors (bARs) play a key role in regulating cardiac function. Loss of surface receptors and desensitization (impaired G-protein coupling) of bARs are hallmarks of a failing heart. Desensitization occurs by phosphorylation of bARs. The bARs are resensitized by protein phosphatase 2A (PP2A) mediated dephosphorylation in the endosomes before recycling to the plasma membrane. While mechanisms of desensitization are well understood, little is known about mechanisms regulating resensitization. Our previous work has shown that PI3Kg phosphorylates an endogenous inhibitor of PP2A (I2PP2A) on serine 9 & 93, which then robustly binds to PP2A inhibiting bAR resensitization. Since it is not known whether resensitization is altered in response to cardiac stress or whether altered bAR resensitization contributes to cardiac hypertrophy and failure, we generated transgenic mice with cardiomyocyte specific overexpression of wild type I2PP2A (WT I2PP2A Tg), I2PP2A phospho-mimetic mutants S9, 93D and mutants with constitutively dephosphorylated S9, 93A state. To test whether resensitization is critical in the development of bAR dysfunction during cardiac hypertrophy, WT I2PP2A Tg mice were subjected to transverse aortic constriction (TAC) for 8 weeks. Echocardiographic analysis post-TAC showed that WT I2PP2A Tg mice had accelerated cardiac dysfunction compared to their littermate controls [HW (mg)/BW(g): Sham: WT - 4.83, WT I2PP2A Tg - 4.82, TAC: WT- 6.47, WT I2PP2A Tg - 7.61; %EF: Sham: WT - 83.53, WT I2PP2A Tg - 74.72, TAC: WT - 70.47, WT I2PP2A Tg - 49.62]. To directly test whether resensitization mechanisms are altered, plasma membranes and endosomes were isolated and in vitro Adenylyl Cyclase activity assessed. Our studies show that compared to littermate controls, WT I2PP2A Tg had altered in vitro adenylyl cyclase activity showing that resensitization mechanisms in the endosomes may in part, contribute to cardiac dysfunction. Mechanistic underpinnings of the resensitization pathways using the I2PP2A S9, 93A and S9, 93D will be presented showing that bAR resensitization a process considered passive is altered in conditions of cardiac stress that in part may contribute to bAR dysfunction leading to cardiac hypertrophy and heart failure.

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Deletion of 12/15 Lipoxygenase and Fatty Acids Interaction Alters Leukocyte Kinetics Leading to Improved Healing Following Myocardial Infarction

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The 12/15 lipoxygenase (LOX) enzyme catalyzes oxygenation of fatty acids to form lipid mediators leading to non-resolving inflammation. However, how 12/15LOX interacts with PUFA (polyunsaturated fatty acids) in post-myocardial infarction (MI) cardiac healing is unclear. Here we assessed the role of 12/15LOX in post-MI cardiac remodeling in a PUFA (10% w/w, 22 Kcal) enriched environment. C57BL/6J wild-type (WT) and 12/15LOX null (12/15LOX$^{-/-}$) male mice of 8-12-weeks age were fed PUFA-enriched diet for 1 month and subjected to permanent coronary artery ligation. Post-MI mice were monitored for day (d)1 or d5 along with standard diet (SD)-fed MI controls. No-MI surgery mice served as d0 controls. 12/15LOX and PUFA+12/15LOX$^{-/-}$ mice reduced infarcted wall thinning with decreased lung edema index at d5 post-MI (all $p<0.05$) despite comparable infarct area (49-52%). PUFA+WT and 12/15LOX$^{-/-}$ mice showed improved ejection fraction with reduced left ventricle (LV) hypertrophy index than SD+WT at d5 post-MI ($p<0.05$). The neutrophil density was decreased in both PUFA+WT and 12/15LOX$^{-/-}$ mice at d1 post-MI ($P<0.05$). The neutrophil clearance was rapid in 12/15LOX$^{-/-}$ alone and PUFA+12/15LOX$^{-/-}$ mice, with increases in expression of *formyl peptide receptor 2* at d5 post-MI. The macrophage density was decreased in PUFA+WT and 12/15LOX$^{-/-}$ mice compared with their respective SD+WT control at d5 post-MI. PUFA+12/15LOX$^{-/-}$ mice displayed an increased expression of *ccl2* (all $p<0.05$) in the infarct area. 12/15LOX deletion stimulated 5-LOX and heme oxygenase-1 in PUFA+12/15LOX$^{-/-}$ mice with increased levels of 9-and 13-hydroxyoctadecadienoic acid indicating improved resolution of inflammation at d5 post-MI. The PUFA+12/15LOX$^{-/-}$ mice displayed higher expression of proresolving macrophages *ym-1, mrc-1* and *arg-1* compared with SD+12/15LOX$^{-/-}$ mice at d5 post-MI (all $p<0.05$). Further, 12/15LOX$^{-/-}$ mice with or without PUFA showed reduced collagen deposition at d5 post-MI compared to SD+WT. In conclusion, deletion of 12/15LOX and short-term PUFA exposure attenuated cardiac remodeling via shortening the pro-inflammatory window, thus leading to an improved resolution of inflammation and LV function post-MI.

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MicroRNA-451 Aggravates Lipotoxic Cardiomyopathy Through Suppression of the LKB1/AMPK Signaling Pathway

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[Rationale]: In some type 2 diabetes mellitus (T2D) patients without hypertension, cardiac hypertrophy and attenuated cardiac function are observed, and this insult is termed “diabetic cardiomyopathy.” Tons of evidence suggests that microRNAs are involved in cardiac diseases. However, the functions of microRNAs in the diabetic cardiomyopathy induced by T2D and obesity are not fully understood.
[Methods and Results]: C57BL/6 mice were fed a high-fat diet (HFD) for 20 weeks, which induced obesity and T2D. MicroRNA microarray and real-time PCR revealed that miR-451 levels were significantly increased in the T2D mouse hearts ($n=4$-5, $p<0.05$). Because excess supply of saturated fatty acids is a cause of diabetic cardiomyopathy, we stimulated neonatal rat cardiac myocytes (NRCMs) with palmitate in physiological albumin concentration and confirmed that miR-451 expression was increased in a dose-dependent manner ($n=6$-12, $p<0.01$). Loss of miR-451 function ameliorated palmitate-induced lipotoxicity in NRCMs ($n=4$, $p<0.05$). Calcium-binding protein 39 (Cab39) is a scaffold protein of liver kinase B1 (LKB1), an upstream kinase of AMP-activated protein kinase (AMPK). Cab39 was a direct target of miR-451 in NRCMs and Cab39 overexpression rescued the palmitate-induced lipotoxicity in NRCMs ($n=4$, $p<0.01$). To clarify miR-451 functions in vivo, we generated cardiomyocyte-specific miR-451 knockout (cKO) mice. HFD-induced cardiac hypertrophy and contractile reserves were ameliorated in cKO mice compared with HFD-fed control mice. Protein levels of Cab39 and phosphorylated AMPK were increased and phosphorylated mammalian target of rapamycin (mTOR) was reduced in HFD-fed cKO mouse hearts compared with HFD-fed control mouse hearts ($n=10$-12, $p<0.05$). We also measured the lipotoxic intermediates, triglyceride and ceramide, in these mouse hearts using HPLC-evaporative light scattering

detector (ELSD). Although there was no difference in triglyceride levels (n=3-5), ceramide level was decreased in HFD-fed cKO mice compared with HFD-fed control mice (n=3-5, p<0.05).
[Conclusions]: Our results indicate that miR-451 exacerbates diabetic cardiomyopathy. miR-451 is a potential therapeutic target for cardiac disease caused by T2D and obesity.
Y. Kuwabara: None. **T. Horie:** None. **O. Baba:** None. **T. Kita:** None. **T. Kimura:** None. **K. Ono:** None.

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The Role of Titin in Cardiac Function: Studies in Two Genetically Engineered Mouse Models With Disparate Titin's Size
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Titin, a myofilament that acts as a molecular spring in the sarcomere, is considered the main contributor to passive stiffness of cardiomyocytes and is responsible for cardiac diastolic function. Increased titin stiffness is related to diastolic dysfunction and HFpEF (Heart Failure with preserved Ejection Fraction). Alteration in size of titin's spring region leads to changes in cardiomyocyte and left ventricular (LV) chamber stiffness.
We tested the effect of alteration in titin's size in two genetically engineered mouse models. We investigated the effect of shortening titin's spring region in a mouse model in which I-band/A-band region of titin's spring has been deleted (TtnΔIAjxn), in comparison to the effect of lengthening titin's spring region in a mouse model deficient in titin splicing factor (Rbm20ΔRRM). Integrative approaches were used from single cardiomyocyte mechanics to pressure-volume analysis and exercise study.
Study of skinned LV cardiomyocytes revealed that cellular passive stiffness was inversely related to the size of titin. Cellular passive stiffness was increased in TtnΔIAjxn homozygous (-/-) (~ 110 % higher than wildtype (WT)) and was reduced in a graded manner in Rbm20ΔRRM heterozygous (+/-) and -/- cardiomyocytes (~61% and ~87% less than WT). This effect was carried through at the LV chamber level which could be demonstrated in pressure volume (PV) analysis as an increased end-diastolic pressure-volume relationship (EDPVR) in TtnΔIAjxn -/- (~110% higher than WT's hearts) and reduced EDPVR in Rbm20ΔRRM +/- and -/- (~57% and ~48% less than WT's hearts). Free-wheel running studies revealed a running deficiency in TtnΔIAjxn -/- mice but an increase in exercise capacity in Rbm20ΔRRM +/– mice.
Conclusions: Functional studies from the cellular to in-vivo LV chamber levels showed that mice with shortening of titin's spring region had increased LV stiffness, diastolic dysfunction and reduced exercise capacity, while mice with lengthening titin's spring region had compliant LV and increased exercise capacity. Thus, our work supports titin's important roles in LV diastolic function and suggests that modification of the size of titin's spring region could be a potential therapeutic strategy for HFpEF.
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Genetic Deletion of Cardiomyocyte Mineralocorticoid Receptors Prevents Cardiac Dysfunction and Premature Death in Mice Lacking Glucocorticoid Receptors in the Heart
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Heart failure is one of the leading causes of death in the Western world, and stress is increasingly associated with adverse cardiac outcomes. Glucocorticoids are primary stress hormones, but their direct role in heart physiology and pathology is poorly understood. The actions of glucocorticoids are mediated classically by the glucocorticoid receptor (GR); however, in cardiomyocytes glucocorticoid occupancy and activation of the mineralocorticoid receptor (MR) may also contribute to the observed glucocorticoid response. To elucidate the *in vivo* function of glucocorticoid signaling in the heart, we generated mice with cardiomyocyte-specific deletion of GR (cardioGRKO). The cardioGRKO mice spontaneously develop cardiac hypertrophy by 2 months of age and left ventricular systolic dysfunction and dilatation between 3-6 months of age, and they die prematurely from heart failure. The mean survival age of the cardioGRKO mice was 9.3 months, and 97% (38 of 39) of the mice died prior to reaching 12 months of age. Loss of GR in the heart was not accompanied by an increase in fibrosis. To investigate whether cardiomyocyte MR signaling contributes to the pathology in the cardioGRKO mice, we generated for the first time mice lacking both GR and MR in the heart (cardioGRMRKO). Despite showing increased expression of classic hypertrophic marker genes, the cardioGRMRKO mice were protected from the development of cardiac hypertrophy, and no major alterations were observed in the function and chamber size of the left ventricle. Moreover, the cardioGRMRKO mice were protected from premature death as 94% (15 of 16) of the mice survived past the 13 month study endpoint. Microarray analysis revealed marked differences in gene expression profiles between the cardioGRKO and cardioGRMRKO hearts. These findings reveal that cardiomyocyte MR signaling, when unopposed by GR signaling, plays a major role in the progression of cardiac disease. Moreover, they suggest that combining GR agonists with MR antagonists may represent an improved therapeutic approach for treating heart failure.
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Anti-fibrotic Function of Relaxin
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Introduction The RELAX in Acute Heart Failure (RELAX-AHF) trial was done in patients admitted for acute decompensated heart failure to evaluate the efficacy of Serelaxin (human recombinant relaxin-2) on dyspnoea relief, as well as its safety and tolerability. Interestingly, the 180-day mortality was significantly better in the Serelaxin group compared to placebo. The pathophysiology of these beneficial effects remains elusive.
Methods and results To induce heart failure in mice, they were treated with Angiotensin II in absence or in presence of relaxin. After 21 days hemodynamic measurements revealed an improved hemodynamic function in relaxin treated animals with AngII-induced heart failure compared to mice without relaxin treatment.
To further investigate the , cardiac fibroblasts were stimulated with pro-fibrotic TGF-β in the presence or in the absence of relaxin. Additionally as a control cardiac fibroblasts were treated with relaxin alone. The treatment with TGF-β induced an increased gene expression of connective tissue growth factor (CTGF)

as well as monocyte chemotactic protein 1 (MCP-1) in cardiac fibroblasts, whereas treatment with relaxin alone decreased the gene expression level of CTGF as well as MCP-1 . To investigate a potential inhibitory function of relaxin during TGF-β induced pro-fibrotic gene expression regulation, cardiac fibroblasts were pre-incubated with relaxin 1 hour prior to TGF-β treatment. After the following 6 hours of TGF-β treatment no decreased gene expression was observed in relaxin pre-treated cardiac fibroblasts compared to TGF-β stimulated fibroblasts without relaxin treatment. **Conclusion** In this study we could demonstrate no inhibitory effect of relaxin during the pro-fibrotic TGF-β-induced signaling pathway. Nevertheless, relaxin alone induced anti-fibrotic function. Therefore, we conclude that the anti-fibrotic function is not due to an influence on the TGF-β induced pro-fibrotic signaling.
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Chemical Induced Reductive Stress Causes Cardiomyocyte Hypertrophy
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Background: Progressive accumulation of misfolded or unfolded proteins is a symbol of impaired proteostasis and proteotoxicity. Such a chronic proteotoxicity is amenable to cell types that are post mitotically matured with lack of further differentiation or proliferation. Our recent discovery using a mouse model of familial human cardiac disease displayed protuberant shift in the redox state towards reductive stress (RS) in association with accumulation of toxic protein aggregates. Further, sustained trans-activation of Nrf2/antioxidant signaling caused RS in the myopathy hearts. Accordingly, we hypothesized that whether profound activation of Nrf2/antioxidant signaling and subsequent RS may cause pathological remodeling in cardiomyocyte. The aim of this study was to investigate the effect of sustained pharmacological activation of Nrf2 on cardiac remodeling. Methods: HL1 cardiomyocytes were used as an in vitro model to study the RS-mediated cardiac remodeling. They were treated with 2-10 μM of potential Nrf2-inducers; sulforaphane (SF), di-methyl fumarate (DMF) and novel small molecules (C-38, C-50, C-63 and C-66) to establish RS by sustained activation of Nrf2/antioxidant signaling. Next, we investigated the implications of RS in cardiomyocyte remodeling by analyzing transcriptional and translational mechanisms using immunoblotting, qPCR, immunofluorescence, GSH and NADPH redox measurements in HL1 cells. Results: Dose dependent effects for individual small molecules including known Nrf2 inducers (SF and DMF) revealed distinct pro-reductive and reductive intracellular (i.e. reductive stress) environments. In fact, the obligatory activation of Nrf2 signaling was associated with significant upregulation of antioxidant enzymes and small molecular thiols including glutathione (GSH). Surprisingly, while pro-reductive condition in HL1 cells was subdued, the RS induced cardiomyocyte hypertrophy was evident from microscopic examination and molecular signature (increased expression of ANF and BNF) after 24-48 hrs of Nrf2 activation. Conclusion: In summary, the chemical induced sustained activation of Nrf2 leading to formation of reductive stress showed hypertrophic remodeling in HL1 cardiomyocytes.
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Diabetes-induced Mitochondrial Dna Damage and Cardiac Dysfunction are Aggravated Due to Low Aldehyde Dehydrogenase 2 (aldh2) Activity in Aldh 2*2 (e487k) Knock-in Mutant Mice
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A prevalent mutation (E487K) in aldehyde dehydrogenase (ALDH) 2, a cardiac mitochondrial enzyme, in East Asians (ALDH2*2) reduces ALDH2 activity and thereby increases aldehyde toxicity. Decreased ALDH2 activity is associated with cardiovascular diseases in humans and animal models. In this study, we hypothesized that reduction in ALDH2 activity in ALDH2*2 mice is sufficient to increase 4-hydroxy-2-nonenal (4HNE) levels and impair mitochondrial respiration and consequently induce cardiac damage in diabetes mellitus (DM). To test the hypothesis, streptozotocin (150 mg/kg i.p.) injected type-1 diabetic ALDH2*2 and C57BL mice as well as corresponding non-diabetic mice were employed. Four experimental groups were C57BL Control, C57BL DM, ALDH2*2 Control, and ALDH2*2 DM. N=6. Data were presented below in the same order. The mice were sacrificed after 3 weeks of DM. Myocardial ALDH2 activity and levels were reduced and 4HNE protein adducts were increased in ALDH2*2 DM mice relative to C57BL DM mice. Decrease in mitochondrial respiration was higher in ALDH2*2 DM mice compared to C57BL DM. Increase in cardiac hypertrophy (207 ± 8, 355 ± 4, 289 ± 22, 370 ± 20 in μm2; $$P<0.01 ALDH2*2 DM vs C57 DM) and fibrosis (4 ± 0.4, 8 ± 0.5, 6 ± 0.7, 9 ± 2.3 in % area of fibrosis; $$P<0.01 ALDH2*2 DM vs C57 DM) were higher in ALDH2*2 DM mice compared to C57BL DM. But the contractile function (56 ± 0.7, 54 ± 1.6, 43 ± 2.7, 48 ± 1.8 in %FS; $p <0.05 ALDH2*2 DM vs C57 DM) was lower only in ALDH2*2 DM, not WT DM. At the molecular level, increased mitochondrial DNA (mtDNA) damage and resultant decrease in MtDNA-encoded respiratory complex proteins were potentiated in diabetic ALDH2*2 mice compared to C57BL DM mice. Based on our data, we conclude that reduced ALDH2 activity in ALDH2*2 mice aggravated diabetes-induced cardiac mitochondrial respiratory dysfunction, ventricular remodeling and dysfunction.
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miR-486 Mediates the Benefits of Exercise in Attenuating Cardiac Fibrosis
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MicroRNAs (miRNAs, miRs), a novel group of small non-coding RNAs, play important roles in cardiac fibrosis. Exercise-induced physiological cardiac growth is associated with hypertrophy and proliferation of cardiomyocytes. In addition, exercise has been shown to inhibit cardiac fibrosis. However, relative little is known about whether exercise could attenuating

cardiac fibrosis via targeting miRNA. miR-486 is a muscle enriched miRNAs, however, its role in heart is relative unclear. The current study aimed to investigate the role of miR-486 in exercise-induced cardiac growth in a 3-week swimming training murine model as well as in the function of cardiac fibroblasts and production of extracellular matrix (ECM) using neonatal rat cardiac fibroblasts in primary culture. Our data showed that exercised mice displayed increased about three-fold expression of miR-486 in hearts as measured by microarray analysis and qRT-PCRs. EdU proliferation assays demonstrated that miR-486 mimics decreased (5.90%±0.57% vs 4.02%±0.27% in nc-mimics vs miR-486-mimics, respectively), while miR-486 inhibitor increased the proliferation of cardiac fibroblasts in vitro (5.87%±0.16% vs 9.60%±0.58% in nc-inhibitor vs miR-486-inhibitor, respectively). Although downregulation of miR-486 had no regulatory effect on α-sma and collagen-1 gene expression in cardiac fibroblasts, overexpression of miR-486 significantly reduced the mRNA level of α-sma (1.01±0.08 vs 0.28±0.04 in nc-mimics vs miR-486-mimics, respectively) and collagen-1(1.02±0.12 vs 0.58±0.09 in nc-mimics vs miR-486-mimics, respectively), indicative of attenuated activation of fibroblasts and reduced production of ECM. These data reveal that miR-486 is essentially involved in the proliferation and activation of cardiac fibroblasts, and might be a key regulator mediating the benefit of exercise in preventing cardiac fibrosis.

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miR-19b Protects Myocardial Ischemia Reperfusion Injury

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Ischemia-reperfusion injury (IRI) following acute myocardial infarction (AMI) has no effective treatment and a poor prognosis. microRNA (miRNA)-19b is a key functional member of miRNA-19-72 cluster family, regulating cellular proliferation, apoptosis, differentiation, and metabolism. Dysregulation of the miR-19b cluster is critically involved in a spectrum of cardiovascular diseases. However, the role of miR-19b in myocardial IRI is unknown. In this study, we found that miR-19b was downregulated in a mouse model of IRI. Meanwhile, about 50% downregulation of miR-19b was detected in H2O2-treated H9C2 cells mimicking myocardial IRI. We also found that overexpression of miR-19b decreased H2O2-induced apoptosis (36.02%±3.92% vs 29.34%±0.79% in nc-mimics vs miR-19b-mimics, respectively) and necrosis (23.11%±1.64% vs 18.76%±0.71% in nc-mimics vs miR-19b-mimics, respectively), and increased proliferation of H9C2 cells in vitro, while downregulation of miR-19b had reverse effects. Furthermore, PTEN, a previously validated target gene of miR-19b, has been found to be negatively regulated by miR-19b at protein levels in H9C2 cells. These data reveal the potential of miR-19b as a therapeutic target for myocardial IRI.

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CCN5 Overexpression Triggers Early Amplification Followed by Regression of Electrical Remodeling in a Pressure Overload Rat Model

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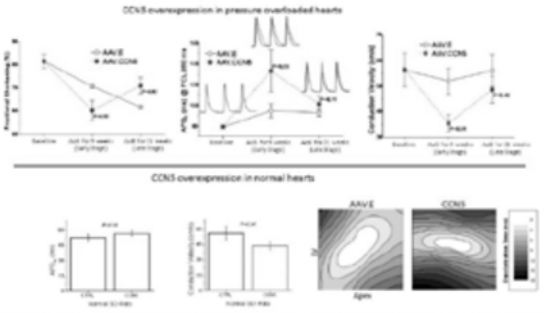
The matricellular proteins CCN2 & CCN5 play opposing roles in pressure overload hypertrophy (PoH). While CCN2 promotes adverse remodeling, CCN5 suppresses fibrosis and therefore may be an important therapeutic target. Since arrhythmias are prevalent during early stages of PoH, we investigated the electrophysiological (EP) effects of CCN5 gene transfer (GT) in a rat model. We hypothesized that CCN5 GT prevents electromechanical dysfunction in PoH.
Methods: 4 wk old rats underwent aortic constriction and were randomized to receive AAV9 CCN5 or an empty vector (E) 3 wks later. In vivo hemodynamic analysis followed by ex vivo EP measurements using high resolution optical action potential (AP) mapping were performed at 9 or 21 wks of PoH.
Results: CCN5 GT did not prevent but rather amplified early electromechanical remodeling as fractional shortening was significantly reduced, AP duration prolonged, and conduction velocity (CV) impaired in AAV9 CCN5 compared to AAV9 E hearts (Fig, top). Remarkably EP remodeling in AAV9 CCN5 hearts was largely reversed by 21 wks of PoH (Fig, top). To probe the basis of these unexpected findings, we tested whether CCN5 GT alters the EP substrate in normal (fibrosis free) hearts. Indeed AAV9 CCN5 GT caused significant CV slowing, consistent with a potent effect on excitability that was independent of fibrosis (Fig, bottom).
Conclusions: CCN5 GT alters myocardial conduction by directly modulating gap junction and/or Na channel function. EP remodeling is amplified during early PoH but undergoes regression at later stages possibly through reversal of fibrosis. CCN5 modulates the EP substrate via fibrosis dependent (late) and independent (early) effects.



J. Hu: None. **D. Jeong:** None. **A.H. Chaanine:** None. **L.J. Motloch:** None. **C. Xie:** None. **R.J. Hajjar:** None. **F.G. Akar:** None.

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Remodeling of K-ATP Channel Expression and Function in Type-2 Diabetes Mellitus

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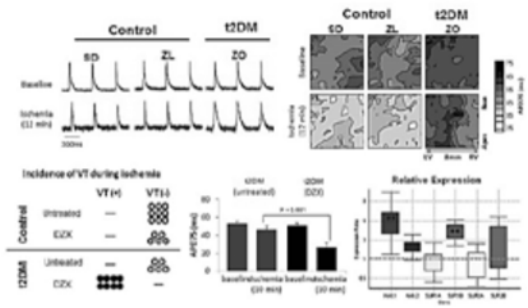
Diabetes mellitus (DM) is associated with enhanced propensity for ventricular tachyarrhythmias (VT). We found that DM hearts exhibit attenuated APD shortening in response to ischemia (Fig). We hypothesized that altered KATP channel expression & function underlie this impaired electrophysiological (EP) adaptation.
Methods: We investigated differential effects of the SUR2a activator Pinacidil (PIN, 100 uM) and the SUR1 activator Diazoxide (DZX, 30 uM) on EP properties in Zucker Diabetic Fatty (ZDF) and control (ctrl) rats at baseline and in response to no flow ischemia. We measured the mRNA expression of KATP channel subunits (Kir6.1, Kir6.2, SUR1a, SUR1b, SUR2a, and SUR2b) in normal and ZDF animals.
Results: PIN caused comparable APD shortening in ctrl & ZDF hearts during baseline perfusion and promoted early onset of sustained VT within 6 min of ischemia in

ABSTRACTS

ctrl & ZDF hearts. In contrast, DZX did not alter APD (p=NS) or cause arrhythmias in either group during baseline perfusion. However, DZX accelerated APD shortening and promoted VT in all (8/8) ZDF hearts during ischemia (Fig 1). The putative molecular mechanism underlying the differential proarrhythmic sensitivity of ZDF vs ctrl hearts to DZX was investigated. We found marked upregulation of Kir6.1 (by 3.7X p<0.001) and SUR1b (by 2.8X p<0.001) mRNA levels with no changes in Kir6.2, SUR1a, SUR2a, or SUR2b expression in ZDF.
Conclusion: Remodeling of KATP channel subunits likely underlies the differential sensitivity of ZDF hearts to DZX. Our findings indicate that this classically cardioprotective agent should be avoided in DM patients at risk of ischemic events. Upregulation of Kir6.1 & SUR1b may be markers of proarrhythmia.



J. Hu: None. **C. Xie:** None. **F.G. Akar:** None.

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Primary Human Cells Isolated From Atria and Bone Marrow Exhibit Comparable Anti-fibrotic Cell Phenotypes Upon Transfection With MicroRNA-301a
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There are many cell types that can contribute to cardiac fibrosis including atrial fibroblasts (AFs) and bone marrow-derived progenitor cells (MPCs). We have previously shown that MPCs display a myofibroblast phenotype in vitro which is linked to altered microRNA(miR)-301a expression, a miR affiliated with maintaining proliferation in numerous cell types. We have also shown that miR-301a influences a dichotomous phenotype in primary human MPCs isolated from patients undergoing open heart surgery. As both MPCs and AFs display a dichotomous phenotype where each cell type displays a phenotype that pathologically contributes to fibrosis, we transfected both MPCs and AFs with miR-301a. AFs were also isolated from patients undergoing open heart surgery. We observed decreases in levels of both mRNA and protein of collagen I, non-muscle myosin IIA, and EDA-fibronectin. These proteins are expressed in myofibroblasts, the cell type predominantly responsible for causing cardiac fibrosis. In addition, transfection of miR301a caused both cell types to increase proliferation, which was analyzed using MTT proliferation assays. These results indicate that miR-301a could be influencing a non-fibrotic phenotype, which could prove useful in cell therapy trials where progenitor cells are injected into scar tissue in order to help heal patients who have suffered from a myocardial infarction. Over-expressing miR-301a in cells used could prevent them from differentiating into pro-fibrotic phenotypes and encourage their proliferation, thereby potentiating their efficacy.
A.L. Müller: None. **D.H. Freed:** None.

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Pulmonary Hypertension Induced Right Ventricle Fibrosis is Associated with Male Gender
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Pulmonary hypertension (PH) is a rare but incurable disease. The primary reason of PH mediated mortality is right ventricle (RV) failure. There is a well-established sexual dimorphism in regard to PH, with females generally associated with higher susceptibility to develop PH, while males possess lower survival rate. We hypothesized that sex difference in PH mediated mortality is associated with sex difference in mechanisms of RV failure development, with males being more prone to develop RV dysfunction. The angioproliferative PH was induced in 8 week old male and female rats by bolus injection of VEGF receptor 2 antagonist, SU5416 (20 mg/kg, s.c.) followed by 4 weeks of exposure to hypoxia (10%±0.5 O2) and 10 weeks of normoxia (21% O2). We found that PH induced a significantly higher mortality in males comparing to females, although the increase in RV peak systolic pressure (RVPSP) was similar in both genders (95.8±13.5 vs. 84.6±18.2; p=0.636; N=5). At the early stage of PH (4 weeks) both males and females had possessed the similar levels of PH induced increase in RV free wall (RVFW) thickness, assessed by Doppler echocardiography. However, starting from 8 week RV hypertrophy continues to progress in males only, while RVFW thickness in females was found to be preserved (RVFW at 12 week, mm: 2.1±0.2 vs. 1.2±0.1; p=0.003; N=5-6). After 14 weeks of study there was a significant impairment of RV relaxation and RV diastolic function in male group only (RV peak diastolic pressure (RVPDP), mmHg: -0.6±0.9 vs. -5.4±0.9; p=0.008; N=4-5). Finally, Masson's trichrome staining revealed severe fibrosis in the RV of male, but not female rats with PH (fibrotic score: 2.5±0.3 vs. 0.7±0.2; p=0.0007; N=5-6), which was associated with endothelial nitric oxide synthase (eNOS) uncoupling, assessed by measuring caveolin expression (fold male Cont: 0.3±0.003 vs. 1.9±0.3; p=0.002; N=4) and eNOS dependent increase in superoxide generation. We conclude that males with PH have a high risk of mortality due to increased cardiac fibrosis development, which, in turn, leads to RV failure. The severe oxidative stress with subsequent injury of myocardial tissue may be responsible for RV fibrotic changes in males, which are lack of female sex hormones known to exert strong anti-oxidant protection.
O. Rafikova: None. **R. Rafikov:** None. **M.L. Meadows:** None. **A. Kangath:** None. **S.M. Black:** None.

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p21-activated kinase-1 (Pak1) Mediates Exercise-induced Cardiac Remodeling Through Calcineurin Signaling
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Aims: Despite its known cardiovascular benefits, the intracellular signaling mechanisms underlying physiological cardiac growth remain poorly understood. Therefore, the purpose of this study was to investigate a novel role of p21-activated-kinase-1 (Pak1) in the regulation of exercise-induced cardiac hypertrophy. Methods & Results: Wild-type and Pak1 KO mice were subjected to six weeks of treadmill endurance exercise-training (ex-training). Cardiac function was assessed via

echocardiography, in situ hemodynamics, and the pCa-force relations in skinned fiber preparations at baseline and at the end of the training regimen. Post-translational modifications to the sarcomeric proteins and expression levels of calcium-regulating proteins were also assessed following ex-training. HW/TL and echocardiography data revealed there was marked hypertrophy following ex-training in the WT mice, which was not evident in the KO mice. Additionally, following ex-training, WT mice demonstrated an increase in cardiac contractility, myofilament calcium sensitivity, phosphorylation of cMyBP-C, cTNT, and TM compared to KO mice. The improvement in contractility with ex-training was accompanied by increased protein levels of SERCA2a and calcineurin along with increased phosphorylation of phospholamban. Conclusions: Our data suggest that Pak1 is essential for adaptive physiological cardiac remodeling and support previous evidence that demonstrate Pak1 signaling is important for cardiac growth and survival.

R.T. Davis: None. **J.N. Simon:** None. **Y. Ke:** None. **B.M. Wolska:** None. **R. Solaro:** None.

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The Effect of PHLPP2 Removal on Cardiomyocyte Hypertrophy

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Crucial cellular decisions that lead to cell growth, metabolism, proliferation, and survival are all dependent on the precise control of the phosphorylation state of proteins. The serine/threonine phosphatase, PHLPP (PH domain leucine-rich repeat protein phosphatase) has been shown to directly dephosphorylate several members of the AGC family of kinases. Knockdown of PHLPP1 by siRNA in neonatal cardiomyocytes potentiates Akt activity and phosphorylation specifically at Ser473 basally and following agonist stimulation while, the removal of PHLPP2 in cardiomyocytes does not affect Akt phosphorylation as previously reported in other cells. We hypothesize that PHLPP2 may target other AGC kinases in cardiomyocytes to regulate cardiac hypertrophy. Preliminary data suggests that removal of PHLPP2 activates fetal gene re-expression at baseline and potentiates phenylephrine (PE) induced gene expression 2 fold over siControl. Recently, G protein-coupled receptor kinase 5 (GRK5), which is an AGC kinase, has been shown to regulate cardiac hypertrophy through HDAC5 phosphorylation and de-repression of gene transcription. We wanted to determine whether PHLPP2 regulates GRK5 phosphorylation and localization in cardiomyocytes. GRK5 translocates to the nucleus following hypertrophic stimulation and we found that removal of PHLPP2 increased GRK5 translocation to the nucleus at baseline and with PE treatment compared to siControl cells. Also, removal of PHLPP2 increased nuclear export of HDAC5 at baseline and following PE treatment. Conversely, overexpression of PHLPP2 blocked nuclear translocation of GRK5 following PE treatment. Ongoing studies will determine whether PHLPP acts as a scaffold or if its phosphatase activity is necessary for inhibition of GRK5 translocation by directly measuring the phosphorylation of GRK5 in the presence and absence of PHLPP2 following hypertrophic stimulation. Our preliminary data is the first to uncover GRK5 as a novel PHLPP2 target in cardiomyocytes. Since little is known about the non-canonical regulation of GRK5, understanding whether phosphorylation and localization is regulated within the cardiomyocyte by PHLPP has potential for new therapeutic targets in the treatment of cardiac hypertrophy and failure.

N.H. Purcell: None. **C. Moc:** None. **G. Birrueta:** None. **A. Taylor:** None. **W. Koch:** None. **C. Zambrano:** None.

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CaMKII as a Link Between Inflammation and Fibrosis Induced by Chronic Isoproterenol and Angiotensin II Treatment

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The Ca2+/calmodulin-dependent kinase, CaMKIIδ, is an established mediator of the development of heart failure and myocardial injury. Cardiac inflammation has been increasingly recognized as an important player in these cardiac pathophysiological changes. We previously demonstrated that CaMKIIδ contributes to cardiac inflammation induced by ischemia/reperfusion through activation of cardiomyocyte NF-kB. In the current study we ask whether angiotensin II (Ang II) and isoproterenol (ISO), both known to activate CaMKII, promote cardiac inflammation through this protein kinase and its effects on NF-kB activation. In addition, chronic ISO and Ang II treatment promote cardiac fibrosis and we hypothesize that this response is initiated through activation of CaMKIIδ and subsequent inflammatory responses. We report on our recent findings that show attenuated inflammatory cytokine expression (e.g. IL-6, MCP1, and TNFα) in response to 7 days Ang II infusion in mice in which CaMKIIδ is specifically deleted in cardiomyocytes (cardiac specific knockout; CKO). In addition the expression of fibrotic markers (e.g. col1a1, col3a1, and CTGF) in response to Ang II infusion is decreased in the CKO mice. This is associated with attenuated fibrosis as evident in histological analysis of CKO vs WT heart sections. Ongoing studies will compare the effects of chronic ISO and Ang II in CKO and WT mice to determine whether inflammation precedes fibrosis and assess the extent to which apoptosis induced by CaMKII activation plays a part in these responses. Currently, we are determining whether Ang II and ISO act through CaMKII to activate NF-kB in the cardiomyocyte compartment to induce proinflammatory and profibrotic factors and whether preventing the expression of these factors block development of further inflammatory and fibrotic responses. Findings from these studies may implicate CaMKII as a promising therapeutic target for attenuating cardiac fibrosis.

A. Willeford: None. **J. Heller Brown:** None.

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Follistatin-like 1 Protects Heart From Post-infarction Rupture by Modulating Fibrogenesis

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Rationale: Stress injured heart secretes several cardiokines, including Follistatin-like 1 (Fstl1). Fstl1 is a member of SPARC (secreted protein, acidic and rich in cysteine) family. Fstl1 is strongly overexpressed in ischemic lesion and secreted to blood circulation in acute coronary syndrome. However, roles and mechanisms of Fstl1 in acute myocardial infarction have not been elucidated yet.
Methods and results: To assess Fstl1 expression after myocardial infarction (MI), C57BL6 WT mice were underwent LAD permanent ligation. Heart and serum Fstl1 protein levels were increased after day 1 and peaked at 7 days after MI. Immunohistochemistry revealed that fibroblasts and myofibroblasts were the major Fstl1 expressing cells in infarcted area and border zone. However, neither cardiomyocytes nor leukocytes (neutrophil, macrophage) produced Fstl1. Fstl1 deleted mice in fibroblasts lineage (Fstl1-CKO mice) were generated by S100a4cre+/- xFstl1flox/flox mice. S100a4cre-/- xFstl1flox/flox mice were used as control. Fstl1 protein overexpression after MI was reduced 50% in Fstl1-CKO mice heart compared to control mice. Mortality rate after MI was significantly higher in Fstl1-CKO mice than control mice (50%;15 of 30 vs 34.8%; 8 of 23, respectively, log-rank test

p=0.031). The majority of causes of death were cardiac rupture in acute period. Cardiac function, blood pressure and severity of inflammation were comparable between Fstl1-CKO and control mice. However, amount of myofibroblasts in Fstl1-CKO heart was significantly less than control mice. Overexpression of extracellular matrix proteins (collagen and fibronectin) in infarcted area was also diminished in Fstl1-CKO mice. Autocrine and paracrine functions of Fstl1 for fibroblasts were assessed in vitro study. Endogenous Fstl1 protein in neonatal rat cardiac fibroblasts (NRCFBs) was knocked down by siRNA fstl1. Migratory activity of NRCFBs was attenuated by Fstl1 ablation. Moreover, Fstl1 augmented TGF-β1 activated Smad2/3 signaling pathway in vivo and vitro.
Conclusions: Increased Fstl1after MI prevents heart from cardiac rupture. This study revealed a novel function of Fstl1, promotes extracellular matrix generation to repair infarcted lesion.
S. Maruyama: None. **K. Nakamura:** None. **K. Walsh:** None.

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Cartilage Intermediate Layer Protein-1 Attenuates Pressure Overload-induced Cardiac Fibrosis by Targeting Transforming Growth Factor-β1
Li Li, Cheng-Lin Zhang, Dan Wu, Li-Ling Wu, Peking Univ Health Science Ctr, Beijing, China

Background: Cartilage intermediate layer protein-1 (CILP-1), a monomeric extracellular matrix glycoprotein expressed mainly in the middle zones of articular cartilage, interacts directly with transforming growth factor-β1 (TGF-β1). Recent studies showed that CILP-1 was upregulated in the heart tissue following cardiac ischemia reperfusion injury. However, the role of CILP-1 in pathological cardiac remodeling is poorly defined. Aims: To explore the effect of CILP-1 on myocardial interstitial fibrosis and reveal the possible molecular mechanism. Methods and Results: We found that CILP-1 was mainly expressed in mouse cardiac fibroblasts (CFs) by using western blot analysis and immunofluorescence. Myocardial expression of CILP-1 was upregulated in mice subjected to transverse aortic constriction (TAC) for 2, 4, and 8 weeks. AAV-9-mediated delivery of CILP-1 into mice increased the binding of CILP-1 with TGF-β1, attenuated interstitial fibrosis, and improved cardiac function. In cultured adult mouse CFs, CILP-1 overexpression inhibited myofibroblast differentiation and expression of profibrotic molecules induced by TGF-β1. Furthermore, CILP-1 attenuated TGF-β1-induced Smad3 phosphorylation and nuclear translocation. Conclusions: CILP-1 alleviates pressure overload-induced cardiac fibrosis and dysfunction. CILP-1 exerts its anti-fibrotic effect through targeting TGF-β1 signaling. This study will offer a new therapeutic strategy for preventing and treating myocardial interstitial remodeling.
L. Li: None. **C. Zhang:** None. **D. Wu:** None. **L. Wu:** None.

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Bendavia, a Novel Mitochondria-Targeting Peptide, Improves Contraction and Relaxation of Failing Cardiomyocytes Isolated From Dogs With Chronic Heart Failure
Hani N. Sabbah, Ramesh C. Gupta, Henry Ford Hosp, Detroit, MI

Background: Mitochondria (MITO) of humans and dogs with heart failure (HF) manifest functional abnormalities characterized by poor respiration, reduced rate of ATP synthesis and increased production of reactive oxygen species (ROS) that adversely impact LV systolic and diastolic function. We previously showed that chronic therapy with Bendavia (BEN, MTP-131), a novel mitochondria-targeting peptide, improves global LV function in dogs with HF without affecting heart rate or blood pressure. This improvement was associated with a reversal of MITO abnormalities and normalization of rate of ATP synthesis. This study tested the hypothesis that the improvement in global LV function seen in dogs with HF during treatment with BEN results primarily from enhanced function of constituent LV cardiomyocytes.
Methods: Cardiomyocytes were isolated from the LV free wall of 8 untreated dogs with coronary microembolization-induced HF (LV ejection fraction <30%). A collagenase-based enzymatic process was used for the isolation and yielded ~70% viable rod-shaped cardiomyocytes that excluded trypan blue. Extent of cardiomyocytes shortening, shortening velocity and lengthening velocity were assessed during 1.0 Hz electrical field stimulation delivered via a MyoPacer (ION Optix). Measurements were made at baseline and were repeated after one hour of gradual exposure of the same cardiomyocytes to BEN at a concentration of 0.1 μM.
Results: At baseline, the extent of cardiomyocyte shortening was 3.7 ± 0.8 μm, shortening velocity was 62.8 ± 16.9 μm /sec and lengthening velocity was -53.8 ± 16.5 μm/sec. Exposure of cardiomyocyte to BEN significantly increased the extent of cardiomyocyte shortening to 5.4 ± 1.1 μm ($p<0.012$), shortening velocity to 94.5 ± 21.9 μm/sec ($p<0.020$) and lengthening velocity to -96.8 ± 21.1 μm/sec ($p<0.016$) compared to baseline.
Conclusions: Exposure of failing isolated cardiomyocytes to BEN elicits improvements in the rate of cardiomyocyte shortening and re-lengthening indicative of improved cell contractility and relaxation. This improvement is likely mediated by increased availability of ATP along with reduced ROS production both secondary to improved mitochondrial function elicited by treatment with BEN.
H.N. Sabbah: 2. Research Grant; Significant; Stealth Peptides, Inc.. 8. Consultant/Advisory Board; Modest; Stealth Peptides, Inc.. **R.C. Gupta:** None.

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Bendavia, a Novel Mitochondria Targeting Peptide, Improves Mitochondrial Function in Circulating Blood Monocytes From Dogs With Chronic Heart Failure
Hani N. Sabbah, Vinita Sing-Gupta, Ramesh C. Gupta, Henry Ford Hosp, Detroit, MI

Background: Mitochondrial (MITO) function is abnormal in heart failure (HF) as evidenced by reduced MITO respiration and rate of ATP synthesis. We showed that MITO dysfunction can be normalized in HF dogs after therapy with Bendavia (BEN), a novel MITO targeting peptide. This study tested the hypothesis that BEN will also reverse abnormalities of MITO function present in blood monocytes (MCs) of HF dogs.
Methods: Blood samples obtained from 6 normal (NL) dogs and 6 dogs with coronary microembolizations-induced HF (LV ejection fraction ~30%) were used to isolate MCs by sequential Ficoll and Percoll density gradients. An XFe/XF96 analyzer (Seahorse Bioscience) was used to measure oxygen consumption rates (OCR) in MCs in the presence and absence of 1 μM oligomycin, 0.5 μM FCCP, or 1 μM each rotenone and antimycin. MITO proton leak, maximal respiration (MAXresp) and spare respiratory capacity (SRC) were measure in the presence and absence of 0.1, 1.0 and 10 μM concentrations of BEN and results expressed in pmols OCR/min/μg protein.
Results: Proton leak, MAXresp and SRC were abnormal in MCs of HF dogs compared to NL. Incubation with BEN had no effect on measures of MCs MITO function of NL dogs but nearly normalized MITO function of MCs of HF dogs as evidenced by a dose-dependent increase MAXresp and SRC and dose-dependent decrease in proton leak (Table).
Conclusions: MITO function is abnormal in blood MCs of HF dogs. BEN had no effect on MITO function of MCS from NL dogs but normalized MITO function in MCsfrom

HF dogs. These findings support the use of circulating blood MCs as means of assessing MITO dysfunction in HF and as a marker of potential benefits derived from treatment with MITO targeted therapies.

Bardoxa and MITO Function in Monocytes

	MCs from NL Dogs				MCs from HF Dogs			
Bardoxa concentration	0.0	0.1 μM	1.0 μM	10 μM	0.0	0.1 μM	1.0 μM	10 μM
Proton Leak (pmols OCR/min/μg protein)	0.49 ± 0.04	0.54 ± 0.22	0.47 ± 0.06	0.48 ± 0.05	2.06 ± 0.23	1.35 ± 0.20*	0.94 ± 0.12*	0.77 ± 0.04*
MAXresp (pmols OCR/min/μg protein)	11.4 ± 0.62	11.1 ± 0.74	12.1 ± 0.84	12.1 ± 0.67	5.5 ± 0.14	6.3 ± 0.23	8.3 ± 0.20*	9.4 ± 0.78*
SRC (pmols OCR/min/μg protein)	6.75 ± 0.55	6.35 ± 0.64	7.04 ± 0.92	7.04 ± 0.58	1.42 ± 0.14	1.95 ± 0.20	3.64 ± 0.36*	4.51 ± 0.70*

*=p<0.05 vs. 0.0 μM Bardoxa

H.N. Sabbah: 2. Research Grant; Significant; Stealth Peptides, Inc.. 8. Consultant/Advisory Board; Modest; Stealth Peptides, Inc. **V. Sing-Gupta:** None. **R.C. Gupta:** None.

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Metabolic Trapping Effects of ACS1 for Fatty Acid Uptake and Intracellular Trafficking are Different in Hearts of Males and Females
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Disruption of cardiomyocyte lipid metabolism has been observed in the responses to both pathogenic and nutrient stresses in human patients and animal models, with distinct sex differences. This investigation examined cardiac acyl CoA synthetase-1 (ACS)-mediated, LCFA uptake and trafficking with hearts of male and female mice. Isolated mouse hearts with cardiac specific overexpression of ACS1 (MHC-ACS) and non-transgenic littermates (NTG) were perfused with buffer containing 13C palmitate, 10 mM glucose, and 1 mM lactate. Dynamic 13C NMR elucidated the two phases of LCFA incorporation into triacylglycerol (TAG). The time constant (τ) of the initial exponential phase, corresponding to LCFA trans-sarcolemmal uptake, was lower, indicating faster LCFA uptake and esterification in male ACS hearts compared to NTG males (ACS male τ = 1.59±0.67 min vs NTG male τ = 2.85±0.90 min, P<0.05). Consistent with greater metabolic trapping, acyl CoA content was 470% higher in male ACS hearts (NTG male 57.5±9.4 vs ACS male 327.4±42 pmol/mg protein. P<0.001). Despite no differences in ACS content, NTG females displayed faster uptake than NTG males at a rate similar to ACS males, which was reversed by ovariectomy (OVX) indicating an estrogen dependent response (NTG female τ = 1.56±0.46 min, P<0.05 vs NTG OVX female τ = 3.02±0.77 min, P<0.05). Despite prior reports of ACS localization distal from the sarcolemma, ACS level and female gender both independently accelerated LCFA uptake without affecting TAG content or turnover. The data are consistent with metabolic trapping to facilitate LCFA uptake due to ACS activity. ACS also leads to higher total ceramide in hearts, with specific elevation of C18 and C22 in both genders, C24 in males, and C20 in females (male P<0.05; female P<0.01). ACS overexpression induced lower content of the cardiac fatty acid transporter (FATP) isoform, FATP6, indicating cooperative regulation of LCFA uptake between membrane transport proteins and intracellular acyl chain activation (P<0.05 male; P<0.001 female and female OVX). These results demonstrate that perturbation of LCFA metabolism though facilitated metabolic trapping by ACS1 affects sarcolemma transporter expression and induces the conversion of the CoA activated LCFA to ceramide.
J.R. Goldenberg: None. **X. Wang:** None. **A.N. Carley:** None. **E. Lewandowski:** None.

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Mitofilin Directly Interacts With Cyclophilin D in the Inner Membrane of Heart Mitochondria
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Introduction: We recently found that acute estrogen treatment delays the mitochondrial permeability transition pore (mPTP) opening and reduces ROS production after ischemia/reperfusion, suggesting that estrogen promotes mitochondrial integrity. As mitochondrial inner membrane protein (mitofilin) has been found to control mitochondrial cristae morphology and function, and cyclophylin-D is a mitochondrial peptidyl-prolyl isomerase known to modulate the opening of the mPTP, we investigated whether a direct interaction between these two proteins exist in the inner membrane of mitochondria.
Methods: Western blot analysis and immunoprecipitation were performed in isolated heart mitochondrial fractions from male WT (C57BL/6NCrL) mice using mitofilin and cyclophilin-D monoclonal antibodies of. Mitofilin and cyclophylin-D distribution in isolated cardiomyocytes as well as in single mitochondria was also visualized using high resolution microscopy.
Results: Western blot analysis shows that both mitofilin and cyclophilin-D proteins were abundantly expressed in the heart mitochondria. Immunoprecipitation with mitofilin antibody pulled down cyclophilin-D, which was detected by Western blot analysis. Co-immunoprecipitation with cyclophylin-D antibody confirms its direct association with mitofilin as mitofilin was detected by Western analysis in the immunoprecipitated product. High resolution microscopy revealed a higher degree of colocalization between mitofilin and cyclophylin-D in both cardiomyocytes and mitochondrial fractions.
Conclusion: Together, these data indicate that mitofilin directly interacts with cyclophylin-D in the inner membrane of heart mitochondria. As mitofilin plays an important role in controlling mitochondrial cristae morphology and cyclophylin-D, known to modulate the opening of the mPTP, this interaction between mitofilin-cyclophilin-D may have an impact in the opening of the mPT pore.
J. Bopassa: None.

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Sarcolemmal Membrane Associated Protein Isoform 1: a Unique Regulator of Glucose Uptake and Metabolism in the Myocardium
Aaraf Dewan, Maysoon Salih, Univ of Ottawa, Ottawa, ON, Canada; Christopher Triggle, Hong Ding, Cornell Medical Sch, Doha, Qatar; Balwant Tuana, Univ of Ottawa, Ottawa, ON, Canada

As one of the leading causes of heart disease, diabetes is a problem which needs a solution. Regulation of glucose uptake and metabolism within skeletal and cardiac muscle has proven capable of altering systemic glucose levels and impacting metabolism to potentially improve patient outcomes. Unfortunately, to date, very few muscle specific metabolic regulators are known which can allow us to achieve blood glucose uptake and metabolism. Sarcolemmal Membrane Associated Protein Isoform 1 (SLMAP1) is a novel protein expressed predominantly within muscle tissue. It has been linked to diabetes through animal models, although its role in metabolism remains to be defined. Here we describe a novel role for SLMAP1 in glucose metabolism within the myocardium. We engineered a transgenic (TG) mouse with cardiac specific expression of SLMAP1. Using neonatal cardiomyocytes (NCMs) collected from these mice we performed glucose uptake assays with 2-deoxy-glucose (2DG), measured glycolytic rate using an Extracellular Flux XF24e Bioanalyzer, and analyzed glucose transporter 4 (GLUT4) trafficking using Western Blots, qPCR, and immunofluorescence imaging.

NCMs extracted from TG hearts expressing SLMAP1 displayed increased levels of 2DG uptake (93% ± 25%, n=5, P<0.01), basal glycolysis (glycolysis (92 ± 40%, n=5, P<0.05), and maximal glycolysis (75 ± 31%, n=5, P<0.05) compared with wildtype littermates. Confocal microscopy revealed both increased localization of glucose transporter 4 (GLUT4) at the cell surface as well as an expansion of GLUT4 early endosomes in TG NCMs. The data here indicates SLMAP1 as a novel regulator of glucose uptake and metabolism in the myocardium. The targeted expression of SLMAP1 in a muscle specific manner may enhance systemic glycemic control and serve to limit cardiovascular disease in metabolic disorders such as diabetes.
A. Dewan: None. **M. Salih:** None. **C. Triggle:** None. **H. Ding:** None. **B. Tuana:** None.

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Cardiac Overexpression of Thioredoxin-1 Delays Mitochondrial Damage and Prolongs Survival in Septic Mice
Juana P Sanchez-Villamil, Veronica D'Annunzio, Ines Rebagliati, Paola Finocchietto, Jorge Peralta, Celina Morales, Ricardo J Gelpi, Juan J Poderoso, Maria C Carreras, Univ of Buenos Aires, Buenos Aires, Argentina

Introduction: Current evidence suggests a main role of thioredoxin-1 (trx1) in the protection against oxidative stress. However, it is unknown yet a putative role of trx1 in the regulation of contractility and mitochondrial function, dynamics and biogenesis in sepsis-induced myocardial dysfunction in which oxidative stress is an underlying cause.
Methods: Transgenic male mice (Tg-trx1) and its wildtype (wt) were subjected to cecal ligation and double puncture or sham operation. After 6, 18 and 24 h, antioxidant enzymes, protein carbonylation and mitocondrial function were evaluated. Hearts were isolated and perfused using the Langendorff technique. Inotropism was evaluated through left ventricular developed pressure (LVDP) and contractile reserve after β-adrenergic stimulus by addition of isoproterenol (ISO) (1 μM). Evaluation of mitochondrial fusion-fission dynamics (Drp1, Mfn2, Opa1) and biogenesis (PGC-1α, NRF-1 and TFAM) were made by real-time qPCR.
Results: Over the time course of sepsis, there was an improvement in average life expectancy in Tg-trx1 (Tg-trx1: 36, wt: 28 h; p=0.0204), and 15 percentages points higher β -adrenergic response at 6 h compared to wt (22.8 vs 7.8%, means of relative percentage differences respectively). Inhibition of complex I activity, protein oxidation, and loss of membrane potential was lower in Tg-trx1, with a sustained MnSOD activity at 24 h. mRNA levels of Opa1 were significantly reduced in sepsis, accompanied by ultrastructural alterations in mitochondrial cristae, while significant Drp1 activation (measured by Ser P616 phosphorylation) was observed in wt mice at 24 h. PGC-1 α mRNA decreased at 6 h in both groups, and then, showed a 2.5-fold increase in Tg-trx1 at 24 h. Autophagy was demonstrated by increased LC3-II/LC3-I ratios in both groups during the progression of sepsis, although the levels were early higher in Tg-Trx1. No indicators of apoptosis were observed.
Conclusions: The results show that mice with cardiac-specific overexpression of Trx1 have higher survival. Trx1 provides protection against oxidative stress, which seems to preserve contractile reserve and keep mitochondrial biogenesis. Associated with mitochondrial autophagy-processes allow mitochondrial renewal and life extension.
J.P. Sanchez-Villamil: None. **V. D'Annunzio:** None. **I. Rebagliati:** None. **P. Finocchietto:** None. **J. Peralta:** None. **C. Morales:** None. **R.J. Gelpi:** None. **J.J. Poderoso:** None. **M.C. Carreras:** None.

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The First Direct Measure of Chylomicron Metabolism in Heart Reveals a Reciprocal Balance Between Mitochondrial Oxidation of Fats From Chylomicron vs Albumin Sources
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Nearly all studies of fat metabolism in healthy and diseased heart are based on albumin sources of fat, even though lipoproteins (chylomicrons, VLDL) are thought to be a major source of fat. If there are distinct differences in uptake, oxidation, and storage of fatty acids (FA) from lipoproteins compared to albumin sources, then our understanding of disease is far from complete. This study provides the very first investigation of the fate of 13C labeled FA from chylomicrons (CM) in heart, with quantified results of the direct competition between CM-FA vs albumin-FA to support mitochondrial ATP synthesis. Chylomicrons were biosynthetically labeled with 13C-oleate by delivering to the duodenum of rat either (a) emulsified in olive oil, or (b) incorporated into micelles. The labeled chylomicrons were collected as lymph via the thoracic duct for six hours. The olive oil approach, used traditionally to prepare CM to contain radiolabeled fats, resulted in a trace fraction of 13C-labeled FA (<2%). The micelle approach enabled 50% enrichment of CM-FA store. Metabolic studies: Isolated rat hearts were perfused with KH buffer containing 13C-chylomicrons, FA free albumin, and glucose; or 13C-chylomicrons, unlabeled oleate complexed to albumin (equimolar FA from CM and albumin; 0.4 mM), and glucose. Hearts were freeze-clamped, and FA oxidation was assessed via high resolution 13C NMR of heart samples. CM-FA provided 50 ± 5 % of the acetyl CoA to support citric acid cycle synthesis of ATP when FA free albumin was used. In the presence of albumin-FA, only 12 ± 2% of acetyl CoA entering the cycle was from the CM-FA. These direct observations of the competition between CM-FA vs albumin-FA counter prior interpretations based on indirect measures. Those studies suggested no change in CM-FA oxidation when albumin-FA was added. Our novel approach enables direct measurements of CM-FA and albumin-FA oxidation in the mitochondria of intact hearts. The uptake and distribution of fat from either exogenous source were found to contribute to a common intracellular source of activated FA, with mitochondrial oxidation being proportional to the availability of the FA source. Importantly, this new evidence for a common pool significantly simplifies our metabolic paradigm of cardiac disease.
J. O'Donnell: None. **E. Lewandowski:** None. **P.V. Subbaiah:** None.

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Molecular Identity of Cardiac Mitochondrial Chloride Intracellular Channel Proteins and Their Role in Mitochondrial Function
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Introduction: Mitochondria are primary target organelles for cardioprotection. Rabbit heart and cardiomyocytes showed increased infarction upon treatment with IAA-94, a known chloride intracellular channel (CLIC) inhibitor implicating role of CLICs in cardioprotection. There are six CLIC homologs (CLIC1-6), but the molecular identity of cardiac mitochondrial CLICs and their functional roles are not deciphered. We aim to determine the identity of cardiac mitochondrial CLICs and establish their role in mitochondrial function to understand the mechanism of CLIC-mediated cardioprotection.
Methods: Hearts were isolated from 2-3 months old SD rats for all the experiments. qPCRs were performed to determine the abundance of CLIC1-6 and corroborated

by Western blot using specific antibodies. Immunofluorescence was performed on neonatal and adult cardiomyocytes. Percoll-purified mitochondria were also probed with Western blot, immuno organelle chemistry and Mass spectrometry to establish the presence of CLICs. In order to determine their role in mitochondrial function, spectrofluorimetric analysis were performed to measure the reactive oxygen species (ROS) production using amplex red and calcium retention capacity (CRC) using calcium green.
Results: We have discovered that CLIC4, CLIC5 and CLIC1 as the most abundant paralogs in rat heart using qRT PCR and corroborated their presence by Western blot. CLIC4 and CLIC5, not CLIC1 were found to be present in mitochondria of neonatal and adult cardiomyocytes. Percoll-purified mitochondria further showed presence of CLIC4 and CLIC5 where they colocalizes with mitochondria (45+/-2 and 74+/-3, n=3, respectively). Over-expressing CLICs in H9C2 cells showed mitochondrial localization of CLIC4 and CLIC5 and not CLIC1 establishing them as mitochondrial anion channels. Blocking of CLICs with IAA-94 modulated the ROS production at both complex I and II/III of electron transport chain of mitochondria. Further IAA-94 reduced CRC of mitochondria by 83+/-6%, n=4.
Conclusions: We have established that CLIC4 and CLIC5 are the cardiac mitochondrial anion channels and their activity regulates the function of mitochondria hence playing a direct role in cardioprotection from ischemia-reperfusion injury.
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Augmented Cardiac Pyruvate Dehydrogenase Complex Flux During Hypoxia Increases Post-hypoxic Damage
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Modulation of cardiac pyruvate dehydrogenase complex (PDC) flux is known to alter post-ischemic myocardial injury. Hypoxia is intrinsic to ischemia, but its specific role in cardiac injury is unknown. We hypothesized that post-hypoxic cardiac injury would be attenuated with activation of the PDC complex during hypoxia.
Mouse hearts were isolated and Langendorff perfused with 0.4mM palmitate and 11mM glucose. Following 30min of normoxia, hearts were subjected to 30min hypoxia (20% of normoxic pO2) followed by 30min of reoxygenation. PDC flux was increased by infusion of 1mM dichloroacetate (DCA), either from onset of hypoxia, or with onset of reoxygenation. Cardiac function was measured using an IV balloon, and fatty acid β-oxidation determined via 3H-labelling. Lactate efflux was assayed from timed buffer collections. Hearts were frozen at end-normoxia, end-hypoxia and end-reoxygenation, and assayed for PDC flux, and phosphate metabolites. Reactive oxygen species formation was assessed by Western blotting of protein carbonyls.
End-hypoxic cardiac PDC flux fell to 41±5% of pre-hypoxic values (0.96 of 2.37 µmol/gdwt, p<0.001) with β-oxidation also lower (25±11%, 0.06 of 0.24 µmol/min/gwwt, p<0.025). Lactate efflux increased 2.3-fold (4.7 to 10.6 µmol/min/gwwt, p<0.028) and AMP/ATP ratio 5.3-fold (0.04 to 0.21 p<0.002). DCA infusion increased end-hypoxic PDC flux (170±16%, 1.64 of 0.96 µmol/gdwt, p<0.003), reducing lactate efflux (47±8%, 5 of 11 µmol/min/gwwt, p<0.021) and AMP/ATP ratio (43±12%, 0.09 of 0.21, p<0.025). Control hearts were damaged by hypoxia, final recovery being 63±7% of pre-hypoxic function (18 of 29 bpm.mmHg.103). Hypoxic DCA infusion impaired recovery (49±4%, 15 of 31 bpm.mmHg.103), compared to DCA infusion during reoxygenation (63±3%, 19 of 30 bpm.mmHg.103, p<0.023). Reactive oxygen species formation was not altered. Increasing PDC flux during hypoxia significantly modified myocardial substrate metabolism. In contrast to the reported beneficial effects of augmented PDC flux during ischemia, increasing PDC flux in the hypoxic heart exacerbated myocardial injury. These results indicate that the potentially therapeutic effects of increasing PDC flux are not dependent on the hypoxic aspect of myocardial ischemia.
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Strategic Distribution of the Mitochondrial Ca2+ Uniporter in Cardiac Mitochondria
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Propagation of SR-derived Ca2+ signals to the mitochondrial matrix is an important tuning tool of cardiac energy metabolism (excitation-bioenergetics coupling). Effective activation of the low-affinity mitochondrial Ca2+ uniporter (mtCU) is supported by high [Ca2+] nanodomains where SR cisternae are tethered to mitochondria. Cardiac mitochondria have large inner membrane (IMM) surface with extensive cristae folding. However, recent comparative patch clamp studies found cardiac mtCU current density extremely low.
We hypothesized that efficient excitation-bioenergetics coupling would require strategic positioning of the mtCU. We thus analyzed the distribution of mtCU's pore protein, MCU in rat and mouse cardiomyocytes and cardiac membrane fractions. Immunofluorescence (IF) using the commercial Sigma antibody showed significant non-mitochondrial (nuclear, cyto/sarcoplasmic) crossreactions as verified in MCU-KO MEFs and cardiomyocytes. To isolate mitochondrial labeling, we performed IF of isolated mitochondria (mtIF) and immunogold electron microscopy of freshly isolated cardiomyocytes. mtIF showed ~50% of MCU-positive structures colocalized with RyR2, in line with a location preference to SR-mitochondria associations. This distribution bias was also reflected in the immunogold TEM analysis. Moreover, WB showed both MCU and calsequestrin being enriched in the same sub-mitochondrial membrane fraction separated on sucrose gradient. The prevalence of MCU and even more the essential MCU regulator (EMRE) was higher in the mitochondria "contaminating" the heavy SR (SR-associated mito membranes) than in the crude mitochondrial fraction. [Ca2+] activation of mitochondrial Ca2+ uptake at similar IMM potentials was as effective in suspensions of SR-associated mito membranes as in the crude mitochondrial fraction. Collectively, our data suggest a location bias of the functionally mandatory mtCU channel components MCU and EMRE toward SR-mitochondrial associations. As the bias is much stronger for EMRE, the component that is required for MCU to function as a channel, it raises the possibility that EMRE availability may be a physiological limiting factor in regional mtCU activity in the cardiac muscle mitochondria.
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Acetylomic and Metabolomic Alterations in Aged Hearts: Role for Silent Information Regulator (SIRT) 3

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Introduction: SIRT3 is a mitochondrial metabolic regulator, and a decline in function of SIRT3 may play a role in age-related mitochondrial alterations. The aim of this study was to investigate the possible down-regulation of SIRT3 activity in aged hearts, and to identify which metabolic pathways in aged hearts may be impaired due to SIRT3 dysfunction.
Methods: Mitochondria were isolated from WT adult (7 mo.), SIRT3$^{-/-}$ adult (7 mo.) and WT aged (18 mo.) hearts. Acetylated proteins in mitochondrial samples were identified using 2D gels and mass spectrometry. Metabolite concentrations and carbon fluxes through core metabolic pathways were determined using ^{13}C-labeled substrates and LC-MS/MS.
Results: Mitochondrial acetylation patterns in the SIRT3$^{-/-}$ adult group matched those found in the WT aged group; the level of acetylation was significantly higher than in WT adult. While the SIRT3$^{-/-}$ samples exhibited zero SIRT3 protein content, no difference in SIRT3 protein level was seen between adult and aged WT hearts. Mechanistically, this suggests that alterations in mitochondrial acetylation during aging were not caused by lower SIRT3 protein levels, but rather by a lower SIRT3 enzymatic activity.
Furthermore, aged myocardium exhibited 40% lower NAD^+ levels, which may underlie compromised SIRT3 activity.
ATP levels were decreased in both SIRT3$^{-/-}$ and WT aged hearts, suggesting possible defects in energy metabolism. Using metabolomics, we demonstrated that alterations of TCA cycle intermediates were similar in SIRT3$^{-/-}$ and WT aged hearts (relative to WT adult), and included a substantial decline of carbon flux through α-ketoglutarate and malate. Furthermore, regulation of energy production might also be impaired at the level of the electron transport chain, where Complex I was significantly inhibited in both SIRT3 deficient and aged hearts.
Conclusions: Collectively these data suggested that acetylomic and metabolomic fingerprints observed in SIRT3$^{-/-}$ hearts were recapitulated in aged hearts.
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Mitophagy is Required for Mitochondrial Biogenesis and Myogenic Differentiation of Myoblasts

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Myogenesis is a crucial process governing muscle development and homeostasis. Differentiation of primitive myoblasts into mature myotubes requires a metabolic switch to support the increased energetic demand of contractile muscle. Skeletal myoblasts specifically shift from a highly glycolytic state to relying predominantly on oxidative phosphorylation (OXPHOS) upon differentiation. We have found that this phenomenon requires dramatic remodeling of the mitochondrial network involving both mitochondrial clearance and biogenesis. During early myogenic differentiation, autophagy is robustly upregulated and this coincides with DNML1/DRP1-mediated fragmentation and subsequent removal of mitochondria via p62/SQSTM-mediated mitophagy. Mitochondria are then repopulated via PPARGC1A/PGC-1α-mediated biogenesis. Mitochondrial fusion protein OPA1 is then briskly upregulated, resulting in the reformation of mitochondrial networks. The final product is a myotube replete with new mitochondria. Respirometry reveals that the constituents of these newly established mitochondrial networks are better primed for OXPHOS and are more tightly coupled than those in myoblasts. Additionally, we have found that blocking autophagy with various inhibitors during differentiation results in a blockade in myogenic differentiation. Together these data highlight the integral role of autophagy and mitophagy in myogenic differentiation.
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The E3 Ubiquitin Ligase Parkin Mediates Clearance of Damaged Mitochondria via Two Distinct Pathways

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Damaged mitochondria release reactive oxygen species and pro-death factors which can lead to loss of cardiac myocytes. To protect against such damage, myocytes have developed several mechanisms of quality control that act both on the protein and organelle levels. We have previously identified the E3 ubiquitin ligase Parkin as an important regulator of mitochondrial clearance via autophagy in the myocardium. Here, we report that Parkin can also mediate clearance of mitochondria via the endosomal-lysosomal pathway. We found that Parkin promotes clearance of damaged mitochondria in both wild type (WT) and autophagy-deficient Atg5 knockout mouse embryonic fibroblasts (MEFs) treated with the mitochondria uncoupler FCCP. Mitochondrial damage leads to rapid activation of the endosomal-lysosomal pathway in both WT and Atg5-/- MEFs. We further observed increased activation of Rab5, a protein involved in early endosome formation, in both WT and Atg5-/- MEFs after treatment with FCCP. In addition, we observed sequestration of damaged mitochondria in Rab5+ and Rab7+ early and late endosomes, respectively. Mitochondria also colocalized with Lamp2+ vesicles in Atg5-/- MEFs indicating that the mitochondria are ultimately being delivered to the lysosomes for degradation. Overexpression of Rab5S34N, a dominant negative of Rab5, reduces FCCP-mediated clearance and increases cell death in Atg5-/- MEFs. Pharmacological inhibition of the endosomal-lysosomal pathway also results in increased FCCP-mediated cell death. Furthermore, we confirmed that FCCP treatment or simulated ischemia reperfusion exposure induces Rab5 activation with subsequent mitochondrial sequestration in early endosomes in neonatal myocytes. Interestingly, the activation of Rab5 is abrogated in the presence of the mitochondrial targeted antioxidant Mito-Tempo, suggesting that mitochondrial ROS is involved in the activation the endosomal pathway. Mitochondrial clearance via this pathway is also dependent on Parkin, as FCCP treatment fails to activate Rab5 and induce mitochondrial clearance in both WT and Atg5-/- MEFS in the absence of Parkin. Thus, our data suggest that Parkin can mediate clearance of damaged mitochondria via two distinct pathways in cells.
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A Novel Method for the Generation of Induced Pluripotent Stem Cells From Human Peripheral Blood

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Background: In terms of the generation of induced pluripotent stem(iPS) cells, one of the important issues for clinical applications is cell source. Human peripheral blood is one of the easily accessible cell sources. However, isolated peripheral blood cells have shown low gene transfection efficiency and inconveniences requiring specific methods to isolate. Here, we report a novel population of peripheral blood-derived stem cells, which can be easily reprogrammed to iPS cells.
Methods and Results: We cultured peripheral blood mononuclear cells (PBMC) from human peripheral blood and seeded on the fibronectin-coated plate. We observed adherent cells from as early as 5 days after the start of culture and those cells gradually formed colonies. We were able to isolate these cells with very high efficiency. Furthermore, we have also confirmed that these cells can be differentiated to osteogenic, adipogenic, and myogenic-lineage cells. Therefore, we named these cells circulating multipotent adult stem cell. We were successful in generating iPS cells with these cells. These cells showed enhanced efficiency of gene transduction, compared to the human dermal fibroblast. We obtained reprogrammed colonies in 8 days after 4 factor virus transduction without feeder cells. We identified our iPS cells had similar features to embryonic stem cell in morphology, gene expression, epigenetic state and ability to differentiate into the three germ layers. We obtained more than 46 iPS cell lines from PBMC of patients with cardiovascular disease and normal volunteers.
Conclusions: Our study showed new methods to isolate stem cells from peripheral blood and to generate iPS cells with high efficacy. This result suggests that our new approach could be one of ideal methods for clinical application of iPS cells in future.
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Assessing Proarrhythmic Potential of Drugs Using Human Induced Pluripotent Stem Cell Derived Cardiomyocytes and Optical Recordings with Voltage-sensitive Dyes

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Background: Drug-induced arrhythmias have been a leading reason for withdrawing drugs from the market and abandoning the development of new drugs. For the past decade FDA has required new drugs to be tested for block of the hERG potassium channel and QT interval prolongation. While these requirements have prevented arrhythmia-related drug recalls, some new drugs affecting multiple ion channels are being dropped from development inappropriately. Human induced pluripotent stem cell derived cardiomyocytes (iPS-CMs) are a new technology for preclinical risk assessment; however they have not been fully characterized or validated.
Methods: We studied 26 drugs and 3 drug combinations that block multiple cardiac ion channels with two commercially available iPS-CM lines from Axiogenesis and Cellular Dynamics using optical recordings of action potentials with voltage-sensitive dyes. Drug-induced action potential duration (APD) prolongation was compared to clinical QT prolongation from two FDA-sponsored clinical trials. The effects of the drugs on multiple individual cardiac ion channels were assessed using manual patch clamp of overexpressed cell lines. Cardiac ion channel gene expression in the iPS-CMs was quantified and compared to primary human heart cell controls.
Results: Of 19 drugs with an FDA label of clinical QT prolongation, 15 exhibited iPS-CM APD prolongation after acute drug exposure. None of the 6 drugs without clinical QT prolongation caused iPS-CM APD prolongation. Of 14 drugs with arrhythmia risk on the FDA label, nine caused arrhythmias in iPS-CMs. iPS-CM response had good correlation with clinical QT data for drugs that block hERG and calcium channels, while drugs blocking the late sodium current had variable response. This was in line with gene expression data, which showed most robust expression of hERG and calcium channels.
Conclusion: Optical recordings from iPS-CMs with voltage sensitive dyes is a promising technology for high-throughput toxicity assessment for drug-induced arrhythmias. This study provides a comprehensive characterization of the cardiac ion channel properties of multiple commercially available iPS-CMs to support a potential new paradigm for assessing the arrhythmia risk of all new drugs.
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Human Induced Pluripotent Stem Cells Reveal Mitophagy as an Essential Process Against Diabetic Cardiomyopathy

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Diabetic cardiomyopathy is a common consequence of diabetes and associated with mitochondrial pathology. Using human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) as an in vitro model of diabetes, we sought to understand the role of mitophagy, a process that selectively degrades mitochondria through the autophagy-lysosome pathway as a crucial quality control pathway against diabetic cardiomyopathy. We first showed that iPSC-CMs exposed to a diabetic milieu demonstrated increased hypertrophy, impaired calcium signaling, and higher oxidative stress. Flow cytometry analysis of iPSC-CMs subjected to diabetic conditions revealed two distinct population of cells (normal and hypertrophied), suggesting a heterogeneous response to hyperglycemia. In these cells, hypertrophied iPSC-CMs were found to have reduced mitophagy compared to normal cells when exposed to hyperglycemia. In addition, we showed that mitochondrial fragmentation was also decreased in the hypertrophied iPSC-CMs compared to normal cells upon exposure to hyperglycemia, demonstrating a link between mitochondrial fragmentation and mitophagy. Surprisingly, pretreatment of iPSC-CMs with a non-selective antioxidant, N-(2-mercaptopropionyl)-glycine, not only failed to limit the deleterious effects of hyperglycemia, but actually led to increased hypertrophy and cell death. We found that mitophagy was significantly reduced in iPSC-CMs following antioxidant treatment, suggesting the need of mild oxidative stress as a trigger for mitophagy. Future studies are warranted to further investigate the association between oxidative stress, mitochondrial fragmentation, and mitochondrial fission as targets against diabetic cardiomyopathy.
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Dose-dependent Role of Inwardly Rectifying Potassium Current on Cardiac Automaticity of Human Embryonic Stem Cell-derived Cardiomyocytes

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The inwardly rectifying potassium current (IK1), encode by Kir2 family, is responsible for maintaining the negative resting potential, and contributes to phase 3 repolarization of the cardiac action potential. IK1 was generally thought to suppress cardiac automaticity, while the suppression of IK1 in adult ventricular cardiomyocytes (CMs) could engineer bio-artificial pacemaker-like cells to spontaneously fire action potential. Our studies also showed that overexpressed the gene of Kir2.1 could facilitate the electrophysiological maturing of mouse and human embryonic stem cell-differentiated CMs (ESC-CMs), which have the high degree of automaticity with nearly 50% of cells that can spontaneously fire action potential. In this study, we extensively analyzed the electrophysiology of mouse and human ESC-CMs, and found that the maximum diastolic potential in spontaneously firing ESC-CMs, -72.1±1.3 mV in atrial cells and -75.0±2.1 mV in ventricular cells, were significantly more hyperpolarized than that in quiescent ESC-CMs (-64.4±2.1 mV in atrial cells and -67.1±3.2 mV in ventricular cells). Applying a small amount of IK1 to hyperpolarize the membrane potential could enable those quiescent ESC-CMs to spontaneously fire action potential, indicating the enhancement of cardiac automaticity, while a large amount of IK1 could quiet those spontaneously firing cells down. By combining computational and experimental analyses, we confirmed that the synergistic interaction of IK1 and pacemaker current (If) could efficiently regulate cardiac automaticity during the differentiation. Our studies disclosed a dose-dependent role of IK1 on cardiac automaticity that a small amount of IK1 enhances and a large amount of IK1 suppresses cardiac automaticity in ESC-CMs during differentiation.

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Aberrant TGFβ Signaling as an Etiology of Left Ventricular Non-compaction Cardiomyopathy

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Left ventricular non-compaction (LVNC) is the third most prevalent cardiomyopathy in children and has a unique phenotype with characteristically extensive hypertrabeculation of the left ventricle, similar to the embryonic left ventricle, suggesting a developmental defect of the embryonic myocardium. However, studying this disease has been challenging due to the lack of an animal model that can faithfully recapitulate the clinical phenotype of LVNC. To address this, we showed that patient-specific induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) generated from a family with LVNC history recapitulated a developmental defect consistent with the LVNC phenotype at the single-cell level. We then utilized hiPSC-CMs to show that increased transforming growth factor beta (TGFβ) signaling is one of the central mechanisms underlying the pathogenesis of LVNC. LVNC hiPSC-CMs demonstrated decreased proliferative capacity due to abnormal activation of TGFβ signaling (Figs A-B). Exome sequencing demonstrated a mutation in TBX20, which regulates TGFβ signaling through upregulation of ITGAV, contributing to the LVNC phenotype. Inhibition of abnormal TGFβ signaling or genetic correction of the TBX20 mutation (Figs C-D) using TALEN reversed the proliferation defects seen in LVNC hiPSC-CMs. Our results demonstrate that hiPSC-CMs are a useful tool for the exploration of novel mechanisms underlying poorly understood cardiomyopathies such as LVNC. Here we provide the first evidence of activation of TGFβ signaling as playing a role in the pathogenesis of LVNC.

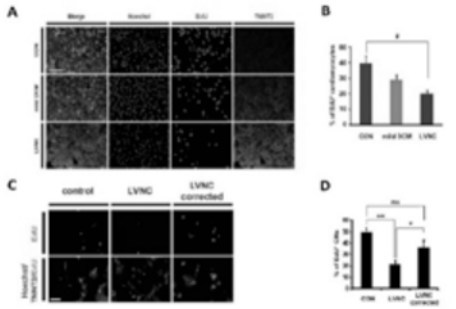

Figure. Proliferation defect in LVNC hiPSC-CMs. A. Immunostaining of nuclear (DAPI), TNNT2 (red), and EdU (green) in 2 weeks-hiPSC-CMs. **B.** Percentage of EdU+ 2 weeks-hiPSC-CMs. **C.** Immunostaining in control, LVNC and TBX20 mutation corrected (LVNC corrected) hiPSC-CMs. **D.** Percentage of EdU+ hiPSC-CMs at 2 weeks in control, LVNC and LVNC corrected group. Statistical analyses were conducted using ANOVA (*p < 0.05, **p < 0.005, #p < 0.001). Error bars represent s.e.m. Scale bars, 100 μm.

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The Role of Trpm4 Ion Channel in Ventricular Remodeling

Miklos Kecskes, Griet Jacobs, Sara Kerselaers, Thomas Voets, Rudi Vennekens, KU Leuven, Leuven, Belgium

Cardiac hypertrophy is characterized by an increase in heart mass and associated changes in the shape of the left ventricle. Pathological hypertrophy is triggered by various stimuli such as hypertension and humoral factors like Angiotensin II. Prolonged cardiac hypertrophy can result in heart failure and sudden death; therefore better understanding of the disease has significant importance.

The Transient Receptor Potential (TRP) superfamily of ion channels form a large family of cation channels related to the product of the trp gene in Drosophila. TRPM4 a member of this family is a calcium-activated non-selective cation channel. It is activated by an increase in intracellular Ca2+ concentrations. The channel is equally permeable to Na+ and K+ but, not permeable to Ca2+. TRPM4 is expressed in the heart both in the atria and ventricle and activated during the cardiac action potential. Our goal was to study the development of hypertrophy in cardiac specific TRPM4 deficient mice.

We have not found significant hypertrophy under basal conditions in these KO mice. However chronic AngII treatment resulted in increased heart weight to body weight ratio in cardiac specific TRPM4 deficient mice compared to WT. Furthermore we have found an increased expression of several hypertrophy marker genes compared to WT, such as atrial natriuretic

peptide, alpha actin and Mcip1 after the Ang treatment. Histological analysis of the hearts showed an increased myocyte size in cardiac specific TRPM4 deficient compared to WT mice. Sarcoplasmic reticulum store depletion experiments showed an increased store-operated calcium entry in TRPM4 deficient myocytes compared to WT. Store-operated calcium entry has been recently recognized as an important mechanism in cardiac hypertrophy by activating calcium dependent pathways. In line with that we have found increased activity and expression of calcineurin - a calcium dependent phosphatase in hypertrophy- in the hearts of AngII treated TRPM4 deficient mice compared to WT
Taken together we propose that TRPM4 play a specific role in calcium homeostasis of the ventricular myocytes and functions as a regulator of cardiac hypertrophy.
M. Kecskes: None. **G. Jacobs:** None. **S. Kerselaers:** None. **T. Voets:** None. **R. Vennekens:** None.

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Elucidating the Role of Platelet Derived Growth Factor Receptor Alpha Signaling in Cardiac Fibroblasts
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Cardiac fibrosis is a major component of heart disease and is a hallmark of decreased cardiac function. Currently, there are no treatments that attenuate fibrosis directly. This major hurdle can be overcome by targeting the resident fibroblast. Preliminary data demonstrates that loss of PDGFRα expression in the adult cardiac fibroblast lineage results in loss of over half of resident fibroblasts. A time course experiment revealed that in as little as 4 days after PDGFRα gene deletion fibroblast loss can observed. Based on the basal level of fibroblast proliferation (0.8%+/-0.9, i.e. 4 of 398 cells), we hypothesize that PDGFRα signaling is essential for fibroblast maintenance and that fibroblasts undergo rapid turnover. We have begun to elucidate which downstream signals of PDGFRα are involved the different roles of the fibroblast. Using a PDGFRα-dependent-PI3K-deficient mouse model, preliminary data indicates that PDGFRα-dependent PI3K signaling is involved in this cell survival response. Future studies will investigate cardiac fibroblast maintenance signals by determining which cell types secrete PDGF ligands. We will also investigate the role of PDGFRα signaling after myocardial infarction. Our lab has genetic tools that enable us to follow fibroblasts after injury, and we have determined both the number of proliferating fibroblasts at different time points, as well as the fraction of fibroblasts that make up the total population of proliferating cells after LAD ligation. Our preliminary data in control hearts shows that fibroblasts reach their peak of proliferation within a week after infarction, although they remain one of the most proliferative cell types as long as three weeks after induction. Our studies will illuminate the role of the fibroblast in tissue homeostasis and after infarction and identify how these cells contribute to overall cardiovascular function and delineate the fine balance between the essential and detrimental functions of the fibroblast.
M.J. Ivey: None. **M. Tallquist:** None.

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Circulating Mir-21, -378, and -940 Increase in Response to an Acute Exhaustive Exercise in Patients With Chronic Heart Failure
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Background: Exercise training is recommended as a useful intervention in cardiac rehabilitation for patients with chronic heart failure. There has been emerging evidence indicating that circulating microRNA (miRNA, miR) could mediate the beneficial effects of exercise in both healthy persons and patients with cardiovascular diseases. The current study was aimed to investigate the regulation of circulating miRNAs in response to an acute exhaustive exercise in patients with chronic heart failure (CHF). Methods: Twenty-eight CHF patients (mean age=59.07±1.79 years, 14 in NYHA Class II and 14 in NYHA Class III) performed a symptom-limited incremental cardiopulmonary exercise test on a bicycle ergometer according to a standardized exercise protocol of revised Ramp10 programs (acute exercise). Serum samples were collected before and immediately after exercise session. The cardiac or muscle specific/enriched miRNAs including miR-1, miR-133a, miR-133b, miR-499, miR-208a, miR-208b, miR-378, miR-486, and miR-940 were determined. In addition, other miRNAs involved in angiogenesis (miR-20a, miR-328, miR-126, miR-221), inflammation (miR-21, miR-146a, miR-155), and ischemia adaptation (miR-210, miR-21, miR-146a) were also investigated. Moreover, inflammatory and muscle damage markers including creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB), troponin T (Tn-T), N-terminal pro-brain natriuretic peptide (NT-ProBNP) and high sensitive C reactive protein (hs-CRP) were determined by enzyme linked immunosorbent assays (ELISA). Results: Serum miR-21, -378, and -940 were found to be significantly increased in response to acute exercise in CHF patients. In addition, no robust correlation was identified between changes of these miRNAs and muscle damage or inflammation, indicating a distinct adaptation by miRNAs. However, a linear correlation between the change of miR-21 and VO2max was observed (R=0.42, P=0.03). Conclusion: Our data suggest that circulating miR-21, miR-378, and miR-940 might be potential biomarkers for the improvement of exercise capacity in patients with chronic heart failure.
T. Xu: None. **Q. Zhou:** None. **L. Che:** None. **G. Li:** None. **J. Xiao:** None.

252
Doxorubicin-induced H9c2 Cell Death is Mediated by Excessive Mitochondrial Fission and Mitophagy
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Doxorubicin (DOX) is a widely used antineoplastic agent that can cause heart failure. DOX cardiotoxicity is closely associated with mitochondrial damage. Mitochondrial fission and mitophagy are quality control mechanisms that help maintain a pool of healthy mitochondria. However, too much mitochondrial fission and/or mitophagy may compromise cell viability. Indeed, Mdivi-1, an inhibitor of the fission protein Drp1, can attenuate DOX-induced cardiac injury, suggesting that mitochondrial fragmentation may play a role in DOX cardiotoxicity. Using genetic gain- and loss-of function approaches, we determined whether mitochondrial fragmentation and/or mitophagy contribute to DOX-induced cardiomyocyte death. H9c2 cardiac myoblast cells were transfected with siRNA targeting Drp-1 before DOX administration. Mitochondrial morphology was examined with confocal microscopy after infection of the cells with the adenovirus encoding mitochondria-targeted fluorescent protein MitoDsRed. Morphometric analysis demonstrated that Drp-1 knockdown markedly diminished DOX-induced mitochondrial fragmentation as shown by form factor, aspect ratio, and mean mitochondrial size. This led to reduced cardiomyocyte death as revealed by the percentage of propidium iodide (PI)-positive cells and the cleavage of caspase-3 and Poly ADP ribose polymerase (PARP). Not

surprisingly, Drp-1 knockdown also attenuated DOX-induced mitophagy flux as assessed by the dual fluorescent mitophagy reporter mt-Rosella. Further, knockdown of Parkin, a key regulator of mitophagy, dramatically diminished DOX-induced H9c2 cell death. Although Drp1 overexpression did not markedly increase DOX-induced cell death, Parkin overexpression predisposed H9c2 cells to DOX toxicity. Together, these results suggest that DOX-induced cardiotoxicity may be due to excessive mitochondrial fragmentation and accelerated mitochondrial degradation through autophagy. Strategies that limit mitochondrial fission and mitophagy within the physiological range may help reduce DOX cardiotoxicity. However, further studies are clearly warranted to make sure that these strategies will not compromise the antitumor efficacy of DOX.

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Restoring GDF11 to Normal Levels in Old Mice Has no Effect on Cardiac Structure and Function

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Rationale: GDF11 (Growth Differentiation Factor 11) is a member of the TGFβ super family of secreted factors, which play an important role in the regulation of cell processes including proliferation, differentiation, death, adhesion, and migration. Recently it was shown that circulating GDF11 levels fall with aging and this change is associated with pathological cardiac hypertrophy (PCH). Restoring GDF11 to normal levels was shown to rescue PCH.
Objective: To determine if we can confirm the hypothesis that correcting the levels of a single factor, GDF11, determines aging related PCH.
Methods and Results: We used the study design described by Loffredo et al, 2013. Investigators were blinded to treatment group. 24 month old C57BL/6 male mice were given a daily injection of recombinant GDF11 at 0 .1mg/kg or vehicle for 28 days. GDF11 bioactivity was confirmed in-vitro. After treatment, GDF11 levels were significantly increased but there was no difference in heart weight (HW) to body weight (BW) ratio (4.74 vs 4.70) between GDF11 and vehicle treated old mice. HW/BW ratios of old mice were not different from12 week-old animals (4.56) and the PCH markers ANP and BNP were not different in young verses old mice. Before GDF11 treatment there were no significant differences in ejection fraction (46 versus 46 %), internal ventricular dimensions (4.33 vs 4.28 mm), and septal wall thickness (0.72 versus 0.78 mm) between GDF11 and vehicle treated groups. All structural and functional parameters remained unchanged at 1, 2 and 4 weeks of treatment. Strain analysis showed no significant difference in radial or longitudinal strain. Invasive hemodynamics performed at time of sacrifice also showed no significant differences in max dP/dt (6499.17 vs 6011.95 mmHg/s) or min dP/dt (-6551.73 vs -6214.33 mmHg/s) between GDF11 and vehicle treated animals respectively.
Conclusions: There is no significant age-related PCH in C57Bl6 mice. GDF11 injections had no effects on cardiac structure or function in old male C57BL/6 mice. Our results question the idea that there is age-related PCH in disease free C57BL6 mice and question the findings that restoring GDF11 in old mice has any effect on cardiac structure or function.

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14, 15-EET Protects Heart Pericytes by Delaying I/R Injury-induced Calcium Overloading

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Background-Pericytes are an important cellular component of the blood vessel wall of the arteries, arterioles, and microvessels of the heart; they provide structural integrity and regulate vessel diameter by contracting and relaxing dynamically in response to vasoactive stimuli. It has been suggested that pericytes contribute to coronary no-flow due to pericyte constriction following myocardial infarction, thus worsening outcome. It has also been demonstrated that intracellular calcium is involved in perciyte constriction. Our previous findings indicate that cardiomyocytes are protected following ischemia/reperfusion injury (I/R) by the eicosanoid 14,15-EET. Since 14,15-EET is protective following I/R and a vasodilator, we tested the hypotheses that I/R injury induces calcium overloading, which injures peciytes, and that 14, 15-EET is able to block this process.
Methods and Results-We isolated and cultured pericytes from the mouse heart ventricle by 3G5 antibody Dynabead sorting. Pericytes were characterized by multiple immunocytochemical markers for contractile proteins, cytoskeletal protein, and cell surface receptors (alpha-smooth muscle actin, calponin-1, NG2, vimentin, CD31,smoothlin, and fibroblast protein-1). Cultured pericytes were subjected to 5 hours of oxygen and glucose deprivation, with or without 14,15-EET, followed by 15 hours of re-oxygenation in the absence of 14,15-EET. Calcium imaging and cell death during re-oxygenation were assessed by Fluo-4 and propidium iodide respectively. Digital images were taken with confocal microscope (Nikon Eclipse Tie-A1RSi). The brightness of the green fluorescent signals represents the relative level of intracellular calcium and the red fluorescent signals represent the cell death. We found that calcium signal peak (overloading) occurred during re-oxygenation, immediately followed by cell death. This process was delayed by 14,15-EET treatment during oxygen and glucose deprivation. The cell death at 5h, 10h, and 15h of re-oxygenation was 57.2%, 71.1%, and 85.3% in control group, and 19.9%, 35.3%, and 58.3% in 14,15-EET treated group.
Conclusions-Our data suggests that 14,15-EET-induced protection in pericytes is mediated through the calcium signaling pathway.

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Hur/ppar-gamma Mediates Posttranscriptional Regulation of Hyperglycemia-induced Pro-inflammatory Response

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Metabolic disorders including diabetes and obesity are associated with persistent activation of the innate immune response, which leads to low-grade inflammation and increased cardiovascular risk. The RNA-binding protein HuR binds to and post-transcriptionally regulates inflammatory cytokines,

mRNA stability and gene expression, although it's exact role in the diabetic condition remains unclear. Bone marrow-derived macrophages (BMM) exposed to high glucose (HG, 25mM D-glucose) displayed elevated expression of HuR and reduced expression of nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR-gamma) as compared to low glucose (LG, 5mM D-glucose)-treated cells. The increase in HuR expression was associated with an increase in the gene expression of various inflammatory cytokines including TNF-alpha, MCP-1, IL-1beta and IL-6. At the molecular level, macrophages activated with LPS showed enhancements in expression of inflammatory mRNAs and also promoted their stability. Conversely, knockdown of HuR induced post-transcriptional silencing, reduced the mRNA stability and expression of inflammatory cytokines. Macrophages treated with selective antagonists of the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR-gamma), GW9662 displayed increases in HuR expression and of various inflammatory cytokines. In an in vivo model of myocardial infarction, mice receiving intramyocardial injection of HuR-specific shRNA displayed a reduced inflammatory cytokine profile in the myocardium as compared to control shRNA treated mice. Taken together, these data highlight the role of HuR as a homeostatic coordinator of cytokine mRNA that regulates innate inflammatory effects and demonstrates the potential of modulating the effects of HuR for clinical benefit against pathologic inflammation in diabetes/obesity.

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Renal Denervation Preconditions the Myocardium by Promoting Nitric Oxide Signaling and Attenuating Oxidative Stress in Spontaneously Hypertensive Rats

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Background: Recent clinical studies suggest that renal denervation (RDN) not only decreases blood pressure (BP) and sympathetic nerve activity, but may also exert additional systemic cardioprotective actions. We investigated whether RDN preconditions the heart against subsequent myocardial ischemia/reperfusion (MI/R) injury in spontaneously hypertensive rats (SHR).
Materials and Methods: SHR (20w old) received either bilateral radiofrequency (RF) RDN or sham RDN (Biosense Webster Stockert 70 RF generator). After 4w of BP recording, Plasma and left ventricle (LV) tissue were collected for measurement of nitric oxide (NO) metabolites, nitrite (NO2-, ion chromatography) and S-Nitrosothiol (RSNO). RT PCR was used to examine transcriptional changes. Colorimetric assays were used to quantify malondialdehyde (MDA) and carbonyl content. In additional studies, 4w after RDN (n = 8) or Sham (n = 9) treatment, SHR were subjected to 30m of left coronary artery ischemia followed by 24h reperfusion and myocardial infarct/area-at-risk (AAR) was determined.
Results: 4w after treatment, mean BP was significantly decreased in RDN compared to Sham SHR (157±2 vs 142±2 mmHg, $p < 0.05$). Plasma NO2- levels were elevated 4w following RDN ($p < 0.01$). Cardiac NO2- levels increased from 2.6 to 3.2 nmol/mg ($p < 0.05$) and RSNO levels increased from 0.5 to 1.1 nmol/mg ($p < 0.05$) following RDN. Moreover, myocardial oxidative stress was markedly attenuated as measured by carbonyl content ($p < 0.05$) and MDA levels ($p < 0.01$). Greater transcription of antioxidants, SOD1 ($p < 0.05$) and GPX-1 ($p < 0.05$) were also observed in the RDN treated group. SHR receiving RDN therapy exhibited a trend in infarct size reduction (42% per AAR reduction, $p = 0.09$) compared to sham following MI/R. We observed 2-fold greater survival in the RDN treated group (88%, 7 of 8) compared to sham (44%, 4 of 9) following MI/R.
Conclusions: RDN produced a sustained elevation in NO bioavailability and signaling in the heart and blood. Additionally, RDN attenuated myocardial oxidative stress and augmented the antioxidant defense system in SHR. Although further studies are warranted, preliminary results indicate that these mechanisms may promote myocardial preconditioning and improve survival in the setting of MI/R injury.

D.J. Polhemus: None. **J. Gao:** None. **D.R. Kapusta:** None. **D.J. Lefer:** None.

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Berbamine Postconditioning Protects the Heart From Ischemia/Reperfusion Injury Through the Regulation of Autophagy During Reperfusion

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Myocardial infarction resulting from coronary atherosclerosis is the leading cause of death in modern society. Reperfusion is an essential treatment to salvage ischemia myocardium from necrosis, while it also leads to additional damage. Therefore, exploring effective medicines to protect the heart from post-ischemic injury is one of the major objectives of cardiovascular research. Berbamine, a nature compound of bisbenzylisochinoline alkaloids from Barberry, protects the heart from ischemia/reperfusion (I/R) injury when given before I/R, but it is unknown whether it has cardioprotective effects when given at the onset of reperfusion (postconditioning, PoC), a protocol with more clinical impact. Therefore, the present study was designed to determine whether berbamine PoC (BMPoC) is able to protect the heart from reperfusion injury by using perfused I/R rat hearts and simulated I/R cardiomyocytes. We found that BMPoC added during the first 5-min of reperfusion concentration-dependently improved post-ischemic myocardial function and limited infarcted area. A similar protection was observed in isolated rat cardiomyocytes characterized by the attenuation of I/R-induced depression of cell contraction and loss of mitochondrial membrane potential. Moreover, autophagy (an intracellular bulk degradation process for proteins and organelles) was significantly stimulated by myocardial I/R, but it was suppressed by BMPoC. Next, we examined how berbamine regulates autophagy and found that BMPoC inhibited the expression of beclin 1, a critical regulator for autophagosome initiation. Then, adenoviral overexpression and knockdown of beclin 1 in ventricles conformed its roles in the BMPoC-mediated cardioprotection. Further, Western blot analysis revealed that BMPoC-suppressed beclin 1 expression was mediated via the activation of PI3K/Akt signaling pathway. Our results suggest that BMPoC confers cardioprotection against I/R injury at least in part by the inhibition of beclin1-dependent autophagy through the activation of PI3K/Akt signaling pathway. These findings reveal new roles and mechanisms of berbamine in the cardioprotection against I/R injury.

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Nanoparticle-mediated Targeting of a Chemical Inhibitor of Drp1 to the Mitochondria Induces Cardioprotection From Myocardial Ischemia-reperfusion Injury

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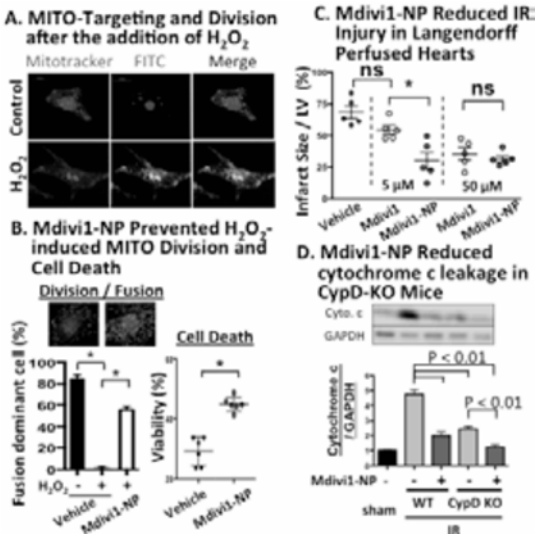
Background: Mitochondria (MITO) injury including MITO permeability transition pore (mPTP) opening plays a major role in the mechanism of ischemia-reperfusion (IR) injury. Intravenous administration of an inhibitor of mPTP opening, cyclosporine A, can reduce IR injury in animals and patients with acute myocardial infarction (MI), however; the power of cardioprotection by cyclosporine A is insufficient. We tested the hypothesis that nanoparticle-mediated targeting of Mdivi1, a chemical inhibitor of Drp1, to MITO enhances cardioprotection from IR injury.
Methods and Results: We formulated poly(lactic acid/glycolic acid) (PLGA) nanoparticles containing Mdivi1 (Mdivi1-NP) or FITC (FITC-NP). In neonatal rat cardiomyocytes, PLGA nanoparticles accumulated in MITO after the addition of hydrogen peroxide (H2O2) that represents oxidative stress during IR (Fig.A). Treatment with Mdivi1-NP reduced H2O2-induced MITO division and cardiomyocyte death (Fig.B). This Mdivi1-NP cardioprotective effect was not seen over adenovirus harboring Drp1 shRNA transduced cardiomyocyte.
In an in vivo and ex vivo murine model, treatment with Mdivi1-NP at the time of reperfusion reduced infarct size more effectively than Mdivi1 alone (Fig.C). Interestingly, Mdivi1-NP inhibited the leakage of cytochrome c to cytosol and MITO swelling, and reduced IR injury in both wildtype and cyclophilin D (a key regulatory molecule for mPTP opening)-KO mice (Fig.D).
Conclusions: Mdivi1-NP enhanced cardioprotection against IR injury through mechanisms independent of mPTP opening. Mdivi1-NP can be a novel cardioprotective strategy in acute MI.



A. Ishikita: None. **T. Matoba:** None. **G. Ikeda:** None. **K. Egashira:** None.

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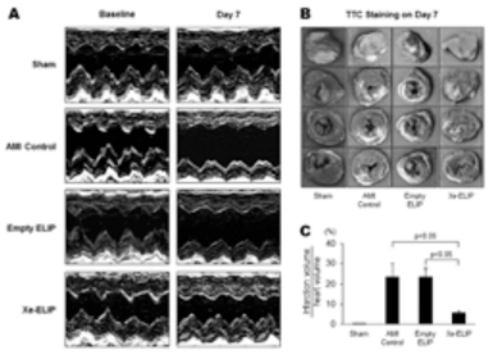
Xenon-loaded Echogenic Liposomes Provide Cardioprotection in Acute Myocardial Infarction
Xing Yin, Yuping Ren, Melvin E Klegerman, Melanie R Moody, Tao Peng, Hyunggun Kim, David D McPherson, Shao-Ling Huang, Univ of Texas Health Science Ctr at Houston, Houston, TX

Background: Acute myocardial infarction (AMI) is a leading cause of sudden cardiac death. Following AMI, left ventricular (LV) remodeling plays an important pathophysiologic role in progression to heart failure (HF). Effective therapeutic approaches to decrease the size of MI can reduce progression to HF. Xenon (Xe), a non-toxic, easily diffusible, noble gas can provide cardiovascular protection. However, delivery of Xe is challenging. Carrier delivery of Xe may be an effective way to deliver this gas to the ischemic area. We have developed Xe-loaded echogenic liposomes (Xe-ELIP) for intravascular delivery of the bioactive gas to the infarct area. Here, we investigate the effect of Xe-ELIP on MI size and LV remodeling in an AMI model.
Methods: Xe-ELIP were prepared by a pressurization-freeze method. Rats were randomly divided into 4 groups; sham, AMI control, empty ELIP, and Xe-ELIP at 45 minutes following AMI. AMI was induced by ligating the left anterior descending artery. Liposomes were intravenously administrated immediately after reperfusion. Cardiac function was evaluated by ultrasound at baseline, after AMI, and on day 7. Infarct size was evaluated by TTC staining.
Results: Gas chromatography-mass spectrometry demonstrated that 1 mg lipid contains 190 μl Xe. Xe-ELIP treatment decreased end-systolic LV volume (Fig. 1A), improved cardiac output, and reduced infract volume (Fig. 1B, 1C).
Conclusion: This novel carrier system allows a therapeutic dose of Xe to reach the ischemic myocardium with resultant all preservation. This methodology can be easily translated for intracoronary injection of Xe-ELIP at the time of coronary intervention at AMI for cellular protection.



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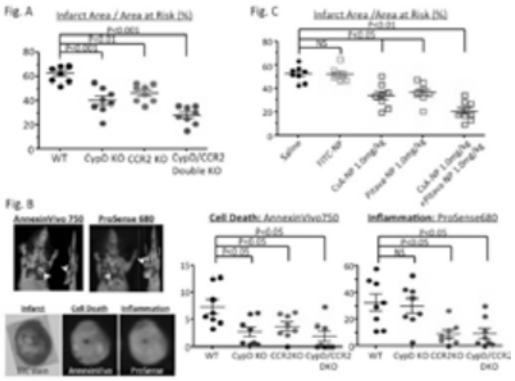
Nanoparticle-mediated Simultaneous Targeting to Mitochondria and Inflammatory Monocytes Confers Additive Cardioprotection against Myocardial Ischemia-reperfusion Injury
Gentaro Ikeda, Tetsuya Matoba, Ayako Ishikita, Kensuke Egashira, Kyushu Univ, Fukuoka, Japan

Background: Targeting one mediator of myocardial ischemia-reperfusion (IR) injury failed to reduce infarct size in clinical trials, suggesting the necessity of the innovative strategy to target more than 2 mediators simultaneously. Previously, we have engineered poly(lactic acid/glycolic acid) nanoparticle containing cyclosporine A (CsA-NP) and pitavastatin (Pitava-NP), and reported that the former inhibits the opening of mitochondrial permeability transition pore (mPTP) and the latter reduces monocyte-mediated inflammation in

IR hearts. Here we tested the hypothesis that nanoparticle-mediated simultaneous targeting to mPTP and monocytes confers additive cardioprotection against IR injury.
Methods and Results: We produced mice deficient with both cyclophilin D (CypD, a key molecule for mPTP opening) and CCR2 (a receptor for monocyte chemoattractant protein-1), and found that the double-KO mice displayed dramatic reduction in myocardial IR injury model (Fig. A). Flow cytometric analysis and fluorescence molecular tomography showed that inflammation was markedly inhibited in CCR2-KO and CypD/CCR2-KO mice while residual inflammation was noted in CypD-KO mice. In CypD mice, Pitava-NP reduced recruitment of Ly6Chigh inflammatory monocytes and showed therapeutic effects (Fig. B). In contrast, CsA-NP reduced IR injury in CCR2-KO mice. Simultaneous treatment with CsA-NP and Pitava-NP at the time of reperfusion showed additive reduction in IR injury in wild-type mice (Fig. C).
Conclusions: Nanoparticle-mediated simultaneous targeting to mitochondria and inflammatory monocytes can be developed as a novel therapeutic strategy in IR injury.



G. Ikeda: None. **T. Matoba:** None. **A. Ishikita:** None. **K. Egashira:** None.

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Foxo4 Promotes Early Inflammatory Response Upon Myocardial Infarction via Endothelial Arg1
Min Zhu, Zhaoning Wang, Robert Luo, Sean Goetsch, Jay Schneider, Joseph Hill, Sidney Morris Jr., **Zhi-Ping Liu**, UT Southwestern, Dallas, TX

Myocardial infarction (MI) can result in a maladaptive remodeling of the heart that leads to heart failure. The post-MI inflammation is necessary for wound healing. Unfortunately it is also a key component of subsequent heart failure pathology. Thus, understanding the mechanism of post-MI inflammation and identifying targets for intervention are of translational interests. To understand the role of FoxO4 in post-MI remodeling, we induced MI in WT and *FoxO4* global knockout (*gKO*) mice by surgical ligation of the left anterior descending coronary artery. *FoxO4*-inactivation resulted in a significantly higher post-MI survival, better cardiac function, and reduced fibrosis and infarct size compared to WT mice. *FoxO4 gKO* mouse hearts had significantly less mount of neutrophils, and reduced expressions of cytokines, MMP9, and Arginase 1 (Arg1). To determine the contribution of cellular FoxO4 to the post-MI phenotype, we generated cardiomyocyte and endothelial cell specific *FoxO4* knockout mice (*FoxO4 cKO* and *eKO*). While *FoxO4 cKO* mice showed similar post-MI phenotype to those of WT littermates, *FoxO4 eKO* mice had better cardiac function with reduced inflammation, neutrophil infiltration, and Arg1 expression similar to those of *FoxO4 gKO* mice. Since Arg1 is a competitive inhibitor of nitric oxide (NO) synthase and NO inhibits lymphocyte adhesion and transmigration across the endothelial barrier, downregulation of neutrophils in post-MI *FoxO4*-null mouse hearts could be due to reduced endothelial Arg1 and thus increased NO. Consistent with this hypothesis, we show that Arg1 is a direct transcriptional target of FoxO4. Endothelial FoxO4 is necessary and sufficient to activate Arg1 expression in response to hypoxia. Knockdown of FoxO4 in human aortic endothelial cells suppressed leukocyte adhesion. Furthermore, chemical inhibition of Arg1 in WT mice had similar cardioprotection following MI as *FoxO4*-inactivation and administration of NOS inhibitor to *FoxO4 gKO* mice reversed the beneficial effects of *FoxO4*-deletion on post-MI cardiac function. Our studies showed that FoxO4 promotes early inflammatory response via endothelial Arg1 and suggest a potential therapy to reduce the early post-MI inflammation and enhance cardiac function in MI-injured hearts.

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Knockout of Type VI Collagen Preserves Mitochondrial Structure and Function Following Myocardial Infarction
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Cardiac remodeling is a dynamic process that is accelerated after myocardial infarction (MI), and the traditional focus of key extracellular matrix proteins that mediate remodeling has been on the fibrillar types I and III collagen. We have previously reported that knockout mice of the lesser-known, non-fibrillar collagen VI (Col6-/-) are protected from MI injury as evidenced by significantly reduced infarct size, fibrosis and apoptosis leading to preserved long-term cardiac function. Determining the mechanisms underlying this cardioprotection is the goal of this study. Interestingly, the Col6-/- mice are a model for Bethlam Myopathy, a rare skeletal muscular dystrophy that is characterized by mitochondrial dysfunction leading to premature apoptosis of skeletal myocytes. We hypothesized that alterations in mitochondrial structure and function in the myocardium of Col6-/- mice may play key mechanistic roles responses to ischemic injury. Mitochondrial morphology was visualized by transmission electron microscopy to compare pre- and post-MI changes. Mitochondria from uninjured Col6-/- LV tissue had similar morphology as WT, and at 3 days post-MI the mitochondrial morphology was similarly compromised in both WT and Col6-/- mice. However, at 14 days post-MI the Col6-/- mitochondria were less swollen (43 ± 5.1% decrease in overall volume) and displayed improved orientation/organization over WT (continuous strands). We measured basal O2 consumption and 24 hours post-MI in mitochondria isolated from the infarcted zones of both genotypes. The respiratory control index (RCI) of the Col6-/- mitochondria was lower in the basal, uninjured hearts (7.2 ± 0.9 in WT vs. 4.9 ± 0.6 in Col6-/-). However, the RCI of mitochondria in the infarcted region of Col6-/- hearts at 24 hours post-MI declined less than WT (post-MI values of 2.7 ± 0.5 in WT vs. 2.8 ± 0.7 in Col6-/-) . These data indicate that Col6-/- mice have preserved mitochondrial morphology and a smaller decline in respiration following MI, which may represent a novel homeostatic mechanism underlying protection from ischemic injury in the Col6-/- heart.

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Therapeutic Potential of a Novel Necrosis Inhibitor in Myocardial Ischemia-reperfusion Injury

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Background: Reperfusion, although essential for salvage of ischemic myocardium, paradoxically causes a wide variety of injuries. Opening of mitochondrial permeability transition pore (mPTP) and Ca^{2+} overload contribute to myocardial ischemia-reperfusion (I/R) injury. We aimed to investigate the protective role of a novel necrosis inhibitor (NecroX-7; NecX) against myocardial I/R injury, using in vitro and in vivo models.
Methods and Results: In H9C2 rat cardiomyoblasts exposed to hypoxia-reoxygenation stress, the main mechanism of cell death was not apoptosis but necrosis, which was prevented mainly by NecX, the necrosis inhibitor, but not by Z-VAD-fmk, the apoptosis inhibitor. The protective effect of NecX was based on its potent ROS scavenging activity, especially on mitochondrial ROS which is one of the major inducers of mPTP opening. NecX preserved mitochondrial membrane potential, mitochondrial structure, through prevention of Ca^{2+} influx and inhibition of the opening of mPTP. Inhibition of necrosis by NecX was accompanied by reduction of phospho-p38 MAPK and phospho-JNK, and decrease of HMGB1. Using Sprague-Dawley rats exposed to myocardial ischemia for 45 minutes followed by reperfusion, we compared therapeutic efficacies of NecX and Ciclosporin A (CsA) with 5% dextrose (control), each administrated 5 minutes before reperfusion. NecX markedly inhibited myocardial necrosis, reduced fibrotic area and attenuated the release of cardiac enzymes, compared to dextrose and CsA. Additionally, NecX preserved systolic function and prevented pathologic dilatory remodeling of left ventricle.
Conclusion: The novel necrosis inhibitor has a significant protective effect against myocardial I/R injury, indicating that it is a promising candidate for cardioprotective adjunctive measure on top of reperfusion therapy.
Clinical implication: We are trying to translate this experimental data into patients. A phase I clinical trial confirmed the safety profiles of NecX [NCT01737424] and a phase II trial for STEMI patients (NEXsteMI trial) is ongoing [NCT02070471].

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Nitrite Therapy Ameliorates Myocardial Injury via H2S and Nuclear Factor-Erythroid 2-Related Factor 2 (Nrf2)-Dependent Signaling in Chronic Heart Failure

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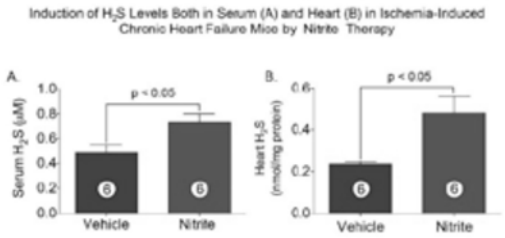
Background: Nitric oxide (NO) and hydrogen sulfide (H_2S) are reduced in congestive heart failure. Recent studies suggest cross-talk between NO and H_2S signaling. We previously reported that sodium nitrite ($NaNO_2$) significantly ameliorates myocardial ischemia-reperfusion injury and heart failure. Nrf2 regulates the expression of antioxidant protein genes and is upregulated by H_2S. We examined the effects of $NaNO_2$ therapy on endogenous H_2S bioavailability and Nrf2 activation in mice subjected to ischemia-induced heart failure.
Materials and Methods: Mice underwent 60 min. of left coronary artery occlusion and 4 weeks (WKS) of reperfusion. $NaNO_2$ (165 μg/kg) or saline vehicle (VEH) was administered at reperfusion and then in drinking water (100 mg/L) for 4 wks. Left ventricular ejection fraction (LVEF) was determined at baseline and 4 wks of reperfusion. Myocardial tissue was collected and analyzed for oxidative stress status and respective gene/protein levels.
Results: $NaNO_2$ therapy preserved LVEF (47 ± 4% vs. 32 ± 4%, $p < 0.01$) and LV diastolic and systolic dimensions (LVEDD/LVESD; 4.0/3.1 mm vs. 4.5/3.9 mm, $p < 0.05$) at 4 wks. MDA and protein carbonyl contents were significantly reduced in $NaNO_2$ treated mice as compared to VEH. $NaNO_2$ markedly increased expression of CuZn-superoxide dismutase and catalase at 4 wks. Furthermore, $NaNO_2$ increased mRNA levels of H_2S producing enzymes and H_2S bioavailabilty. Cardiac Nrf2 activation was also observed with $NaNO_2$ therapy.
Conclusions: Our results demonstrate that $NaNO_2$ therapy significantly improves left ventricular function via by increasing H_2S bioavailability, activation of Nrf2, and increased antioxidant defenses.



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K.N. Islam: None. **E. Donnarumma:** None. **S. Bhushan:** None. **D.J. Lefer:** 7. Ownership Interest; Significant; is a participant of a pending U. S. patent filed on 15 nov. 2007 (# 61/003150) regarding the use of nitrite salts in chronic ischemia.. 8. Consultant/Advisory Board; Significant; Is on the Scientifc advisory board of Theravasc, Inc. Theravasc is currently developing sodium nitrite for the treatment of cardiovascular diseases..

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CITED4 Induces Physiologic Hypertrophy and Improves Cardiac Remodeling After Ischemic Injury

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Cardiac hypertrophy is an adaptive response to increased hemodynamic stresses, which can be either physiologic, as with exercise, or pathologic, as with valvular heart disease. Recent data suggest that physiologic hypertrophy secondary to exercise may be mediated by the transcription factor CEBPβ and the p300-interacting protein CITED4. We sought to investigate the cardiovascular effects of CITED4 expression in vivo.
Using a cardiac-specific and inducible transgenic mouse (Tg) model, we determined the effects of CITED4 expression on cardiac parameters including heart weight, cell size, cardiac function and gene expression. Expression of CITED4 for 3 weeks induced increases in heart weight (22% in HW/TL, $p < 0.01$) and cardiomyocyte (CM) size (24.5% in cell area, $p < 0.001$) with normal systolic function and without evidence of fibrosis. Gene profiling demonstrated increased expression of cardiac troponin, a favorable αMHC/βMHC ratio and a reduction in BNP consistent with physiologic hypertrophy. Genome-wide expression profiling of neonatal rat ventricular myocytes (NRVMs) over-expressing CITED4 demonstrated the activation of

a unique set of genes including BCL2, ATP12a, Efemp1, Ifi204 and Tcf19.
To further examine the potential beneficial role of CITED4, we induced ischemia by transient occlusion of the left anterior descending (LAD) coronary (30 min) followed by reperfusion for 24 hours, 6 days or 4 weeks. At 24 hrs after ischemia-reperfusion injury (IRI), neither cardiac dysfunction on echo nor infarct sizes were different between CITED4 Tg and controls. However, CITED4 Tgs showed substantial recovery of cardiac function at 4 weeks (FS: CITED4 Tg 51%, Control 34%, $p < 0.01$) and a 3.4-fold reduction in fibrosis ($p < 0.005$). Analysis of possible cellular responses responsible for the functional recovery demonstrated enhanced autophagic flux with reduced accumulation of LC3II (down 71%, $p<0.05$) and p62 (down 54%, $p<0.005$). Further examination of the involved signaling pathway revealed direct activation of mTORC1 and its effectors consistent with a growth phenotype.
We conclude that CITED4 expression is sufficient to induce physiologic cardiac hypertrophy and improves cardiac remodeling after ischemic injury likely through activation of mTORC1.
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Screening and Validation of Small Molecule Inhibitors of Serum and Glucocorticoid Regulated Kinase-1 as Novel Therapy for Cardiac Arrhythmia Disorders

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Alterations in sodium flux (INa) play an important role in the pathogenesis of cardiac arrhythmias and may also contribute to the development of cardiomyopathies. Recent data demonstrates a critical role for the serum and glucocorticoid regulated kinase-1 (SGK1) by modulation of INa in the heart, by regulating the voltage-gated sodium channel NaV1.5. To better understand and pharmacologically probe the significance of SGK1 in cardiac dysrhythmias, we have used computer aided drug discovery (CADD) to identify small molecule inhibitors of SGK1.
Expression of a constitutively active form of SGK1 (SGK1-CA) increased INa (1.7 fold, $p <0.005$) in a stable line of HEK cells expressing NaV1.5. Conversely, expression of a dominant negative form (SGK-DN) decreased NaV1.5 channel activity (2.8 fold, $p <0.005$). We examined the effects of SGK1 inhibition in a LQT model, by quantifying the ability of SGK1 inhibition to rescue the 2:1 AV block phenotype of the potassium channel zebrafish mutant, breakdance (bkd). Morpholino injection or expression of SGK1-DN significantly rescued the 2:1 AV block phenotype as compared to controls ($p < 0.05$).
Using CADD partnered with iterative empirical screens we identified several hit chemical scaffolds. Our lead compound inhibits the phosphorylation of the SGK1 target gene, GSK3-β in a dose dependent manner in cardiomyocytes (CMs) expressing SGK1-CA at the lowest effective concentration of 0.5μM. There was no significant inhibition of AKT dependent phosphorylation of GSK3-β up to a concentration of 50μM, demonstrating specificity of the inhibitor for SGK1. Incubation of bkd zebrafish mutants with the inhibitor rescued the 2:1 AV block in a dose dependent manner (60% rescue with 45μM, $p < 0.05$). Acute application of the inhibitor dramatically inhibited INa with either expression of SGK1-CA (90.8% reduction, $p <0.05$) or with RFP only (77.5% reduction $p < 0.005$). The half-time of inhibition was 200s with resulting current densities that were not statistically different than those observed with genetic inhibition by expression of SGK1-DN.
We conclude SGK1 activity regulates INa and speculate that structure activity relationship (SAR) derivatives of our lead compound might have a role in treatment of human cardiac arrhythmias.
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Preserved Cardiac Contractility and Venous Return in Beta-arrestin2 Transgenic Mice During Sepsis With Insufficient Resuscitation
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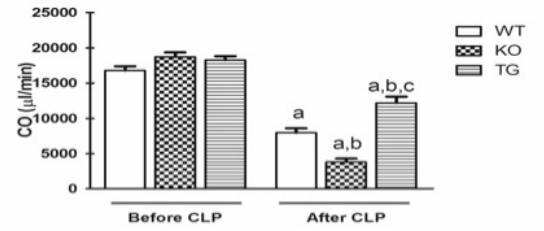
β-arrestin 2 is a negative regulator of inflammation and a protective signaling transducer in acute heart injury. In this study, using echocardiography and Millar Pressure-Volume systems, we found that heart dysfunction accompanied with hemodynamic instability occurred rapidly after experimental sepsis with insufficient resuscitation in wild type and β-arrestin 2 knock out mice but not in β-arrestin 2 transgenic mice. β-arrestin 2 overexpression is associated with preserved preload, cardiac output, systolic contractility and diastolic elasticity after cecal ligation and puncture (CLP). Furthermore, β-arrestin 2 overexpression up-regulated IL-6/IL-6R/gp130/STAT3 anti-apoptotic signaling through suppressing p38 activation and subsequently inhibiting phosphorylation of membrane bound gp130, the signal transducer part of IL-6 receptor complex. In conclusion, β-arrestin 2 is a crucial cardiac function regulator in sepsis.



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Myofilament Tyrosine Phosphorylation by Src Kinase is upregulated in ErbB2 Transgenic Mice
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Objectives: Tyrosine (Tyr) phosphorylation of the myofilament is an emerging, and potentially important,

post-translational modification in cardiomyopathy. ErbB2, a Tyr receptor kinase, was overexpressed in transgenic mice (ErbB2-Tg) resulting in significant cardiac hypertrophy. We hypothesize that the development of cardiac hypertrophy in ErbB2-Tg is associated with increased myofilament Tyr phosphorylation and may implicate myofilament Tyr phosphorylation in cardiac hypertrophy.
Methods: Proteins were isolated from ErbB2-Tg and Ntg heart homogenates (n=4 per group). Reduction/alkylation was followed by trypsinization. Resulting peptides were desalted in C^{18} columns and lyophilized. Phosphorylated Tyr (p-Tyr) enrichment was performed on 20 mg of peptides using a p-Tyr Mouse mAb kit (Cell Signaling). Immuno-precipitated and desalted peptides were analyzed by LC-MS/MS (Orbitrap Elite, Thermo). Raw data were searched with Mascot 2.3. Label-free quantification with MS1 extracted ion chromatograms was performed using Skyline. Western blot analysis for total and phosphorylated Src kinase was performed per manufacturer's protocol (Cell Signaling).
Results: We found a total of 286 p-Tyr modified peptides in ErbB2-Tg compared to 226 in control NTg mice. Over 70 p-Tyr sites on myofilament protein were up-regulated in ErbB2-Tg, including troponin I, myosin heavy chain, titin, α-tropomyosin, myosin-binding protein-C3, myosin regulatory light chain-2 and myosin light chain-1. We used PhosphoMotif Finder to search the potential responsible kinase. Most of the p-Tyr sites were consistent with Src kinase motifs. Furthermore, Western blot analysis showed that total, and phospho-Src (Y416) expression was increased in ErbB2-Tg mice.
Conclusion: We concluded that these novel p-Tyr sites on myofilament proteins are increased in ErbB2-Tg mice and correlate with up-regulated Src kinase activity. Thus increased tyrosine myofilament phosphorylation may be involved in the development of cardiac hypertrophy. Since ErbB2 is a therapeutic target of trastuzumab therapy this may also have translational implications to ameliorate off target effects of cancer treatment.
M. Xu: 2. Research Grant; Modest; Natural Science Foundation of China, No. 81300124. 3. Other Research Support; Modest; Shen Zhen Scientific Plan, No. JCYJ20140416141331544. **G.A. Ramirez-Correa:** None. **Z. Fu:** None. **P.S. Shah:** None. **F. Belmonte:** None. **K. Gabrielson:** None. **A.M. Murphy:** None.

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Sirt-6 Deficiency Impairs Wound Healing in Diabetic (db/db) Mice Through Enhancement of Nf-kb Dependent Transcriptional Activity

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Delayed wound healing is one of the major complications in diabetes and is characterized by chronic proinflammatory response, and abnormalities in angiogenesis and collagen deposition. Sirtuin family proteins regulate numerous physiological processes, including those involved in promotion of longevity, DNA repair, glycolysis and inflammation. However the role of sirtuin 6 (SIRT6), a NAD+-dependent nuclear deacetylase, in wound healing specifically under diabetic condition remains unclear. To analyze the role of SIRT6 in cutaneous wound healing, paired 6 mm stented wound were created in diabetic db/db mice and injected siRNA against SIRT6 in the wound margins (transfection agent alone and non-sensed siRNA served as controls). Wound time to closure was assessed by digital planimetry, and wounds were harvested for histology, immunohistochemistry and western blotting. SIRT6-siRNA treated diabetic wound showed impaired healing, which was associated with reduced capillary density when compared to control treatment. Interestingly, SIRT6 deficiency decreased VEGF expression in the wounds. Furthermore, SIRT6 ablation in diabetic wound promotes NF-kB activation resulting in increased expression of proinflammatory marker and oxidative stress. Collectively, our findings demonstrate that loss of SIRT6 in cutaneous wounds promotes proinflammatory response by increasing NF-kB activation, oxidative stress and decrease in angiogenesis in the diabetic mice. Based on these findings, we speculate that activation of SIRT6 signaling might be a potential approach for promoting wound healing in diabetics.
R.A. Thandavarayan: None. **D. Joladarashi:** None. **S. Suresh Babu:** None. **P. Jeyabal:** None. **S. Krishnamurthy:** None. **V.N. Garikipati:** None. **S.K. Verma:** None. **R. Kishore:** None. **P. Krishnamurthy:** None.

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The Eya4/six1 Signalling Cascade is Activated in Acquired Heart Disease

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Rationale:We previously identified a mutation in the human transcriptional cofactor Eya4 as cause of familial dilated cardiomyopathy (DCM). We now provide evidence that the Eya4/Six1 signalling cascade also is crucial in acquired heart disease.
Hypothesis:We hypothesize that the transcriptional complex Eya4/Six1 regulates targets relevant in normal cardiac function. We speculate it, amongst others, regulates expression of p27kip1, a known inhibitor of hypertrophy in adult cardiomyocytes, upon hypertrophic stimuli.
Methods and results: We first examined the correlation of Eya4 and p27 in regards to phosphorylation and cellular distribution in failing and normal human hearts. Immunhistology revealed Eya4 is mainly distributed in the cytoplasm while p27 predominantly resides in the nucleus of healthy myocardial tissue. In sections of failing human hearts, Eya4 accumulated in the perinuclear and nuclear region; nuclear p27 levels were significantly diminished, phosphorylated p27 was evenly distributed in the cytoplasm. In a murine model of MI, IH showed Eya4 translocates in a time-dependent manner. WB analyses for p27 showed an age dependent decrease in p27 protein levels upon MI compared to control littermates. We generated transgenic mice with constitutive myocardial overexpression of Eya4 and E193. As judged by MRI, hemodynamic and morphometric analysis both transgenic mouse models developed cardiac phenotypes compared to age-matched wildtype littermates already under basal conditions in an age dependent manner. p27 expression and downstream factors were also altered in both transgenic lines as a result of Eya4, and accordingly, E193 overexpression. In summary, we provide evidence that the Eya4/Six1 signalling cascade is not only relevant in a rare version of heritable DCM but also in more common forms of acquired heart disease. Eya4/Six1 seems to regulate p27, which was shown to be an important regulator of cardiac physiology in postmitotic cardiomyocytes.
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Nos1ap Alters Qtc Intervals Upon Overexpression in Mice

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Rationale: The QT interval duration (QTc) reflects cardiac depolarization. It may predispose individuals to ventricular tachycardia and sudden cardiac death if prolonged (long QTc), shortened (short QTc) or otherwise unregularly. Whole-genome association studies have linked genetic variations in the neuronal nitric oxide synthase adapter protein NOS1AP to variations in QTc intervals and sudden cardiac death.
Hypothesis: We hypothesize NOS1AP functions as an L-type-Ca2+ channel modulator via its interaction with the neuronal nitric oxide synthase NOS1. Therefore, alterations in myocardial NOS1AP expression should temper with QTc intervals and increase susceptibility to rhythm disorders.
Methods and results: We generated conditional double transgenic mice by crossbreeding pTRE-6xHN-Nos1AP animals with α-MHC-tTA mice; NOS1AP expression is therefore restricted to cardiomyocytes and under control of doxycycline (Tet-Off system). Double transgenic animals were investigated with the main focus upon electrical alterations. Heart rates were similar in NOS1AP overexpressing and non-induced animals. Atrial programmed stimulation repeatedly caused atrial tachycardia, while ventricular programmed stimulation caused VT in NOS1AP overexpressing mice. There was a clear decrease of QTc intervals in NOS1AP overexpressing mice paralleld by a significantly reduced survival (only 56% after 12 weeks vs 100% in non-induced mice. Induced QTc alterations and accompanied deaths subsided upon readministration of doxycycline.
We also investigated the functional effect of the human SNP rs16847548 (T/C). We found that this SNP decreased NOS1AP transcriptional activity in vitro and therefore suggest this leads to a decrease in NOS1AP expression in humans.
Conclusion:: Myocardial overexpression of NOS1AP leads to short QTc syndrome with increased susceptibility to atrial and ventricular rhythm disorders and cardiac death. SNP rs16847548 in NOS1AP resulted in less NOS1AP promoter activity in vitro which could explain the alteration in QTc intervals.
In summary, not only mutations in ion channels themselves but also genetic alterations in the expression of ion channel modulators such as NOS1AP, have an impact on QTc intervals.
T. Williams: None. **D. Oppelt:** None. **A. Arias-Loza:** None. **M. Abesser:** None. **J. Schmitt:** None. **K. Schuh:** None. **O. Ritter:** None.

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Hyperhomocysteinemia Potentiated the Impairment of Hydrogen Sulfide-induced Endothelium-derived Hyperpolarization-mediated Vascular Relaxation in Diabetic Db/db Mice

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Background: Cumulative evidence indicates that plasma homocysteine (Hcy) level is positively correlated with cardiovascular mortality in Type 2 diabetic patients.
Aims: To explore the effects and mechanisms of elevated plasma Hcy level— hyperhomocysteinemia (HHcy) on endothelial function in db/db mice.
Methods and Results: HHcy was induced in diabetic db/db and non-diabetic db/+ mice fed with a high methionine diet (HM, 2% methionine) for 8 weeks (plasma tHcy=54.31±5.4 and 34.21±4.15 μM). Endothelial function was examined in small mesenteric arteries (SMA) using myographs. In non-diabetic mice, HHcy did not change vascular relaxation to acetylcholine (Ach); whereas, nitric oxide (NO)- and prostacyclin (PGI2)-mediated relaxation to Ach were impaired. Interestingly, endothelium-derived hyperpolarization factor (EDHF)-mediated relaxation to Ach was improved. In diabetic mice, HHcy potentiated the impairment of NO-, PGI2- and EDHF-mediated relaxation to Ach. Moreover, sodium hydrogen sulfide (NaHS), a donor of hydrogen sulfide (H2S), induced EDHF-mediated relaxation which was impaired in diabetic mice and potentiated by HHcy. NS309, a non-specific calcium-activated potassium channel (Kca) activator, significantly improved H2S- and Ach-induced EDHF-mediated relaxation in diabetic mice with HHcy. Tram-34, an intermediate conductance Kca (IK) blocker, but not small conductance Kca blocker apamin, diminished HHcy-induced impairment of EDHF-mediated relaxation in diabetic mice, suggesting that IK inactivation plays a major role. Free sulfide was decreased in plasma and SMA of diabetic mice which was potentiated with HHcy. Superoxide generation was increased and potentiated by HHcy in lung ECs from diabetic mice. Moreover, PEG-SOD improved vascular relaxation in diabetic mice with HHcy. Finally, tyrosine nitration of IK was increased in human cardiac microvascular endothelial cells (ECs) treated with either D-glucose (25 mM) or DL-Hcy (500 μM) for 48h, which was potentiated by a combination of D-glucose and DL-Hcy.
Conclusions: H2S is a major EDHF in resistant arteries in mouse. H2S-contributed EDHF-mediated vascular relaxation was impaired in diabetes and was potentiated by HHcy via oxidative stress and IK inactivation.
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Phosphoinositide 3-Kinase γ Regulates Cardio-protective ERK by Kinase Independent Mechanism

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Phosphoinositide 3-kinase (PI3K) enzymes are critical in many cellular processes including survival. PI3Kγ, a member of the PI3K family activated by G-protein coupled receptor (GPCR), is known to be a critical player in activation of extracellular regulated kinase (ERK) signal transduction cascade, a cell survival pathway. However, the exact mechanism by which PI3Kγ plays a role in ERK activation is not clearly understood. Our studies show that PI3Kγ plays a crucial role in enhancing the tone of ERK activation as use of PI3K inhibitors reduced GPCR stimulated ERK phosphorylation in HEK293 cells. siRNA knockdown of PI3Kγ resulted in loss of ERK phosphorylation through GPCRs (β-adrenergic) as well as receptor tyrosine kinases. The role of PI3Kγ in ERK activation was further corroborated by loss of insulin stimulated ERK phosphorylation in PI3Kγ-knockout (KO) mouse embryonic fibroblasts (MEFs). Surprisingly, ERK activation in KO MEFs post-insulin stimulation was completely rescued by expression of kinase-dead PI3Kγ mutant in KO MEFs suggesting a kinase-independent role of PI3Kγ in regulating ERK function. Indepth mechanistic studies showed that PI3Kγ mediated

activation of ERK by inhibiting ERK dephosphorylation following stimulation, thus stabilizing the ERK phosphorylation. PI3Kγ physically disrupts the interaction between ERK and ERK dephosphorylating phosphatase PP2A as evidenced by increase in phosphatase association with ERK in KO MEFs. Consistent with this observation, ERK activation was completely abolished in KO MEFs following carvedilol suggesting an essential role for PI3Kγ in cardio-protective ERK activation pathway. In this context, it is known that transverse aortic constriction (TAC) in mice leads to increase in ERK activation in the hearts and is also associated with concurrent up-regulation of PI3Kγ suggesting a key role for kinase-independent function of PI3Kγ in activating and maintaining the ERK signaling cascade. These indepth cellular studies and observation from our TAC studies led us to believe that kinase-dependent function of PI3Kγ may contribute to pathology while kinase-independent function may be cardio-protective through inhibition of PP2A by PI3Kγ. This novel signaling mechanism by PI3Kγ will be presented.

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Carvedilol Stimulated Gαi-β-Arrestin Biased β1 Adrenergic Receptor Signaling

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The β1 and β2 adrenergic receptors (βARs) are the predominant G-protein-coupled receptors (GPCRs) subtypes expressed in the heart. It is now appreciated that ligands can induce multiple distinct “active” receptor conformations with unique downstream functional signaling profiles or efficacies. Our current understanding of GPCR signaling is that ligands can be biased toward activating either a G protein or a β-arrestin-signaling pathway, a concept known as biased ligand signaling. To identify novel biased signaling pathways mediated by β1- and β2ARs, we performed a proteomic interactome study of β1AR and β2AR using stable isotope labeling by amino acids in cell culture (SILAC). We identified several hundred proteins that distinctly bind to the β1AR or β2AR following stimulation with the unbiased full agonist, isoproterenol, or the β-arrestin-biased ligand carvedilol. We found that stimulation by the β-arrestin-biased agonist carvedilol of only β1ARs resulted in the recruitment of Gαi. No of the other ligand tested promoted Gαi recruitment, suggesting that carvedilol may be unique in its ability to activate Gαi-biased signaling. The Gαi inhibitor pertussis toxin blocked β-arrestin-dependent extracellular signal-regulated kinase (ERK) activation and epidermal growth factor receptor (EGFR) transactivation stimulated by carvedilol, suggesting the involvement of Gαi in carvedilol-induced β-arrestin biased signaling of β1ARs. Using β1AR/β2AR chimeric receptors we show that the C-terminal tail of the β1AR is required for carvedilol stimulated Gαi recruitment, but is unable to rescue the lack of Gαi recruitment by the β2AR. Since our current conceptual framework for biased signaling is based on the “bar code hypothesis”, ongoing phosphoproteomic experiments are testing whether recruitment of Gαi by carvedilol induces a distinct phosphorylation pattern on the c-tail of the β1AR. Our study shows that activation of β-arrestin-biased signaling requires G proteins for signaling and provides a new mechanistic understanding of how biased β-blockers can activate signaling.

J. Wang: None. **K. Xiao:** None. **H.A. Rockman:** None.

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275

The Effect of High Salt-diet on Pioglitazone Treated-db/db-/- mice

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The objective of this study was to evaluate the impact of high-salt-diet on pioglitazone treated db/db mice. Groups of 6 weeks-old db/db mice obtained from Jackson Laboratory were given either pioglitazone (0.02%) in diet or pioglitazone (0.02%) plus high salt-diet (8% NaCl) for 6 to 12 weeks. Control groups received either pioglitazone or normal salt diet. During the course of treatment, urine volume, urine sodium, creatinine and blood glucose were measured in the animals. For urine collection, mice were placed in metabolic cages. In order to evaluate whether differences exist between diabetic animals that received pioglitazone or not in the handling of a sodium load and to characterize the transport mechanisms involved, at the end of experimental period the animals were given an acute sodium load (physiological saline), 1.25 ml/100 g body weight by intraperitoneal route. The sodium load was repeated in mice that was treated 15 minutes earlier with hydrochlorothiazide (40 mg/kg i.p.) or furosemide (8 mg/kg i.p.), or amiloride (1.65 mg/kg). The animals were placed in metabolic cages and urine voided was collected over 5 hr. for determination of urine volume and sodium. Sodium was determined by flame photometer. No significant changes were observed in mean arterial blood pressure in all the groups (at p 0.05). There was significant increase in UNav with all the diuretics in db/db mice that received high salt diet at 6 weeks. The blood pressure did not increase with the sodium diet. This might be due to natriuresis resulting from polyurea in the diabetic condition of the mice.

P.O. Obih: None. **M.C. Ezebuenyi:** None. **A.P. Jimoh:** None. **J.A. Obih:** None.

276

Systemic Delivery of Microrna-146a Improves Endothelial Function and Ameliorates Atherosclerosis in Apolipoprotein E-deficient Mice

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Rationale: Endothelial inflammation is an early event in the development of atherosclerosis. The microRNA (miR)-146a showed anti-inflammatory effects in cultured endothelial cells. In this study, we investigated the therapeutic role of miR-146a in endothelial function and atherosclerosis in apolipoprotein E (ApoE)-deficient mice.

Methods and Results: The miR-146a was packaged into a multistage vector (MSV) that was conjugated with an E-selectin-targeting thioaptamer (ESTA) to form an ESTA-MSV microparticle. The ApoE-deficient mice were fed with Western diet and injected through tail vein with 15μg of miR-146a loaded ESTA-MSV microparticles or vehicle vectors biweekly for 12 weeks. The expressions of miR-146a in aortic tissue was increased by five times at two weeks after injection. However, the expressions of miR-146a in heart, lung, liver, spleen, kidney, and skeletal muscle were not increased. The acetylcholine-induced endothelium-dependent relaxations in both carotid arteries and aortas were significantly improved in mice from miR-146a treated group compared with vehicle group. In addition, the endothelium-dependent contractions of carotid arteries were also improved by miR-146a treatment. The en face oil red O staining of

whole aortas showed the plaque area was decreased in miR-146a-treated mice. Application of miR-146a also decreased the plaque size, macrophages, and T-lymphocytes, but increased the collagen deposition and vascular smooth muscle cells in the sections of aortic roots. The PCR results showed that expressions of chemokine (C-C motif) ligand (CCL)-2, CCL-5, and CCL-8 were decreased by miR-146a.
Conclusions: E-selectin-targeting delivery of miR-146a improves endothelial function and inhibits atherosclerosis.
S. Ma: None. **X. Tian:** None. **C. Mu:** None. **H. Shen:** None. **Y. Zhang:** None. **J. Bismuth:** None. **W. Wong:** None.

277
High Consistency in Left Ventricular 31P-MR Spectra in Rats in vivo Allows Follow-up Detection of Small Variations in Energy Status and Early Energy Defects
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Introduction: Phosphorus magnetic resonance spectroscopy (31P-MRS) makes a unique and valuable contribution to understand how mitochondrial energy supply responds to ATP demand in beating hearts. Changes in PCr/ATP allow detecting energy dysbalance that often predict disease and mortality. However, MR techniques in vivo are challenged by tissue contamination and poor signal-to-noise (S/N) ratio, especially in hearts of rodent models.
Methods: Here we optimized acquisition of localized cardiac 31P-MR spectra, obtained with a surface probe at 9.4T in vivo from left ventricle in control and SHR rats. MR spectra (192s) and short-axis cine-MRI frames were obtained during 9-weeks in sinus rhythm and during tachycardia induced by acute injection of isoproterenol (10μg/kg) at week 10.
Results: Highly reproducible spectra with low S/N ratio helped demonstrating healthy PCr/ATP despite progressive ventricular hypertrophy in SHR rats. Isoproterenol induced similar tachycardia (425bpm ± 7) but higher drop in PCr/ATP (-17.1% vs. -4.7%, $P<0.05$) associated with higher recovery time constant (23.9 vs. 7.3 min, $P<0.05$).
Discussion/Conclusion: Our results indicate that SHR rats maintain energy homeostasis during early development of pathology but present a blunted capacity of mitochondrial energy supply to respond to high ATP demand during strong ß-adrenergic activation of the myocardium. The optimization in localized 31P-NMR analysis offer exciting possibilities to better investigate primary precursors detection of cardiac failure in rodent models.
V. Deschodt-Arsac: None. **J. Magat:** None. **J. Naulin:** None. **B. Quesson:** None. **P. Dos Santos:** None. **L. Arsac:** None.

278
Cardiac Med1 is Necessary for Postnatal Survival in Mice
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Alterations in gene transcription are commonly associated with cardiovascular disease pathogenesis characterized by cardiomyocyte hypertrophy. The phenotypic responses result in diminished cardiac contractility, ventricular dilation, fibrosis and ultimately sudden death. The mediator complex is a crucial facilitator of gene transcription; however, few studies have investigated the role of mediator in cardiovascular disease initiation and progression. A key subunit of the Mediator complex, MED1, interacts with nuclear receptors to target gene-specific transcription. To determine the role of MED1 in regulating cardiac function, we generated a heart specific knockout of MED1 (cMED1KO). Postnatal deletion of MED1 in mice results in lethality between 3 to 6 weeks of age. The cMED1KO mice display a marked increase in heart mass compared to floxed controls. Furthermore, echocardiography and histological analysis of hearts taken at 3 weeks showed that the cMED1KO animals had decreased cardiac function, increased fibrosis and a dilated left ventricle. Transcriptional changes were observed for key markers of cardiac disease including MYH7, ANF, ACTIN1. We performed RNAseq analysis to identify changes in the transciptome between cMED1KO and floxed control hearts. The analysis unveiled changes in expression of genes regulating cardiac development, metabolism and function. Taken together these results reveal a critical role for MED1 in postnatal cardiac growth and development due to altered gene expression in adult cardiomyocytes.
K.M. Spitler: None. **J.M. Ponce:** None. **D.D. Hall:** None. **C.E. Grueter:** None.

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Etv2-mir-130a Network Regulates Mesodermal Precursors Through Inhibition of Pdgfra Signaling
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MicroRNAs (small non-coding RNAs) are known to regulate critical developmental stages during embryogenesis. Here, we defined a novel Etv2-miR-130a cascade that regulates mesodermal specification and determination. Ablation of *Dicer* in the *Etv2*-expressing precursors resulted in altered mesodermal lineages and embryonic lethality by E12.5. We identified miR-130a as a direct target of Etv2 and demonstrated its role in the segregation of bipotent hemato-endothelial progenitors towards the endothelial lineage. Loss- and gain-of-function experiments demonstrated that miR-130a is an important regulator of the endothelial program at the expense of cardiac program without impacting the hematopoietic lineages. Mechanistically, miR-130a directly suppresses expression level of *Pdgfra* and promotes the endothelial program by blocking Pdgfra signaling. Inhibition or activation of Pdgfra signaling phenocopied the miR-130a over-expression and knockdown, respectively. This is the first report of a miRNA that specifically promotes the divergence of the common hemato-endothelial progenitor to the endothelial lineage.
B.N. Singh: None.

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Cardiac Disease Status Dictates Functional mRNA Targeting Profiles of Individual microRNAs
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Background: Gain- and loss-of-function experiments have demonstrated that microRNAs are key players in cardiac stress responses, but the mRNAs whose abundance and/or translational potential are primarily affected by changes in cardiac microRNAs are not well characterized. Stimulus-induced, large-scale alterations in the cardiac transcriptome, together with consideration of the law of mass action, further suggest that the mRNAs most substantively targeted by individual microRNAs vary between unstressed and stressed conditions. To test the hypothesis that microRNA target profiles differ in health and disease, we traced the fate of miR-133a and miR-378 targets in mouse hearts undergoing pressure-overload hypertrophy.
Methods and Results: Ago2 immunoprecipitation with RNA-sequencing (RISC-sequencing) was used for unbiased definition of microRNA-dependent and -independent alterations occurring amongst ~13,000 mRNAs in response to transverse aortic constriction

(TAC), in response to the microRNA overexpression needed to define miR-133a and miR-378 targets and to track their fate, and in response to the combined stimuli. Of 51 direct targets of miR-133a defined in unstressed hearts (fold-change ≥25%, FDR<0.02), only 8 (16%) continued to be targeted by miR-133a during TAC, while for miR-378 direct targets, 17 of 28 (61%) targets were maintained during TAC. Similarly, only 9% (for miR-133a) and 58% (for miR-378) of hundreds of indirectly affected mRNAs underwent comparable regulation, demonstrating that the significant effect of TAC on microRNA direct target selection resulted in wider alterations of signaling function. Numerous microRNA-mediated regulatory events occurring exclusively during pressure overload revealed signaling networks likely responsive to the endogenous decreases in miR-133a and miR-378 during TAC. Conclusions: Pressure overload-mediated changes in overall cardiac RNA content alter microRNA targeting profiles, reinforcing the need to define microRNA targets in tissue-, cell- and status-specific contexts. Such considerations need to be taken into account for tailoring of therapeutic manipulation of microRNAs toward the most appropriate microRNAs active under defined stimuli.

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Loss of Krueppel-like Factor 15 (KLF15) Leads to Altered Wnt-dependent Gene Regulation in Hearts with Systolic Dysfunction

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Background & Aim: Developmentally conserved pathways, such as the Wnt/β-catenin pathway, are upregulated in adult heart diseases. Here, we aimed to elucidate the role of Wnt regulation via KLF15 for adult heart homeostasis.
Results: We identified the transcription factor KLF15, a factor previously identified to contribute to cardiac fibrosis and hypertrophy, as a cardiac specific Wnt/β-catenin inhibitor. Accordingly, a constitutive Klf15 KO mouse model revealed specific cardiac upregulation of the Wnt target genes Tcf7l2 and cMyc (n=6, p<0.001). Serial echocardiography showed reduced systolic function starting at 16 weeks in the Klf15 KO hearts (n=10, p<0.05). Genome-wide sequencing studies of Klf15 KO vs. WT hearts at the postnatal age day P10, week 4 and 20 showed that gene expression differs increasingly at 4 and 20 weeks of age, respectively. This is in line with the increasing KLF15 expression after birth, reaching significant level at P13 (n=3/embryonal, fetal, and neonatal stage; each 5-8 pooled hearts). Accordingly, gene set enrichment analysis revealed activation of the Wnt signaling in Klf15 KO hearts at 4 and 20 weeks but not at P10 (p<0.001). This activation was milder in 20 weeks vs. 4 weeks, which may be explained by a concomitant upregulation of Wnt repressors, as Shisa3 and Dact3 with so far unknown function in the mammalian heart (n=9, p<0,01). Wnt activation was accompanied by upregulation of developmental genes and transcriptional regulators at 4 weeks and followed by upregulation of stress factors such as ANP, BNP, and Ankrd1 at 20 weeks. Moreover, we found the Wnt targets TCF7L2 and AXIN2 significantly upregulated in human ventricular biopsies from dilated (n≥5, p<0.05) and ischemic cardiomyopathy (n≥5, p<0.001) vs. non-failing, further underscoring the relevance of the Wnt pathway for human cardiac cellular homeostasis as well. Conclusion: A Wnt-dependent gene signature may precede the expression of stress factors and functional deterioration due to deletion of its cardiac repressor KLF15. Our data indicate that KLF15 is an important regulator of the Wnt pathway and is relevant for cardiac homeostasis and function in the postnatal heart.

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Dynamics of 5-hydroxymethylcytosine During Cardiac Maturation and Disease

Paolo Kunderfranco, Carolina M Greco, Roberto Papait, Gianluigi Condorelli, Humanitas Clinical and Res Ctr, Rozzano, Italy

The epigenome -the sum of chemical modifications on both DNA and histone proteins associated with the genome- is a key regulatory mechanism through which gene expression changes take place in heart development and disease. DNA methylation (5-mC), a key repressive mark, has emerged in the last years as a determinant of myocardial gene expression regulation. Historically, DNA methylation was considered a stable epigenetic mark. However, high-resolution genome wide mapping in pluripotent and differentiated cells has uncovered the dynamic nature of DNA methylation. In fact 5-methylcytosine can undergo oxidation to 5-hydroxymethylcytosine (5-hmC), catalysed by ten-eleven translocation (TET) enzymes. While in embryonic stem cells and cancer the role of 5-hydroxymethylation in modulating gene expression programs has been extensively investigated, whether this epigenetic modification is of relevance in myocardial physiology and disease is still unknown. We performed hydroxymethylated DNA immunoprecipitation coupled with high-throughput sequencing (hMeDIP-seq) in cardiomyocytes to elucidate the function of this modification in this cell type in normal state and under stress, in particular cardiac hypertrophy induced by pressure overload. Our data indicate that the presence of 5-hmC within the gene-body strongly correlates with gene expression and cooperates with activating histone marks marking cardiac specific genes. Moreover, during hypertrophy a profound genomic re distribution of 5-hmC on distal regulatory regions such as enhancers and repetitive elements takes place. We then dissected the functional role of enhancer-associated hydroxymethylation in hypertrophy, defining a subset strongly enriched for 5-hmC. Enhancers harboring 5-hmC strongly influenced the expression of the associated genes and were found in the proximity of many transcription factors known to play a pivotal role in cardiac cells (i.e. Mef2c, Hif1a, Nkx2.5, Hand2).
These results provide the first genome-wide analysis of 5-hmC in cardiac cells and strongly support a regulatory function of this epigenetic modification in the specification of the transcriptional profile of cardiomyocytes.

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Chaer Lncrna Negatively Regulates Polycomb Repressive Complex 2 During Cardiac Hypertrophy

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Sciences, David Geffen Sch of Med, Univ of California at Los Angeles, Los Angeles, CA; Guangping Li, Dept of Cardiology, Tianjin Inst of Cardiology, Second Hosp of Tianjin Medical Univ, Los Angeles, CA; Yibin Wang, Div of Molecular Med, Depts of Anesthesiology, Physiology and Med, David Geffen Sch of Med, Univ of California at Los Angeles, Los Angeles, CA

Long non-coding RNAs (lncRNAs) emerge to be critical regulators of cellular processes, but only a few out of thousands have been functionally characterized. We identified a novel heart-specific lncRNA, named cardiac hypertrophy associated epigenetics regulator (Chaer), which was both necessary and sufficient for hypertrophy of neonatal rat ventricular cardiomyocytes. RNA deep-sequencing revealed that Chaer contributed to the global transcriptome reprogramming during phenylephrine (50 μM)-induced hypertrophy, and regulated imprinted gene H19 expression independent of DNA methylation but dependent on histone tri-methylation at H3K27 (H3K27me3). RNA immunoprecipitation assay found that Chaer directly interacting with and negatively regulating PRC2 function on H3K27me3. Tagged RNA pull-dwon and RNA EMSA assays confirmed that Chaer directly bound to the catalytic subunit Ezh2 with a conserved 66-mer motif near its 5' end in competition with and functionally interrupting other PRC2-binding lncRNAs. Interestingly, Chaer-PRC2 interaction was transiently enhanced at the onset of hypertrophy and responsible for hypertrophy fetal gene induction which was sensitive to Ezh2 inhibitor GSK126 (1 μM). Moreover, mTOR inhibitor rapamycin (20 nM) completely blocked the enhanced Chaer-PRC2 interaction, reversed the decrease of global H3K27me3, and abolished phenylephrine-induced expression of hypertrophy fetal genes. Finally, Chaer silence in vivo using chemically modified siRNA and nanoparticle transfection reagents significantly reversed the development of cardiac hypertrophy, pathological remodeling and H3K27m3-modification-mediated fetal gene induction under transaortic-constriction-induced pressure overload. The findings unveil Chaer as an epigenetic determinant of cardiac hypertrophy, and shed a light into the early molecular events under cardiac stress.
Z. Wang: None. **X. Wang:** None. **I. Chen:** None. **C. Gao:** None. **T. Yokota:** None. **H. Wang:** None. **S. Ren:** None. **A. Cass:** None. **X.G. Xiao:** None. **G. Li:** None. **Y. Wang:** None.

300
A Novel Targeted Angiogenesis Technique Using Magnetic Nanoparticles
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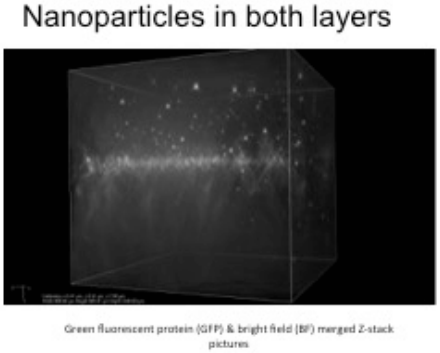
Aims: To develop a novel technique for angiogenesis which could be potentially useful for therapeutic purposes using magnetic nanoparticles.
Methods: Magnetic nanoparticles were synthesized and were conjugated with vascular endothelial growth factor. The particles were tested in tissue culture microfluidic chips for angiogenesis. Four layers were generated for the experiment respectively - the nutrient layer, hydrogel layer, HUVEC spheroids and another hydrogel layer in the bottom. The particles were inserted in a layer of collagen hydrogel and were guided to desired location using a magnet. The extent and the direction of angiogenesis were studied using 3D confocal microscopy.
Results: Angiogenesis was observed compared to the controls when nanoparticles were interfaced between the nutrient and HUVEC layer. When the nanoparticles was placed below the HUVEC spheroids angiogenesis occurred in the basal layer predominantly, which showed the effect of the nanoparticles with growth factor on angiogenesis more than the nutrients. In another experiment in microfluidic chip, the nanoparticles were placed in nutrient layer and HUVEC cell monolayer was placed underneath the nutrient layer. The nanoparticles was observed to cross endothelial cells and reach lower hydrogel layer, which was observed by Z stack confocal microscopy.
Conclusion: Angiogenesis happen by the effect of magnetic nanoparticles conjugated with growth factors. These nanoparticles can be controlled with a magnet. Also, these nanoparticles have potentials to cross endothelium.



M. Arokiaraj: None.

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A Novel Tram Stent Method in Treatment of Coronary Bifurcation Lesions
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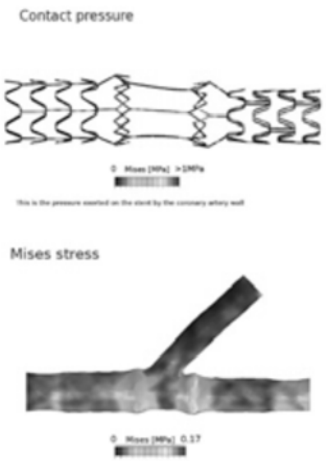
AIMS A novel coronary stent was designed for the treatment of coronary bifurcation lesion, and it was investigated for its performance by finite element analysis.
METHODS AND RESULTS A coronary bifurcation model was created with the proximal vessel of 3.2 mm diameter, and the distal vessel after the side branch (2.3 mm) was 2.7 mm. A novel stent was designed with connections that had a profile of a tram. Laser cutting and shape setting of the stent was performed, and thereafter the stent was deployed over a balloon. The contact pressure, stresses on the artery wall, stresses on the stent, the maximal principal log strain of the main artery and the side-branch were studied. The finite element study was performed in Abaqus, Simulia.The stresses on the main branch and the distal branch were minimally increased after deployment of this novel stent. The side branch was preserved, and the stresses on the side branch were lesser. At the confluence of the bifurcation on either side of the side branch origin the Von Mises stresses were marginally increased. However, the stresses at the bifurcation were significantly lesser than the stresses of the currently existing techniques used in the treatment of bifurcation lesions. Further, the tram area was studied parametrically to reduce the stresses. A firm lesion model with a stenosis of 80% was then created with a discrete lesion in side-branch and the main vessel. The stent was deployed in the main branch and the side branch was stented at the ostium. The stresses at the bifurcation and the main vessel was further reduced and the stent deployed well.
CONCLUSIONS There is a potential for a novel Tram-stent method in the treatment of coronary bifurcation lesions.



M.C. Arokiaraj: None.

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Four-and-a-half Lim Domain Protein-1 Upregulation via an Alternate Start Site Prepares the Left Atrium for Birth

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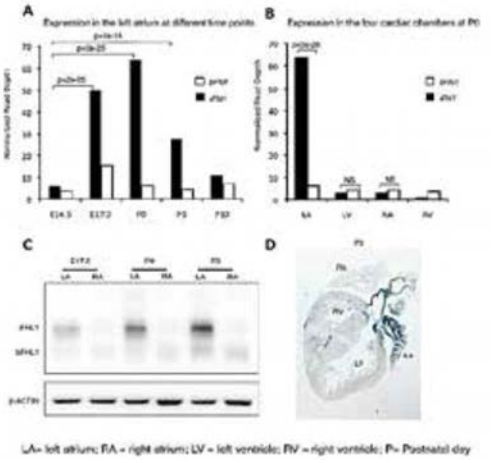
Four-and-a-half-LIM-domain protein-1 (FHL1) participates in the heart's response to biomechanical stress during pathological states. Normally, the basal isoform, *bFhl1*, predominates in non-stressed states. In pathology, a change in start site leads to expression of the induced isoform, *iFhl1*, which is 16 amino acid residues longer than bFHL1. We have studied the expression and role of *Fhl1* during normal cardiac development. The shift from fetal to neonatal circulation signifies new stress for the heart, and provides a unique opportunity to study the role FHL1 in the setting of physiological stress. We hypothesize that selective *iFhl1* upregulation during this transitional period prepares the heart for the stress involved. Using 5' RNA-seq, we demonstrate that *iFhl1* was selectively upregulated by 10-fold in the left atrium by embryonic gestation day 17.5 (E17.5; $p=2 \times 10^{-5}$) but remained unchanged in the other cardiac chambers (see B). The preferential increase in expression in the left atrium was confirmed with X-gal staining of $Fhl1^{Lacz}$ mice hearts (see D, blue stain), and the selective expression of the induced isoform at the protein level was confirmed by Western blot (see C). Assessment of left atrial volume by magnetic resonance imaging in mice at 2 weeks of age showed that Fhl1-null mice have significantly larger left atria than wild-type littermates (0.63±0.10 µl vs. 0.45±0.04 µl, p=0.004). The demonstrated selective upregulation of *iFhl1* prior to birth and the increase in left atrial volume that follows genetic ablation of *Fhl1* suggests that *Fhl1* is central in the heart's ability to respond to physiological stress as represented by normal birth.



I.M. Cubero Salazar: None. **A. Axelsson:** None. **H. Wakimoto:** None. **D. DeLaughter:** None. **D. Christodoulou:** None. **J. Jiang:** None. **M. Burke:** None. **C. Benson:** None. **J. Gorham:** None. **S. Depalma:** None. **J. Chen:** None. **S. Mukundan:** None. **M. Jerosch-Herold:** None. **C.E. Seidman:** None. **J.G. Seidman:** None.

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Mirna-17-3p is Required for Exercise-induced Cardiac Growth and Protects Against Myocardial Ischemia-reperfusion Injury

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Aims: Little is known about the role of microRNAs (miRNAs, miRs) in exercise induced cardiac growth. This study aims at investigating the role of members of the miR-17-92 cluster and their star miRNAs in exercise-induced cardiac growth.
Methods and Results: miRNAs including miR-17-3p, -17-5p, -18a-3p, -18a-5p, -19a-3p, -19a-5p, -19b-1-5p, -19b-3p, -20a-3p, -20a-5p, -92a-3p and -92a-5p were determined by quantitative reverse transcription polymerase chain reactions (RT-PCRs) in heart samples from mice undergoing 3-week swimming training. miR-17-3p and miR-18a-3p were found to be increased in swimming mice models while only miR-17-3p was further found to be increased in voluntary cage wheel exercise mice models. Interestingly, miR-17-3p was decreased in ventricular tissues of DCM patients and TAC mice. In isolated cardiomyocytes, forced-expression of miR-17-3p induced cardiomyocytes hypertrophy and proliferation as determined by α-actinin and EdU or Ki-67 staining. miR-17-3p was found to indirectly increase the AKT/PI3K pathway, contributing to cardiomyocytes hypertrophy. TIMP-3 was identified as a target gene responsible for cardiaomyocyte proliferation by western blotting, luciferase assays and rescue experiments. In vivo, using heart weight-to-body weight (HW/BW (mg/gm)) ratio, heart weight-to-tibia length (HW/TL (mg/mm)) ratio and EdU/α-actinin staining, mice with miR-17-3p inhibition (n=9) showed milder cardiac growth and less cardiomyocyte proliferation compared with control group (n=8) after the 3-week swimming exercise model. Moreover, subjected to ischemia-reperfusion injury, mice with forced-expression of miR-17-3p (n=10) showed significantly less impairment of cardiac function measured by echocardiography than negative control group (n=11). Also, cardiac apoptosis was also significantly decreased in the miR-17-3p agomiR treated mice as assessed by TUNEL staining.

Conclusions: miR-17-3p is required for exercise-induced cardiac growth and protects against myocardial ischemia-reperfusion injury. miR-17-3p represents a novel therapeutic target for cardiac repair and regeneration.
J. Shi: None. **H. Wang:** None. **Y. Bei:** None. **Q. Xuan:** None. **W. Sun:** None. **H. Liu:** None. **X. Li:** None. **J. Xiao:** None. **X. Kong:** None.

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Efficient Generation of Cardiac Purkinje Fiber-like Cells From ESCs by Activating cAMP Signaling
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Dysfunction of the cardiac conduction system (CCS) significantly impacts pathogenesis of arrhythmia, a major cause of morbidity and mortality. Strategies to derive cardiac conduction cells including Purkinje fiber cells (PC) would facilitate models for mechanistic studies and drug discovery, and also provide new cellular materials for regenerative therapies. A high-throughput chemical screen using CCS:lacZ and Contactin2:eGFP (Cntn2:eGFP) reporter embryonic stem cell (ESC) lines was used to discover a small molecule, sodium nitroprusside (SN), that efficiently promotes the generation of cardiac cells that express gene profiles and generate action potentials of PC-like cells. Imaging and mechanistic studies suggest that SN promotes the generation of PC from cardiac progenitors initially expressing cardiac myosin heavy chain, and that it does so by activating cAMP signaling. These findings provide a novel strategy to derive scalable PC, along with insight into the ontogeny of CCS development.
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Single Cell RNA Sequencing of Adult Cardiac c-kit+ Cells in a Murine Lineage Tracing Model
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A subset of adult cardiac resident cells defined by the stem cell factor tyrosine kinase receptor termed c-kit, are believed to have myogenic potential and are now being delivered via intracoronary infusion to presumably promote cardiac regeneration and improve ventricular function after ischemic cardiac injury. However, recent studies have shown that despite these benefits, c-kit+ progenitor cells in the adult murine heart are more inclined to take on an endothelial rather than cardiomyocyte lineage. To better define the factors involved in early differentiation of these resident cardiac progenitor cells and to distinguish distinct cell subpopulations, we performed single cell RNA sequencing on c-kit+ cells from Kit-Cre lineage traced GFP reporter mice versus total mesenchymal cells from the heart that were CD31- and CD45-. Cells were isolated by cardiac digestion and FACS was performed, positively sorting for the c-kit+ lineage while negatively sorting for CD31 and CD45 to eliminate endothelial and leukocyte progenitor contamination, respectively. Following this isolation, cells were examined to determine GFP reporter status and then submitted for single cell RNA sequencing using the Fluidigm A1 system. Clustering of 654 genes from this data identified 6 distinct subpopulations indicating various stages of early differentiation among CD31- and CD45-negative cardiac interstitial cells. Furthermore, comparison of GFP+ c-kit cells to the total non-GFP mesenchymal cells identified 75 differentially expressed transcripts. These unique gene signatures may help parse the genes that underlie cellular plasticity in the heart and define the best molecular lineages for transdifferentiation into cardiac myocytes.
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Sympathetic Reinnervation is Required for Mammalian Cardiac Regeneration.
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Rationale: Established animal models of limb and tissue regeneration with re-vascularization demonstrate a critical dependence on concurrent reinnervation by the peripheral nervous system. Objective: Considering the significant abundance of autonomic nerves in the mammalian heart we tested the hypothesis that reinnervation is required for neonatal mouse cardiac regeneration. Methods and Results: Crossing Wnt1:cre transgenic mice with a double-tandem (td) tomato reporter strain identifies all neural crest-derived cell lineages including the peripheral autonomic nerves in the heart. Whole mount epi-fluorescence microscopy facilitated the clear resolution of subepicardial autonomic nerves in the mouse ventricles providing unprecedented detail of the subepicardial neuroanatomy of the mouse heart. Sympathetic nerve bundles envelop the entire heart and extend to the tip of the ventricular apex. Our data demonstrate that during regeneration of the resected ventricular apex of the neonatal mouse heart, sympathetic nerves fibers undergo concurrent re-growth into the injury site resulting in complete sympathetic reinnervation of the regenerated tissue. Sympathectomy of the heart, induced by administration of 6-OHDA, was sufficient to block innate cardiac regeneration in the neonatal mouse. Conclusions: We report that the innate ability of the neonatal mouse heart to undergo regeneration in response to injury is dependent on sympathetic innervation of the ventricular myocardium. Ablation of post-ganglionic sympathetic nerves blocks the innate regenerative capacity of neonatal mouse hearts suggesting that sympathetic reinnervation is critical for ventricular regeneration. This finding has significant implications for adult regeneration following myocardial infarction where nerve growth is hindered by age related influences and scar tissue.
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Quantitative Characterization of the Contractile Architecture of Human Stem Cell Derived Cardiomyocytes
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Human induced pluripotent stem cell derived cardiomyocytes (hiCMs) exhibit a fetal phenotype, but tools to quantify their relative immaturity are scarce. We reasoned that, during myocyte specification, cells progress through myofibrillogenesis as force-generating units, known as sarcomeres, self-assemble along the cell cytoskeleton. Therefore, we developed image processing techniques to quantitatively score myocyte structural phenotypes by the increasing degree of organization and alignment that sarcomeres acquire during myofibrillogenesis. Since this is a highly conserved process, quantifications obtained from α-actinin immunostains in rodent and hiCMs can be compared. Utilizing these metrics we quantitatively showed that hiCMs patterned on square fibronectin islands had significantly under-developed contractile architecture, in agreement with the qualitative

observation that these cells retain a more migratory cytoskeleton. Furthermore, we trained thee independent machine learning algorithms on over 100 α-actin immunostains from engineered primary cardiac tissues at 6, 24 and 48 hours after seeding. These preparations were taken to represent differentiated, immature and mature structural architectures, respectively. After training, α-actinin immunostains of hiPS-derived cardiac tissues were unbiasedly analyzed by these classifiers. The results indicated that ~30% of cells exhibited cytoskeletal architectures similar to those of mature myocytes and that treatment with commercially available small molecules influence hiCM structural maturation. In conclusion, we provided metrics to assess the organization of the contractile cytoskeleton in primary and stem cell-derived cardiomyocytes and to unbiasedly quantify their maturation.

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An in vitro Model of Cardiac Stem Cell Therapy to Study the Coupling of Primary and Stem Cell-derived Cardiomyocytes

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For cardiac cell therapy to be effective, newly formed immature cardiomyocytes need to structurally and functionally integrate with the existing myocardium. Unfortunately, testing the electro-chemo-mechanical coupling of mature and immature cardiomyocytes in vivo is difficult. Here we engineered two cell μtissues containing combinations of mouse neonate, ES-derived, and iPS-derived cardiac myocytes on flexible substrates and utilized ratiometric calcium detection and traction force microscopy to measure excitation-contraction coupling in individual cells and in the pairs. We found that SC-derived cardiac myocytes can structurally couple with neonate cardiomyocytes to functionally support synchronous contraction, yet diastolic calcium levels were reduced in SC-derived cardiomyocytes. Consistently, neonate cardiomyocytes exerted peak systolic forces that were ~1.5-fold higher than that generated by SC-derived myocytes, yielding an imbalance in tension within the pair that was dissipated by focal adhesion-like structures at the cell-cell boundary. Finally we developed a finite element model of two-cell tissue contraction to demonstrate that an imbalance in isometric tension is sufficient to limit force transmission across cell-cell boundaries. Taken together, these results suggest that reduced force transmission between poorly coupled immature and native cardiomyocytes may explain the incomplete repair of ejection fraction observed in several clinical studies of cardiac cell therapy.

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MiR-31a-5p Controls Cardiomyocyte Proliferation in Postnatal Hearts

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Backgroud: MicroRNAs (miRNAs, miRs) are a class of endogenous non-codingRNAs, participating in a variety of essential biological processes including development, differentiation, proliferation and apoptosis. Rodents have the capacity to regenerate their hearts in response to injury while the capacity would be lost 7 day after birth, suggesting that mammals gradually lose their regenerative potential during postnatal development. The roles of miRNAs in regulating cardiomyocyte proliferation in postnatal hearts are largely unclear.

Methods and Results: Cardiomyocytes were isolated from rat at day 0 and day 10. Agilent rat miRNA arrays were performed to determine the dysregulated miRNAs in cardiomyocytes between day 0 and day 10. A total of 32 miRNAs were found to be dysregulated between day 0 and day 10 (Fold change over 2 and P values less than 0.05). As determined by quantitative reverse transcription polymerase chain reactions and functional assays using EdU staining and Ki-67 staining, miR-31a-5p was found to be able to promote neonatal cardiomyocyte proliferation. Moreover, the expression of proliferation maker- Proliferating Cell Nuclear Antigen (PCNA) was also increased in cardiomyocytes transfected with miR-31a-5p mimics as determined by PCRs and Western blotting analysis. Tumor suppressor RhoBTB1 was found to be negatively regulated by miR-31a-5p in cardiomyocytes and also was responsible for the pro-proliferation effects of miR-31a-5p in neonatal cardiomyocytes.

Conclusions: These studies demonstrate that miR-31a-5p regulates cardiomyocytes proliferation in postnatal hearts by targeting RhoBTB1. miR-31a-5p represents a therapeutic target for cardiac repair and regeneration.

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Vascular Endothelial Growth Factor and Endothelial Nitric Oxide Synthase-mediated Regulation of Cardiomyocyte Proliferation During Development in Humans and Mice

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Our previous studies in early mouse embryonic development (E8.2) showing that vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) are expressed in embryonic endothelial cells, but not in embryonic cardiomyocytes, together with the findings by others indicating that NO is required for cardiomyocyte proliferation in mice, led us to investigate the relevancy of the VEGF/eNOS signaling pathway during cardiovascular development. First, wild type and NOS3 knockout mouse embryos between E8.0 and E17.0 were stained with antibodies against smooth muscle α-actin, phospho-histone H3 (PH3), VEGFR2 and PECAM, markers of cardiomyocytes, proliferating, progenitor and endothelial cells, respectively. Our confocal analysis showed hearts of E8.0 NOS3 nulls develop normally. However, E8.5 and E9.5 NOS3 nulls have reduced cardiomyocyte proliferation and impaired heart development. As consequence, hearts of E17 NOS3 nulls were approximately 20% smaller compared to wildtype hearts. To translate our findings to humans, we stained human heart specimens with antibodies against VEGFR2, eNOS, PH3 and sarcomeric α-actinin. Confocal analyses showed for the first time that VEGFR2 is highly expressed in the perinuclear region of human cardiomyocytes of a young donor. They also showed a correlation between eNOS expression and cardiomyocyte proliferation in humans. Consequently, we developed an in vitro three-dimensional co-culture model of human endothelial cells, cardiomyocytes and fibroblasts: “human cardiac tissue spheroids” (HCTSs). Our data showed that laminin and collagen type IV synthesis is increased in VEGF-treated HCTSs

generated using CMs from an older donor compared to untreated cultures, suggesting a role for VEGF and eNOS in postnatal human heart development. In conclusion, our data showed that VEGF and eNOS play a similar role in mediating cardiomyocytes proliferation and heart regeneration in both mice and humans. Current studies are focusing on evaluating molecular targets of the VEGF/eNOS signalling pathway in human proliferating cardiomyocytes, which may have significant therapeutical impact for stem cell differentiation, and prevention of cardiovascular complications such as myocardial infarction.
C. Gentile: None. **C.Y. Chuang:** None. **C.J. Drake:** None. **M.J. Davies:** None.

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Single-Dose Isoproterenol does not Depress Cardiac Function in Mice
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Rationale: Myocardial injury after repeated or continuous administration of isoproterenol (ISO) in preclinical models has been widely described in the literature by our lab and others. Recent controversial reports using a one-time dose of ISO, to mimic a Takotsubo-like cardiomyopathy, demonstrated pronounced and reversible depression of cardiac function at one-week post injection with widespread myocyte death followed by robust myocardial regeneration and recovery of cardiac function. Hypothesis: Single-dose ISO does not produce depression of cardiac function
Methods and Results: C57Bl/6 mice were given a single subcutaneous injection of vehicle (saline) or 5, 200, or 300 mg/kg of ISO. Cardiac function was measured using transthoracic echocardiography with cardiac strain analysis at baseline prior to ISO injection and after 1, 7, 14, and 28 days post-injection. Animals were sacrificed after 1, 7, and 28 days post-injection for evaluation of gross heart weight (HW), HW/body weight (BW) and HW/tibia length (TL). Left ventricular (LV) functional measurements revealed no significant differences in global systolic function (ejection fraction and fractional shortening) between vehicle- or ISO-treated animals at any concentration. Additionally, no significant differences were detected between vehicle- or ISO-treated animals in any cardiac dimensions measured by echocardiography (LV cross-sectional area, internal diameter, end-diastolic or end-systolic volumes) or in gross HW, HW/BW or HW/TL. LV global cardiac strain was also not significantly different between vehicle and ISO-treated animals at any time point. When apical regions of the LV endocardium (the area most predominantly affected by Takotsubo cardiomyopathy) were specifically examined using strain analysis, no significant differences could be detected between vehicle and ISO-treated animals at any time point.
Conclusion: Single-dose ISO injury in a mouse model does not produce any depression of cardiac function at 1 week post injection.
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Molecular Characterization and Prospective Isolation of Human Secondary Heart Field Progenitors From Pluripotent Stem Cells
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The secondary heart field (SHF) progenitors ultimately contributes to diverse cardiovascular cell types through the formation of an early, multipotent heart progenitor pool and are marked by expression of ISL1, a LIM-homeodomain transcription factor. Human SHF can be derived from human pluripotent stem cells but their characterization has been limited due to the inefficiency of the differentiation protocols and lack of a proper reporter or surface marker based purification system. Using genetic tools and antibiotic selection we were able to purify ISL1+ cells for global gene expression analysis to identify key pathways that SHF identity. Genetic and small molecule based manipulation of these pathways alter ISL1 induction in differentiating cultures. Further proteomic analysis of enriched ISL1+ cells identified a hit surface marker that enables prospective isolation of ISL1+ secondary heart field progenitor cells with more than 90% purity. Purified SHF cells were multipotent and differentiate into pacemaker cells, endothelial and smooth muscle cells as well as mature beating cardiomyocytes. Finally transplantation of hPSC-derived purified SHF progenitors using this surface marker, restored myocardial function and regenerated infarcted area in mice myocardial infarction model.
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Twist Contributes to Cardiomyocyte Renewal and is Required for Maintenance of Adult Cardiac Function
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Cardiomyocyte renewal occurs very slowly in adult mammals, and little is known of the genetic basis of cardiac regeneration. Twist is a highly conserved bHLH transcription factor responsible for Drosophila mesoderm formation during embryogenesis. Recent studies have shown that Twist protein is essential for muscle regeneration in adult Drosophila, but the potential role of Twist in the mammalian heart has not been explored. There are two Twist genes in vertebrates, Twist-1 and -2. We show that Twist-1 and -2 are expressed in epicardium and interstitial cells but not in differentiated cardiomyocytes in mice. To understand the potential function of Twist-dependent lineages in the adult heart, we generated inducible Twist2CreERT2; ROSA26-tdTomato reporter mice. By treating these mice with tamoxifen at 8 weeks of age, we observed progressive labeling of various cell types, such as epithelial cells, cardiac fibroblasts, and cardiomyocytes in the heart. We isolated Tomato-positive nonmyocytes from these mice and found that these cells can differentiate into cardiomyocytes and other cell types in vitro. Furthermore, cardiac-specific deletion of both Twist1 and Twist2 resulted in an age-dependent lethal cardiomyopathy. These findings reveal an essential contribution of Twist to long-term maintenance of cardiac function and support the concept of slow, lifelong renewal of cardiomyocytes from a Twist-dependent cell lineage in the adult heart.
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Allogeneic Cortical Bone Stem Cells Preserve Cardiac Structure and Function by Inhibition of Cell Death and Enhancing Angiogenesis in the Swine Heart After Myocardial Infarction

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Introduction: Novel therapies are needed to improve cardiac function after MI; one strategy is to replace lost myocardium. Despite the success of bone marrow- and cardiac- stem cell clinical trials, we're still searching for the optimal stem cell type most suitable for cardiac regeneration. Previously, we described a novel cell population derived from the cortical bone (CBSCs) which repaired the heart post MI via transdifferentiation and paracrine signaling mechanisms in a mouse model. In the present study, we evaluate the translational potential of allogeneic CBSCs in swine MI model.
Hypothesis: Intramyocardial injection of CBSCs into the MI border zone immediately after reperfusion will preserve cardiac structure and pump function by enhancing endogenous repair through secretion of paracrine factors.
Methods & Results: Female Göttingen minipigs received MI via occlusion of the left anterior descending coronary artery (LAD) for 90 minutes, followed by reperfusion. Animals received either 20 million CBSCs (via 10 intramyocardial injections) or saline injections. Cardiac structure and function was evaluated using echocardiography at baseline and 4 weeks post-MI. During the 4 weeks after MI there was depression if pump function in saline treated group (EF: 69.27% ± 1.92 [baseline] to 46.305% ± 3.53 [4 weeks post MI] *p<0.0001*) and dilation of the LV (EDV: 39.01 ml ± 3.47 [baseline] to 51.5 ml ± 5.67 [4 weeks post MI] and ESV was 13.71 ml ± 2.47 to 27.72 ml ± 3.86 (*p= 0.0136*). The MI + CBSC group had no significant change in ventricular pump function from baseline to 4 weeks post-MI; while demonstrating preservation of cardiac structure indicating a decrease in ventricular remodeling/scar formation. A decrease in TUNEL+ cells and an increase in angiogenesis was also observed in CBSC's treated animals.
Conclusion: CBSC's appear to reduce infarct expansion by reducing post MI myocyte death and by increasing angiogenesis. These data show that administration of CBSCs immediately after reperfusion of an infarcted region of the heart can reduce adverse cardiac remodeling and improve cardiac function.
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Stromal Cell-derived Factor 1 Protects From Diabetic Cardiomyopathy

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We have demonstrated that stromal cell-derived factor 1(SDF-1) protects against palmitate-induced cardiac apoptosis, which is mediated by NOX-activated nitrosative damage and endoplasmic reticulum stress, via CXCR7, to activate AMPK/p38 MAPK-mediated IL-6 generation (Diabetes 62:2545-2558, 2013). Whether SDF-1 prevents diabetic cardiomyopathy has not been addressed. Here we evaluated the preventive effects of SDF-1 from diabetic cardiomyopathy in a high fat diet plus streptozotocin (HFD/STZ)-induced type 2 diabetic model in C57BL/6J mice. After 1 month on HFD, cardiac function was assayed by echocardiography, and then HFD-fed mice were injected with one low dose STZ (100mg/kg body weight, ip). Five days after STZ injection, mice with blood glucose levels ≥250 mg/dl were defined as diabetic. In parallel, the age-matched normal diet-fed mice injected with a same volume of citrate buffer (pH4.5) were used as control. After onset of diabetes, the mice were maintained on HFD or normal diet for another 4 months with or without SDF-1 treatment. Then cardiac function was assayed again, and the mice were sacrificed and cardiac tissue collected for cardiomyopathic index assay. We found that 1 month HFD feeding induced a significant insulin resistance without effect on cardiac function, but continued HFD feeding after STZ injection significantly impaired cardiac function, which were accompanied by increased insulin resistance and blood glucose, as well as blood insulin, triglyceride and cholesterol levels. Treatment with SDF-1 dose-dependently prevented diabetes-induced cardiac dysfunction but without significant effects on the above mentioned other pathophysiological parameters. These results indicate that SDF-1 possibly prevents diabetic cardiomyopathy via a direct cardiomyocyte action, which needs to be further defined in future study.
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Enhanced Cardiac Proteasome Function in the Naked Mole-rat, the Longest-lived Rodent

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The ubiquitin-proteasome system (UPS) is responsible for the recycling of misfolded proteins. Dysfunction of the UPS has been implicated in the pathophysiology of multiple heart disorders, including heart failure and reperfusion injury, but the basic science of cardiac UPS function remains unclear. An attractive mode of inquiry into the cardiac proteasome is the long-lived naked mole rat (NMR), which maintains intact cardiac reserve and diastolic function exceptionally late into its lifespan; equivalent to a 90 year old human with a 30 year old's heart. In this study, we investigated whether the long-lived and healthful NMR had upregulated aspects of UPS function in comparison to the short-lived well-characterized mouse. NMR hearts have more than twofold ($p<0.001$) greater proteasome-mediated chymotrypsin-like activity than mouse hearts. NMR hearts also have significantly greater levels of proteasome subunits than mice, including $\alpha 7$ and Rpt5, suggesting that the greater numbers of proteasomes could contribute to the high chymotrypsin-like activity, alternatively, the naked mole-rat heart may also have more immunoproteasomes which are more efficient. The UPS is energy-dependent, with its activity significantly influenced by available ATP. Interestingly, basal ATP levels were 40 to 50 fold higher in NMR hearts than in those of mice. This is consistent with the much larger pools of mitochondria observed in the NMR heart than in the mouse heart. Considering that both high and low ATP levels are associated with a decline in proteasome activity, we next asked whether the remarkably high basal ATP levels of the NMR heart caused a qualitative difference in UPS function between NMRs and mice. Levels of ubiquitinated protein were significantly lower in the NMR heart than in the mouse heart, suggesting that the NMR cardiac UPS system is more effective at destroying ubiquitin-tagged damaged proteins than that of the mouse, and that the NMR heart's elevated ATP levels may play a physiological role in maintaining this enhanced UPS functionality. Overall these data suggest a high basal level of proteasome activity in the NMR heart that may be of paramount importance in this animal's ability to withstand and/or prevent age-related cardiovascular functional declines.

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Physiologic Cardiac Hypertrophy in Mice Reduces mRNA Levels of Myostatin and Autophagy Genes

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Myostatin and autophagy are involved in muscle growth regulation. However, there are few studies exploring their role in physiologic cardiac hypertrophy. We evaluated myostatin and autophagy in mice subjected to a swimming protocol to induce physiologic cardiac hypertrophy. *Methods:* Adult (8 weeks-old) male BALB/c mice (*n*=52) were divided in sedentary (S) and trained (T) groups, which were evaluated in 7 (S7 or T7, initial hypertrophy) and 28 (S28 or T28, stablished hypertrophy) days after the start of the protocol. Left ventricular/tibial length ratio (LV/TL) and cardiomyocyte diameter were used to assess cardiac hypertrophy. Gene expression was evaluated by RT-qPCR, while protein expression was analyzed by western blot. Bioinformatic analysis was performed by TargetScan to predict potential miRNAs' targets and Genemania to create an interaction network between miRNAs and genes. All results were expressed as mean ± SEM and comparisons were performed using the Student T test. *Results:* Myocardial hypertrophy was confirmed in trained group both by the increase in LV/TL ratio in 28 days (13%, p=0.0001) and cardiomyocyte diameter in 7 days (20%, p=0.04) and 28 days (30%, p=0.002). There was a decrease in myostatin gene expression levels in T7 compared to S7 group (0.8 ± 0.1 *vs* 1.2 ± 0.1, p=0.01) without changes at day 28. However, there was no difference in mTOR phosphorylation at T7, although it was increased in T28 compared to S28 (397±95 *vs* 90±23 AU; p=0.02). Autophagic genes showed reduced expression levels in trained groups at both time points (reductions of 19% and 10% for *Lc3,* 22% and 11% for *P62*, 19% and 10% for *Beclin1* in T7 and T28, respectively; p<0.05 for all analyses compared to sedentary groups), but there was no difference at protein level. Bioinformatics analysis showed that miR-30a, - 221, -27a/b and 208a/b are possible regulators of autophagic and myostatin genes. *Conclusions:* Taken together, reduced myostatin during initial hypertrophy and increased mTOR phosphorylation in the established hypertrophic phenotype might favor muscular growth and reduce basal autophagy. Candidate miRNAs identified through bioinformatic analysis might regulate this process, and should be further validated in this scenario.

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Targeting of Tp53inp1 by Mir-221 to Reduce P62-mediated Autophagy is Cardioprotective in Ischemia / Reperfusion Injury

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Purpose: Tumor protein 53-induced nuclear protein 1 (Tp53inp1) acts as a tumor suppressor by inducing cell death. Tp53inp1 mRNA is a predicted target of miR-221. Whether targeting Tp53inp1 plays a role in miR-221-mediated cardioprotection has not been investigated. We hypothesized that miRNA-221 directly targets Tp53inp1 to reduce ischemia/reperfusion (I/R)-induced autophagy. **Method:** Myoblast H9c2 cells underwent 16 hours 0.2% O_2 hypoxia followed by 2 hours re-oxygenation (H-R, simulating I/R). H9c2 were transfected with miRNA-221 mimic (25 nmol) and scrambled mimic control (miR-221 and MC). Cell count/viability, WST assay, cell injury-induced LDH release, and GFP-LC3 labeled autophagosome formation were measured. Cells were collected for RT-qPCR and western blot (WB) analyses. pCMV-Myc-Tp53inp1 and pcDNA3.1-Flag-p62 plasmids were cloned and transfected into H9c2 for recovery and immuno-precipitation (IP) studies. The effects of miRNA-221 inhibitor in H9c2 were also assessed. **Results:** miR-221 significantly reduced H-R injury as indicated by higher cell count/viability and WST activity, and reduced LDH (miR-221 vs. MC p<0.05). qPCR confirmed that (1) miRNA-221 expression was reduced in H-R; (2) RISC-loaded (IP pull-down Ago-2) miRNA-221 increased by ~80 fold and reduced by 95% following mimic and inhibitor transfection respectively; (3) Increased Tp53inp1 following H-R was reversed by miR-221. miR-221 inhibited H-R induced autophagosome formation (GFP-LC3). WB indicated (1) increase of LC3-I/II ratio and p62, indicators of reduced autophagy, and (2) decrease of Tp53inp1 by miR-221. IP pull-down Myc-Tp53inp1 indicated the formation of p62-Tp53inp1 complex. The protective effect of miR-221 was abolished by Tp53inp1 overexpression (pCMV-Myc-Tp53inp1 and miRNA-221 mimic co-transfection). The protective effect was corroborated in neonatal rat ventricular myocytes (NRVM). MiRNA-221 inhibitor induced reverse effects. **Conclusion:** The cardioprotection of miR-221 entails direct targeting of Tp53inp1 which reducing p62-Tp53inp1 complex formation and inhibiting H-R-induced autophagy.

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Mirna-125b Reduces Ischemia / Reperfusion (i/r)-induced Apoptosis Through Targeting Multiple Targets in Intrinsic and Extrinsic Apoptosis Pathways

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Apoptosis is mediated through extrinsic and intrinsic pathways, both play a role in ischemia/reperfusion (I/R) injury. Predicted targets for miRNA-125b include extrinsic pathway mediators Traf6 and Tnfrsf1b, and intrinsic mitochondria regulators Bcl-2 family pro-apoptotic effectors Bak1 and BH3-only facilitators Bim, Bmf, Puma. We hypothesized that miRNA-125b directly targets multiple genes to reduce I/R-induced apoptosis. Myoblast H9c2 cells underwent 16 hours 0.2% O_2 hypoxia followed by 2 hours re-oxygenation (H-R, simulating I/R) and were transfected with miRNA-125b mimic vs. scrambled mimic control (25 nmol, miR-125b-M vs. MC) and miR-125b inhibitor vs. inhibitor control (miR-125b-I vs. IC). Cell count/viability, WST assay, cell injury-induced LDH release and apoptotic marker Casp3/7 were measured. Cells were trypsinized for assessment of apoptosis (7-AAD and annexin V double staining) and lysed for RT-qPCR and western blot (WB) analyses. pCMV-Myc-Bak1 plasmids were cloned and transfected into H9c2 for recovery studies. The effects were verified in neonatal rat ventricular myocytes (NRVM). miRNA-125b-M significantly reduced H-R injury as indicated by higher cell count/viability and WST activity, and reduced LDH (miR-125b-M vs. MC p<0.05). qPCR confirmed that (1) miR-125b expression was reduced in H-R; (2) RISC-loaded (immunoprecipitation pull-down Ago-2) miR-125b increased by ~35 fold and reduced to ~3% following

mimic and inhibitor transfection respectively; (3) multiple apoptosis-related genes were reduced by miR-125b-M, Bak1, Bmf, Bim, Puma, Traf6 and Tnfrsf1b. All changes were confirmed by WB. Luciferase reporter assays indicated miR-125b bound to the 3'-UTR of all genes tested except Traf6. Total apoptotic cell numbers and Casp3/7 release were significantly reduced by miR-125b-M. The protective effect of miRNA-125b was partially abolished by Bak1 overexpression (pCMV-Myc-Bak1 and miR-125b co-transfection). Protective effects of miRNA-125b were further verified in NRVM. MiRNA-125b inhibitor reversed protective effects and target changes at mRNA and protein level. miR-125b is powerfully cardioprotective in I/R injury due to directly targeting multiple genes in the extrinsic and intrinsic apoptotic pathways.

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Selective Estrogen Receptor Modulator Retards Arterial Senescence and Atherosclerosis via Upregulation of Sirt-1 and Autophagy in Post-menopause Model Mouse

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Background Incidence of cardiovascular disease (CVD) increases with development of arterial senescence and atherosclerosis, which are accelerated after menopause in women. This study aims 1) to clarify the mechanism of which post-menopose facilitates arterial senescence and atherosclerosis, and 2) to develop optical treatment.

Methods and results Female Apolipoprotein E deficient (ApoE KO) mice underwent ovariectomy at 8 weeks old were used as post-menopausal model mice (PM). Age-matched female mice with sham operation were used as control (Ctr). We confirmed that serum estrogen concentration was significantly lower in PM than in Ctr. Twelve weeks after surgery, aortas were harvested to examine molecular biological analysis. Sirt-1 and the ratio of LC3II/LC3I protein expressions were significantly lower in PM than in Ctr. Ratio of senescence associated β galactosidase (SA-β Gal) positive cells and expressions of acetyl (Ac)-p53, p21, and PAI-1 were significantly higher in PM than in Ctr. Aortic atherosclerotic lesions assessed by Oil Red O staining was significantly greater in PM than in Ctr. Overexpression of either Sirt-1 or autophagy retarded arterial senescence in vitro. These results suggest that ovariectomy accelerates arterial senescence and atherosclerotic development through downregulation of Sirt-1 and autophagy. Next, we administrated selective estrogen receptor modulator (SERM) to PM mice. Sirt-1 and the ratio of LC3II/LC3I protein expression were significantly higher in SERM-treated PM mice than in untreated mice. Ratio of SA-β Gal positive cells, protein expressions of Ac-p53, p21, and PAI-1, and aortic atherosclerotic lesions were significantly lower in SERM-treated PM mice than in untreated mice. The effect of SERM on augmentation of autophagy and inhibition of arterial senescence and atherosclerotic development was attenuated by administration of sirtinol, Sirt-1 inhibitor.

Conclusion SERM retards development of arterial senescence and atherosclerosis by activation of Sirt-1 and autophagy in postmenopause mice.

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Macrophage Elavl1 Regulates Fibrogenesis Through Post-transcriptional Mechanism Under Diabetic Conditions

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Patients with diabetes are predisposed to increased risk of cardiovascular diseases. Persistent interaction of infiltrating macrophages and resident fibroblasts play a critical role in cardiac fibrosis. However, the signaling mechanism is not clear. We hypothesized that macrophage ELAV1 (mRNA stabilizing protein) modulates profibrotic mediators and extracellular matrix turnover by binding to 3'UTR and regulating the mRNA stability of TGF-beta and MMP-9 in hyperglycemic conditions. Mice receiving intramyocardial injection of HuR-specific shRNA showed significant reduction in infarct size and fibrosis area. Reduced fibrosis was associated with decrease in TGF-beta and MMP-9 expression in the myocardium. Conditioned media (CM) from high glucose (HG) treated macrophages significantly increased profibrogenic response (increased mRNA expression of Col1a1, Col3a1 and fibronectin) in fibroblast cell line as compared to fibroblasts incubated with CM from low glucose (LG)-treated macrophages. Knockdown of ELAV1 in HG-treated macrophages abrogated the profibrotic effects in fibroblasts. Indirect immunofluroscence of bone marrow-derived macrophages (BMM) demonstrated that HG increases nuclear ELAV1 export to the cytoplasm. Pharmacological inhibition of Protein kinase C-delta (PKCd) blocked HG-induced ELAV1 nuclear to cytoplasmic translocation. In vitro, stable knockdown of ELAV1 in mouse macrophage cell line RAW 264.7 reduced mRNA expression of TGF-beta and MMP-9 following LPS challenge, accompanied by a marked reduction in the mRNA stability of these genes. Our study here establishes an ELAV1/TGF-beta/MMP-9/PKC-delta signaling axis in the macrophages controlling the profibrogenic responses in fibroblasts, the major contributor in the pathogenesis of fibrosis. Therefore, targeting this signaling pathway might be of therapy value for cardiac fibrosis in diabetic patients.

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Reversal of Intramuscular Lipotoxicity is Mediated by a Novel p62-lipophagy Pathway

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Background and Hypothesis: In the mammalian heart, autophagy is considered an adaptive mechanism promoting the removal of protein aggregates or damaged cell organelles. A common feature of non-ischemic heart failure is lipid accumulation. We hypothesize that autophagy is impaired in the muscle in chronic lipid overload. Therefore, we investigated whether activation of autophagy protects myocytes from lipotoxicity. Methods: We incubated rat L6 myocytes over a period of 6 days with or without long

chain fatty acids (equimolar mixture of oleate and palmitate, 1.0mM). At day 6, an autophagic inhibitor (bafilomycin A1, 200 nM), or an autophagic activator (rapamycin, 1 μM), or both, were added for 48 hours. Following the pharmacologic treatments, glucose uptake was measured using [3H] 2-Deoxy-D-glucose, and insulin sensitivity was assessed. Intracellular triglyceride (TG) accumulation was assessed by Oil Red O staining and immunofluorescence and was quantified enzymatically. Protein markers of autophagic flux (LC3 and p62) and cell death (Caspase 3 cleavage) were measured by immunoblotting. Results: Inhibition of autophagy using bafilomycin increased TG accumulation and also increased fatty acid-mediated cell death. Conversely, activation of autophagy using rapamycin reduced both intracellular lipid accumulation and cell death. Unexpectedly, treatment with both rapamycin and bafilomycin resulted in a decrease in lipid accumulation. Immunoblotting indicated p62 degradation (autophagic flux), while immunofluorescence revealed the colocalization of p62 with lipid droplets in these cells. These findings indicate the potential association of p62 with lipid droplet turnover, which is a novel pathway for the breakdown of lipid droplets in muscle cells. In the same cells, rapamycin treatment increased glucose uptake, in response to insulin (100mM). Conclusions: Autophagy promotes the clearance of lipids from myocytes, improves insulin sensitivity, and switches to an alternative p62 mediated pathway of lipophagy in the context of chronic lipid overload. Moreover, lipophagy promotes metabolic adaptation and myocyte survival.
T.N. Lam: None. **R. Harmancey:** None. **H. Vasquez:** None. **N. Patel:** None. **B. Gilbert:** None. **H. Taegtmeyer:** None.

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MicroRNA208a Silencing Attenuates Doxorubicin Induced Cardiac Toxicity and Dysfunction
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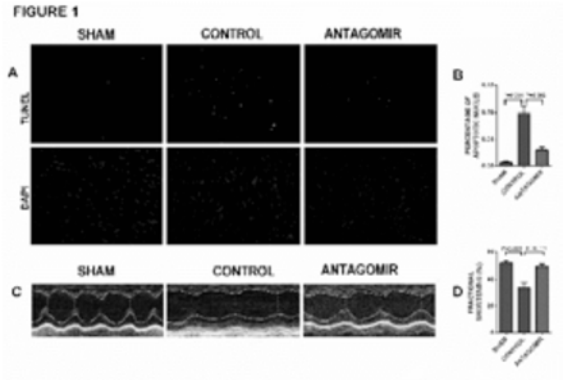
Aims Salvaging GATA4 expression mitigates doxorubicin-induced myocyte apoptosis and cardiac dysfunction. We investigated if therapeutic silencing of miR-208a, a heart specific microRNA known to target GATA4, could attenuate doxorubicin-induced myocyte apoptosis and improve heart function.
Methods Eight weeks old female Balb/C mice were randomly assigned to Sham, antagomir and Control groups. Antagomir group mice were pre-treated with 50nmols of miR-208a antagomir 4 days prior to giving doxorubicin. At day 0, control and antagomir group got 20mg/kg of doxorubicin while sham mice received phosphate buffered solution. Echocardiography was done at day 7, after which animals were sacrificed, and hearts assessed for apoptosis and expression of miR-208a, GATA4 and Bcl-2 by quantitative PCR.
Results Doxorubicin significantly upregulated miR-208a P=0.008 , downregulated GATA4 P=0.025 , and increased myocyte apoptosis P=0.001. Therapeutic silencing of miR-208a mitigated the doxorubicin-induced increase in miR-208a, P= 0.003 and salvaged GATA4 expression, with noted increase in Bcl-2 levels compared to controls, P=0.033. Doxorubicin significantly increased cardiomyocyte apoptosis P= 0.001, and this effect was attenuated by pretreatment with miR-208a antagomir, P=0.002 (Figure 1A and B). Doxorubicin also caused significant cardiac dysfunction, P=0.005, while antagomir treatment attenuated doxorubicin-induced cardiac dysfunction as assessed by fractional shortening P=0.011 (Figure 1Cand D)
Conclusion Therapeutic silencing of miR-208a salvages GATA4 and attenuates doxorubicin-induced myocyte apoptosis with subsequent improvement in cardiac function.

FIGURE 1



H. Tony: None. **Q. Zeng:** None. **K. Yu:** None.

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Knockout of the Pro-apoptotic Er-stress Gene Chac1 in Mice Results in Embryonic Lethality and Activation of the Notch Pathway
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The ER-stress pathway is activated by oxidized phospholipids in human aortic endothelial cells (HAECs), and may play a role in the development of atherosclerosis. Systems genetics screens of transcriptome wide RNA co-expression in populations of human cells can broadly define novel functional gene-gene relationships. Using this method, we predicted a role for the human CHAC1 gene downstream of ATF4 in the ER-stress pathway in HAECs. Targeted cell culture and genetic perturbations refined the ATF4 dependent ER-stress induction of CHAC1 and a role in regulating apoptosis. Herein we report the generation of a mouse model with the Chac1 gene deleted (Chac1-KO), and heterozygous insufficiency (Chac1-Het). We obtained transgene positive C57BL6 founders that were bred to subsequent generations producing Chac1-Het mice. Intercrossing Chac1-Het mice revealed embryonic lethality, as no Chac1-KO progeny were generated from 25 litters. Chac1-KO embryos were produced at 13 days post coitus and used to make Mouse Embryonic Fibroblasts (MEFs), and the absence of Chac1 expression in these cells was validated. Since Chac1 is an ER-stress inducible gene activated by ATF4, we examined the expression of this pathway in Chac1-KO MEFs, versus controls. We noted increased basal and ER-stress induced expression of ATF4 and its target CHOP accompanying Chac1 knockout, highlighting a novel role for Chac1 in feedback regulation of the ATF4 branch of the ER-stress pathway. We also noted increased expressions of Parp1 and Caspase3, in Chac1-KO MEFs, supporting a role in the regulation of apoptosis pathways. Additionally, Chac1 cells had a higher proliferation rate, displayed a distinct morphology, and grew to a higher density. Since recent reports show a role for Chac1 in blocking Notch maturation, we examined expression of the Notch pathway in Chac1-KO MEFs, versus controls. We noted increased expression of the Notch target gene Hes1 in Chac1-KO MEFs at baseline. Following treatment with the proteasome inhibitor MG132, Chac1-KO MEFs had increased expressions of Hes1, Hey2 and Nrarp, target genes of the Notch pathway. These data reveal activation of the Notch pathway in Chac1-KO cells treated with MG132, a hyper proliferative phenotype and embryonic lethality at dpc 13.
I. Mungrue: None. **E.T. Prescott:** None. **C.F. Sylvester:** None. **R.R. Crawford:** None.

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miR-223 Negatively Regulate Ischemia/Reperfusion-induced Cardiac Necroptosis

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It is well known that myocardial ischemia/reperfusion (I/R) causes myocyte apoptosis and necrosis. For many years, apoptosis was considered to be the only form of gene-regulated cell death, whereas necrosis was thought as a passive accidental cell death. Recent studies, however, clearly indicate that necrosis can be controlled by multiple genes, and RIPK1/3-regulated necrosis, called necroptosis, has gained well attention. We and others previously showed that miR-223, an anti-inflammatory miRNA, was greatly up-regulated in the infarcted heart. To test whether miR-223 regulates I/R-induced cardiac necroptosis, transgenic (TG) mice with cardiac-specific overexpression of miR-223 and miR-223 knockout (KO) mice were used and underwent global no-flow I/R (30min/1h). We observed that TG hearts displayed the better recovery of contractile function (+dP/dt: 92±4%), compared with wild-type (WT) hearts (65±3%). This improvement was accompanied with a 2.4-fold decrease in lactate dehydrogenase (LDH), a marker of necrosis, released from TG hearts, comparable to WTs. By contrast, KO-hearts showed the worse recovery of contractile function (+dP/dt: 41± 3%) than WTs (+dP/dt: 70±4%), and increased LDH release (3-fold). Notably, both TUNEL-staining and DNA fragmentation analysis for cardiac apoptosis showed no difference between groups. Western-blotting assays showed that protein levels of RIPK1, RIPK3 and MLKL, three known mediators in the necroptotic pathway, were reduced in TG hearts, whereas they were increased in KOs, compared to respective WT controls upon I/R. Furthermore, pre-injection of NEC-1s (1.65mg/kg), a specific inhibitor of necroptosis, into miR-223-KO mice, significantly improved cardiac function recovery during I/R, compared to saline-injected KOs. To elucidate the mechanisms underlying the miR-223-mediated cardiac necroptosis, we performed a series of experiments (bioinformatics, luciferase report assay, and western-blotting). Our results showed that miR-223 negatively regulated the expression of TNFR1 and death receptor 6 (DR6), two activators of the necroptotic pathway. Put together, this study indicates that miR-223 could control I/R-induced cardiac necroptosis via targeting the DR6/TNFR1-RIP1/3-MLKL pathway.

G. Fan: None. **D. Qin:** None. **X. Wang:** None. **L. Yang:** None. **W. Huang:** None. **Y. Wang:** None.

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Postural Orthostatic Tachycardia Syndrome and its Associated Chronic Pain Symptoms

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Background and Purpose: The Postural Orthostatic Tachycardia Syndrome (POTS) affects primarily young women. POTS is a form of dysautonomia that is estimated to impact between 1,000,000 and 3,000,000 Americans, and millions more around the world. The symptoms of POTS are widespread because the autonomic nervous system plays an extensive role in regulating functions throughout the body. The aim of this study is to determine the chronic pain symptoms in Postural Orthostatic tachycardia syndrome (POTS) patients.
Method: Two hundred fifty-five (255) POTS patients were randomly selected from our clinic (January 2014 to March 2015), reviewed the medical records of 255 POTS patients for chronic pain symptoms and performed data analysis.
Results: Two hundred thirty-three of the 255 (91%) patients are females (n=233, age 29.20 ± 10.32), Twenty-three of the 255 (9%) patients are males (n=23, age 29.70 ± 14.52).63% (161 of the 255) had Joint Pain/aches, 51% (131 of the 255) had Chronic headache, 40% (102 of the 255) had chest pain, 31% (80 of the 255) had Migraine, 30% (76 of the 255) had Chronic back pain,16% (42 of the 255) had Heartburn,8%(20 of the 255) had Chronic pleurisy, Rheumatoid arthritis, Muscle aches, Chronic regional pain syndrome and Hip aches.
Conclusion: Our study is the first to characterize that Patients with postural orthostatic tachycardia syndrome (POTS) have a very high prevalence of chronic pain symptoms.

C. Ashangari: None. **A. Suleman:** None.

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A Comprehensive Echocardiogram Study in Postural Orthostatic Tachycardia Syndrome

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Background and Purpose: The Postural Orthostatic Tachycardia Syndrome (POTS) affects primarily young women. POTS is a form of dysautonomia affecting millions around the world. The aim of this study is to determine the echocardiographic findings in POTS patients.
Methods: 173 POTS patients were randomly selected and underwent echocardiogram results were reviewed and performed cumulative distribution of echocardiogram results in to top 10 percentile to see the abnormalities.
Results: Out of 173 patients, 96% are females (n=166; age 29.20 ± 9.26), 4% are males (n=7; age 28.50 ± 13.64)
Our findings (mean ± SD).
RVDD (2.37 cm ± 0.30 cm), LVED (4.34 cm ± 0.35 cm), IVS (0.82 cm ± 0.12 cm), LVEF (63.7%±2.61%), LVES (2.93 cm ± 0.25 cm), LVPW (0.83 cm ± 0.13 cm) , LVOT Dia (1.82 cm ± 0.20 cm) AO Root (2.70 cm ± 0.31 cm) , LA Dia (3.13 cm ± 0.36 cm) ,AV Vel (1.12 m/sec ± 0.13 m/sec), LVOT Vel (0.83 m/sec ± 0.31 m/sec), Mean Gradient (2.81mmHg ± 0.68 mmHg), Peek Gradient (4.91 mmHg ± 1.40 mmHg), AVA (2.65 cm2 ± 0.44 cm2),E Vel (0.77 m/sec ± 0.13 m/sec), A Vel (0.54 m/sec ± 0.10 m/sec), E/A Ratio (1.45 ± 0.36), Mean Gradient (0.83 mmHg ± 0.29 mmHg), Peak Gradient (2.21 mmHg ± 0.82 mmHg), PHT (48.25 m/sec ± 11.13 m/sec), MVDT (151.40 m/sec ± 31.44 m/sec) , MVA (4.85 cm2 ± 1.12 cm2), MR Mild in 82 % patients ,TV Vel (0.61 m/sec ± 0.13 m/sec), RAP (est) (10 mmHg ± 0 mmHg), TR Vel (20.78 m/sec ± 5.62 m/sec), RSVP (est) (29.04 ± 4.54),TR Mild in 86 % Patients.PV Vel (0.72 m/sec ± 0.16 m/sec).
Top 10 percentile parameters are follows
RVDD 2.75 cm, LVED 4.79 cm, IVS 0.97 cm, LVEF 67.04 %, LVES 3.25 cm, LVPW 0.97 cm, LVOT Dia 2.08 cm, Ao Root 3.10 cm, LA Dia 3.59 cm AV Vel 1.29 m/sec , LVOT Vel 1.23 m/sec , Mean Gradient 3.68 mmHg, Peek Gradient 6.70 mmHG,AVA 3.21 cm2 .
E Vel 0.94 m/sec , A Vel 0.67 m/sec , E/A Ratio 1.91 , Mean Gradient 1.20 mmHg, Peak Gradient 3.26 mmHg, PHT 62.51 m/sec , MVDT 191.69 m/sec, MVA 6.29 cm2, TV Vel 0.78 m/sec, RAP (est) 10mmHg, TR Vel 27.98 m/sec, RSVP (est) 34.81,PV Vel 0.93 m/sec.
Conclusion: POTS patients have normal echocardiographic parameters however even the top 10 percentile of these patients had smaller LVEDD. Similarly the internal dimensions of cardiac chambers does appear to be smaller though still within accepted normal parameters. Further studies should be taken to better define this subgroup.

C. Ashangari: None. **A. Suleman:** None. **A. Brown:** None.

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Effects of Sympatho-excitation on Directional Anisotropy in Ventricular Myocardium

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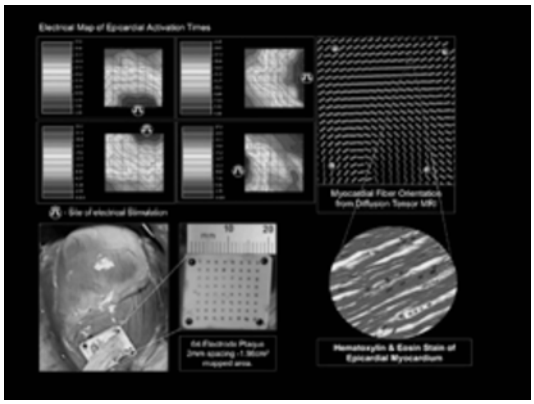
Introduction: Control of electrical propagation exerted by the sympathetic nervous system is has not been quantified in-depth.
Methods: High-resolution mapping (64-electrode plaque, 8x8, 1.96cm^2) of the anterior LV myocardium in porcine model (n=6) was performed before & during left stellate ganglion stimulation (LSGS). Activation times (AT), activation recovery intervals (ARIs), conduction velocities (CV), and CV anisotropy were obtained during pacing. Ex vivo diffusion tensor MRI and histology were performed to define myocardial fiber orientation in the mapped regions.
Results: LSGS shortened ARI (314.3±7.8ms vs. 287.8±6.6ms, p<0.001). At baseline, longitudinal CV (CV_L) was greater than transverse CV (CV_T) (1.2±0.2m/s vs 0.7±0.1m/s, p<0.001). LSGS did not increase CV_L (1.16±0.16m/s vs 1.17±0.15m/s, p=0.2), or CV_T (0.67±0.07m/s vs 0.7±0.04m/s, p=0.2). However, CV in the retrograde direction along fiber orientation was significantly increased by LSGS (0.7±0.05m/s vs 0.9±0.06m/s, p<0.01). This resulted in a significant reduction in directional anisotropy ($CV_{antegrade}/CV_{retrograde}$) along fiber direction (1.6±0.3 vs 1.3±0.3, p<0.001), but not CV_L/CV_T (1.78±0.2 vs. 1.66±0.2 for BL and LSGS, respectively, p>0.2). Heterogeneity of activation (AT_{disp}) was greater in the transverse than longitudinal direction (20±2ms^2/cm^2 vs. 13±1.5ms^2/cm^2, p= 0.022). LSGS decreased transverse AT_{disp} to 18±1.8 ms^2/cm^2 (p=0.015), but not longitudinal AT_{disp}.
Conclusion: Cardiac sympatho-excitation modulates CV and AT_{disp} in a fiber orientation-dependent manner. These uneven changes may contribute to arrhythmogenic mechanisms seen during sympatho-excitation.



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The Human Pericardial Fluid is Enriched With Cardiovascular-expressed Micrornas and Exosomes Able to Elicit Therapeutic Responses

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Cells release functionally active microRNAs (miRs) into extracellular vesicles (EVs: exosomes and microparticles). In vitro, EVs shuttle miRs and other molecules between cells. The functional relevance of EVs in the context of human physiopathology is still debated.
We hypothesize that the pericardial fluid (PF) mediates myocardium cell-to-cell communication through EVs exchanges. Here, we aimed to (1) characterize human PF EVs and exosomal miRs; (2) investigate if PF EVs exert a biological function.
PF, plasma, thoracic aorta (TA) and right atrium appendage (RAA) samples were collected as leftovers from aortic valve replacement surgery. A miR array identified PF miRs expression. The top expressed cardiovascular miRs were measured (RT-qPCR) in TA and RAA (to validate expression in patients cardiovascular tissues), PF and plasma, and exosomes enriched from PF and plasma. EVs concentration and size distribution in PF and plasma were quantified by a nanoparticle tracking analysis system. The exosome functional activity was tested in human endothelial cells (ECs) and in a mouse model of limb ischemia (LI).
A pool of cardiovascular miRs (including miR-21 and -29a), were enriched in the PF in comparison to plasma, suggesting that the PF/plasma concentration gradients of cardiovascular miRs were caused by the trafficking of these miRs from the myocardium and thoracic vessels to the PF. This gradient was reverted for the negative control miR-122 (produced by the liver and undetectable in the PF). Exosomes were abundant in the PF, even if less concentrated than in plasma. PF-exosome treatment of ECs exposed to hypoxia significantly decreased cell apoptosis and increased cell proliferation (P<0.01 vs PBS). Local PF-exosomes delivery increased post-LI ischemic blood flow recovery (P<0.01 vs PBS). By contrast, plasma-exosomes were not effective (P=NS vs PBS).
We have provided the first characterization of EVs and exosomal miRs in the human PF and for first identified a therapeutic potential of PF- exosomes from cardiovascular patients. Future studies will be tailored to exploit the properties PF-exosome in regenerative medicine.
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Serum Mir-129 and Mir-21 Predict 100-day Mortality in Acute Heart Failure Patients

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Background: Heart failure is a common disease worldwide and it could be divided as chronic heart failure (CHF) and acute heart failure (AHF). Circulating microRNAs (miRNAs, miRs) have been reported to be novel biomarkers of diagnostic, prognostic and predictive values in cardiovascular diseases. However, little is know about using circulating miRNAs as biomarkers for mortality in AHF patients.
Methods and results: A total of 151 AHF patients were enrolled in this study. Ten miRNAs involved in the regulation of AHF including miR-129, miR-675, miR-622, miR-146a, miR-155, miR-21, miR-18b, miR-92b, miR-126 and miR-22 were determined by reverse transcription polymerase chain reactions using total RNA isolated from serum of those 151 patients with AHF enrolled in our center. After a follow-up period of 100 days, 16 patients died and based on that, we found that expression levels of serum miR-129 (p=0.032) and miR-21-5p (p=0.001) were significantly

lower in those patients died within 100 days. The kaplan cumulative survival analysis confirmed that patients with higher levels of miR-129 (p=0.036) and miR-21 (p=0.001) had significantly higher survival rate. Conclusion: Serum low levels of miR-129 and miR-21 predict 100-day mortality in AHF patients.
L. Tao: None. **Y. Bei:** None. **S. Shen:** None. **J. Li:** None. **R. Gao:** None. **X. Wu:** None. **J. Xiao:** None. **X. Li:** None.

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Mir-433 Controls Cardiac Fibrosis

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Background: Cardiac fibrosis, a result of multiple injurious insults in heart, is a final common manifestation of chronic heart diseases and can lead to end-stage cardiac failure. MicroRNAs (miRNAs, miRs) participate in many essential biological processes and their dysfunction has been implicated in a variety of cardiovascular diseases including fibrosis. miR-433 has recently been implicated in renal fibrosis, however, its role in cardiac fibrosis is unclear.
Methods and results: miR-433 was increased in heart samples from dilated cardiomyopathy patients as determined by qRT-PCRs. In addition, miR-433 was also consistently upregulated in mice model of cardiac fibrosis after myocardial infarction or heart failure. Additionally, miR-433 was found to be enriched in fibroblasts compared to cardiomyocytes. In neonatal cardiac fibroblasts, forced expression of miR-433 promoted cell proliferation as indicated by EdU and Ki-67 staining. Moreover, miR-433 overexpression promoted the transdifferentiation of fibroblasts into myofibroblasts as determined by qRT-PCR and western blot for α-SMA and collagen whether in the presence of TGF-β or not, indicating that miR-433 is sufficient to induce fibrosis. In addition, knockdown of miR-433 inhibited proliferation and the transdifferentiation into myofibroblasts, indicating that miR-433 is required for cardiac fibrosis. Interestingly, miR-433 did not affect the migration of cardiac fibroblast. Importantly, miR-433 antagomir could partially attenuate cardiac fibrosis induced by myocardial infarction in mice.
Conclusion: both in vitro and in vivo. Inhibition of miR-433 represents a novel therapeutic strategy for cardiac fibrosis.
L. Tao: None. **X. Wu:** None. **P. Chen:** None. **S. Li:** None. **X. Zhang:** None. **Y. Bei:** None. **T. Xu:** None. **J. Xiao:** None. **X. Li:** None.

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Exercise Training Protects Against Acute Myocardial Infarction via Improving Myocardial Energy Metabolism and Mitochondrial Biogenesis

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Acute myocardial infarction (AMI) represents a major cause of morbidity and mortality worldwide. Exercise has been proved to reduce myocardial ischemia-reperfusion (I/R) injury. However it remains unclear whether, and (if so) how, exercise could protect against AMI. Methods: Mice were trained using a 3-week swimming protocol, and then subjected to left coronary artery (LCA) ligation, and finally sacrificed 24 h after AMI. Results: Exercise training reduces myocardial infarct size and abolishes AMI-induced autophagy and apoptosis. MI leads to a shift from fatty acid to glucose metabolism in the myocardium with a downregulation of PPAR-α and PPAR-γ. Also, AMI induces an adaptive increase of mitochondrial DNA replication and transcription in the acute phase of MI, accompanied by an activation of PGC-1α signaling. Exercise abolishes the derangement of myocardial glucose and lipid metabolism and further enhances the adaptive increase of mitochondrial biogenesis. Conclusion: Exercise training protects against AMI-induced acute cardiac injury through improving myocardial energy metabolism and enhancing the early adaptive change of mitochondrial biogenesis.
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AAV9 Serca2a Gene Transfer Reverses Some but Not All Electrophysiological Deficits in a Chronic Model of Congestive Heart Failure

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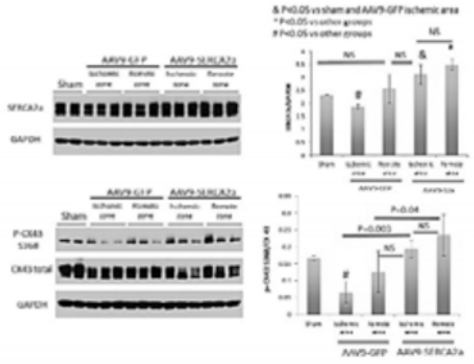
SERCA2a gene transfer (GT) to the failing heart improves it mechanical function. The electrophysiological (EP) consequences of SERCA2a GT are less clear. We investigated the EP substrate, total (t) and phosphorylated (p) Cx43 expression in a chronic model of heart failure (HF) with & without SERCA2a GT.
Methods: After 2 mo of aortic banding rats underwent 30min LAD occlusion & reperfusion for another 2 mo. Rats developed widespread proliferation of reactive fibrosis in ischemic & remote zones and were randomized to receive AAV9 GFP (HF) or AAV9 SERCA2a (HF treatment) at the time of LAD occlusion/reperfusion. Hearts from normal (Sham, N=6), HF (AAV9.GFP, N=7), and HF treatment (AAV9.SERCA2a, N=9) rats were studied using optical mapping. Cx43 levels (t & p) were measured.
Results: HF rats exhibited significant APD prolongation (by 50%) & CV slowing (by 30%). More importantly APD heterogeneity was increased 2.5 fold (p=0.006). The ratio of S368 p-to-t Cx43 was reduced in the ischemic zone of HF rats where SERCA2a expression was decreased (Fig). AAV9 SERCA2a GT increased SERCA2a levels in the ischemic and remote zones. Surprisingly, APD & CV were comparable (p=NS each) in HF and HF treatment groups. In contrast SERCA2a GT reduced APD heterogeneity by 30% relative to untreated rats (p=0.03), fully restored pCx43 expression to sham levels in the ischemic zone and abolished regional differences in p-to-t Cx43 (Fig).
Conclusions: AAV9 SERCA2a GT reverses key EP and molecular deficits that are causally related to arrhythmias in HF. This therapy, however, fails to reverse other hallmark features of HF, which are likely dependent on widespread ion channel and structural remodeling, namely fibrosis.



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An Inotropic S100A1-derived Peptide Enhances Contractile Performance and Survival of Post-ischemic Failing Hearts

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S100A1 gene therapy was suggested as therapeutic for cardiomyopathies since myocardial levels of S100A1 are decreased in heart failure. S100A1 increases systolic and diastolic performance of cardiomyocytes through enhanced Ca2+-induced SR Ca2+ release and augmented SR Ca2+ re-uptake. These effects are due to enhanced systolic Type 2 Ryanodine Receptor (RyR2) and diastolic Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase 2a (SERCA2a) activity. The S100A1 C-terminus (S100A1ct) was recently shown to be the bioactive domain of the protein. The aim of this study was therefore to characterize the effects of an S100A1ct-derived peptide on cardiomyocyte function and to test its therapeutic action in a mouse model of heart failure. An S100A1ct-derived peptide encompassing aa75-85 of the human S100A1 protein and preceded by a hydrophilic motif (DKDDPP) was named S100A1ct6/11. S100A1ct6/11 was cell permeable and accumulated in the intracellular space of intact rat ventricular cardiomyocytes in a striated pattern, similar to endogenous S100A1. S100A1ct6/11 exerted a time- and dose-dependent positive inotropic effect in electrical field stimulated rat ventricular cardiomyocytes. This effect was associated with the regulation of the SR calcium content. Peptides encompassing aa75-85 derived from S100 paralogs A4 and B did not mimic S100A1ct6/11-mediated inotropy. S100A1ct6/11 protected cardiomyocytes from pro-arrythmic store overload-induced calcium leak as well as from chronic caffeine exposure-induced apoptotic cell death. Mice intravenously injected with S100A1ct6/11 exhibited a significant enhancement of LV contractile performance under basal and βAR-stimulated conditions. Daily intraperitoneal administration of S100A1ct6/11 for two weeks to mice with post-ischemic contractile dysfunction resulted in significantly improved contractile performance and survival due to reduced myocardial apoptosis. Moreover, this treatment protected failing hearts with caffeine-induced leaky RyR2 channels from βAR-triggered lethal ventricular tachyarrhythmias. In conclusion, administration of the S100A1ct6/11 peptide may represent a novel option for the treatment of cardiomyopathies without apparent side effects.

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Regulation of Muscle Contractility by a Family of SERCA-Inhibitory Micropeptides

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Functional micropeptides can be concealed within RNA transcripts that have been putatively annotated as non-coding. We recently discovered a muscle-specific micropeptide, named myoregulin (MLN), that inhibits the activity of SERCA, the membrane pump that controls muscle relaxation by regulating Ca2+ uptake into the sarcoplasmic reticulum (SR). Genetic deletion of MLN in mice enhances Ca2+ handling in skeletal muscle and improves exercise performance. MLN shares structural and functional similarity with phospholamban (PLN) and sarcolipin (SLN), two well-studied micropeptides that regulate cardiac contractility and disease. Here we identify an additional member of this micropeptide family, named endoregulin (ELN), that specifically overlaps with the expression of SERCA3, the dominant Ca2+ ATPase in endothelial cells that controls the contractility of vascular and visceral smooth muscles. ELN encodes a single transmembrane alpha helix that localizes to the endoplasmic reticulum (ER), where it forms a stable complex with SERCA3. In cell based assays, ELN inhibits SERCA-dependent Ca2+ uptake into the ER and controls ER calcium levels. Due to the essential role of SERCA3 in regulating vascular smooth muscle contractility, ELN represents a potential regulator of vascular tone and novel therapeutic target for the treatment of cardiovascular disease.

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Conservation of Cardiac L-type Ca2+ Channels and Their Modulation in Drosophila: a Novel Genetically Pliable Channelopathic Model

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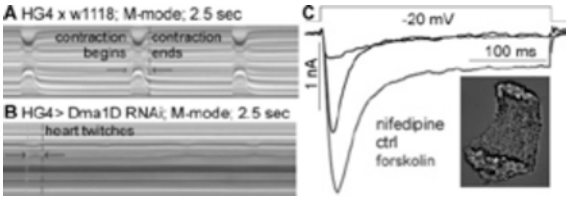
Misregulation of L-type Ca^{2+} channels (LTCCs) underlies numerous cardiac pathologies. For example, heart failure features blunted protein kinase A (PKA) upregulation of the LTCCs. However, studying LTCC regulation in mammalian systems is challenging due to complex physiology and lack of genetic manipulability while recombinant channels expressed in heterologous systems, which lack key auxiliary elements, poorly represent the native context. *Drosophila*, however, has a simple heart with many conserved genes and is genetically pliable. Thus, we pinpointed the signature of Ca^{2+} channels (CCs) in the fly cardiac tubes. First, RNAi-mediated selective knockdown of Dmca1D, homologous to the human LTCCs, abolished cardiac contraction (B, as compared to ctrl in A), establishing this channel isoform as the primary CC in fly hearts. Second, we successfully isolated viable single fly cardiomyocytes (C, inset) and recorded robust Ca^{2+} currents using patch clamp electrophysiology (C), a feat never before

accomplished for the fly cardiac system. Moreover, recording Ca^{2+} currents in distinct hypomorphic CC lines, we confirmed the CC type in the fly heart. We also observed pharmacological responses of the CCs that were strikingly similar to those seen in humans. Current through Dmca1D is blocked by a dihydropiridine, nifedipine (C), and is readily upregulated by forskolin, a downstream activator of the PKA pathway (C). In all, we have established the existence of a conserved compendium of cardiac CCs in fly heart tubes, suggesting that *Drosophila* may serve as a robust and effective platform to study the pathophysiology of diseases involving cardiac CCs and to devise therapeutic strategies.



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Reversal of Pathological Responses to Pressure Overload by Inhibition of Phospholipase Cbeta1b

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The immediate downstream effector of Galpahq is the early signaling enzyme phospholipase Cbeta1b (PLCbeta1b), which is selectively elevated in failing human myocardium. When delivered to the adult mouse heart, expression of PLCbeta1b causes rapid contractile dysfunction. PLCbeta1b targets to the sarcolemma for activation through an interaction with Shank3/Homer1C/TrpC4α using a specific C-terminal sequence of 32aa. The targeting/activity of PLCbeta1b can be inhibited by expression of a sarcolemmal targeted mini-gene composed of this 32aa sequence (lyn-FLAG-PLCbeta1b-CT, b-CT). rAAV6-b-CT, or blank virus, was delivered IV (1011vg/mouse) and trans-aortic-constriction (TAC) or sham-operation was performed 8 weeks later. TAC induced maximal hypertrophy by 8 weeks after TAC, followed by contractile dysfunction and lung congestion from 16 weeks. Expression of rAAV6-b-CT prior to TAC reduced the hypertrophic response and prevented the contractile dysfunction and lung congestion. Expressing a modified b-CT peptide that does not inhibit PLCbeta1b signaling had no effect on hypertrophy, contractility or lung congestion following TAC. We conclude that PLCbeta1b can be effectively targeted by preventing its binding to the sarcolemma and that this inhibition ameliorates pathological responses following acute pressure overload. The binding of PLCbeta1b to its sarcolemmal scaffold provides the basis for the development of a new class of inotropic agent.

rAAV6-construct	blank		lyn-FLAG-PLCbeta1b-CT (b-CT)	
	Sham	TAC	Sham	TAC
LV/tibia (mg/mm)	5.8±0.2	13.4±1.8*	6.9±0.4	10.9±0.1*†
FS%	41.2±1.4	16.9±3*	40.5±2.3	35.5±1.6†
Lung/tibia (mg/mm)	8.5±0.2	12.7*	9.3±0.6	9.2±0.2†

*=p<0.01 SO vs. TAC, †p<0.01 relative to blank virus

D.R. Grubb: None. **H. Kiriazis:** None. **X. Du:** None. **J. McMullen:** None. **E. Woodcock:** None.

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Cardiac Overexpression of Creatine Kinase Improves Cardiomyocytes Function in Heart Failure and During Increased Redox Stress

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AIMS. Several studies suggest that abnormal energy metabolism contributes to heart failure or that the failing heart is energy starved. Here we aim at testing whether an increase in intracellular CK improves myocellular contractility in experimental myocardial dysfunction and protects from increased oxidative conditions. METHODS-RESULTS. We tested the response to the β-agonist isoproterenol (2.5 nM, ISO) in field-stimulated (.5 Hz, RT) adult cardiomyocytes isolated from wild-type (WT) mice and mice overexpressing cardiac myofibrillar or mitochondrial CK (CK-M or CK-mito) from sham and failing (8 wk transverse aortic constriction (TAC)) hearts, to dissect whether overexpressing CK alters myocyte function at baseline and during increased energetic demand. There were no differences in sarcomere fractional shortening (FS) or Ca2+ transients at baseline and with ISO among sham WT, CK-M or CK-mito myocytes. However, ISO effects were significantly reduced in WT TAC myocytes, consistent with prior reports. Conversely, in CK-M or CK-mito TAC myocytes, ISO-induced inotropy was fully preserved. Interestingly, incubation with the AMPK-stimulator AICAR (1mM for at least 90') did not have any effect on WT TAC, but increased FS in TAC CK-M (+82%) and CK-mito (+42%) myocytes significantly, supporting the important metabolic role played by enhancing CK in failing hearts. To test whether overexpressing CK-M or CK-mito confer protection against acute oxidative stress, sham myocytes were exposed to H2O2 (50μM, 10') and the interval (seconds) between the beginning of H2O2 superfusion and the appearance of irreversible arrhythmias was measured. WT and CK-M myocytes had a similar response (416±91s vs 411±68s), whereas in CK-mito this interval was significantly prolonged (600±64s). Similarly, upon acute infusion of the anticancer TKI sunitinib (2μM), whose cardiotoxic properties have been linked also to an increase in ROS, irreversible arrhythmias appeared after 657±43s in CK-mito (p<.5 vs 561±66 for WT and 467±88 for CK-M). CONCLUSIONS. Overexpressing CK-M and CK-mito under failing-TAC conditions improves myocyte function likely through better preserved Ca2+ handling, whereas only the up-regulation of CK-mito is more effective in buffering ROS effects.

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ABSTRACTS

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DWORF: a Novel Cardiac Micropeptide That Enhances SERCA Activity and Cardiomyocyte Contractility

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Background: Our lab has discovered a novel conserved micropeptide of 34 amino acids encoded by a muscle-specific RNA that was previously annotated as a putative long non-coding RNA (lncRNA). This micropeptide, which we named DWORF (DWarf Open Reading Frame), shares structural similarity with the known SERCA modulators phospholamban (PLN), sarcolipin (SLN) and myoregulin (MLN).
Objective: To define the functional role of DWORF in the heart and elucidate its mechanism of action.
Methods and Results: Immunofluorescence staining and imaging in isolated adult mouse cardiac myocytes indicates that DWORF specifically localizes to sarcoplasmic reticulum (SR) membranes where it co-localizes with SERCA. Co-IP experiments performed in COS or HEK cells co-transfected with DWORF and SERCA show that DWORF forms a stable complex with various SERCA isoforms. Further biochemical analysis indicates that DWORF binds to the same site on SERCA as PLN, and overexpression of DWORF is capable of competing with PLN for SERCA binding. Co-expression of GFP-tagged DWORF and PLN in the presence of SERCA followed by SERCA pull-down and GFP Western indicates that PLN and DWORF have similar affinities for SERCA binding. Transgenic mice with cardiac specific over-expression of DWORF show enhanced cellular contractility as evidenced by increased peak Ca2+ transient amplitude and faster cytosolic Ca2+ decay rates (reduced Tau values). Furthermore, Ca2+-dependent Ca2+-uptake assays performed in homogenates from DWORF transgenic and littermate control hearts show a statistically significant leftward shift of the Ca2+-dependence curve for SERCA indicating a higher affinity of SERCA for Ca2+. Intriguingly, DWORF expression is silenced in the heart in response to pathological calcineurin signaling, implicating this micropeptide in the cellular pathway for calcineurin action.
Conclusions: We have identified a novel muscle-specific micropeptide, DWORF, which is expressed in the heart at high levels and has the capacity to bind SERCA and modulate its function to increase contractility in transgenic mice. Our findings provide exciting data in support of DWORF as a previously unrecognized endogenous SERCA activator that plays a role in cardiac contractility.

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Single Nucleotide Polymorphism of MLX Gene Plays a Crucial Role in the Pathogenesis of Takayasu Arteritis Through Facilitating Inflammasome Formation of the Aorta

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We have identified that single nucleotide polymorphisms (SNPs) of both *IL12B* gene which encodes IL-12p40 and *MLX* gene, which encodes a basic helix-loop-helix leucine zipper transcription factor, were significantly associated with clinical manifestations of Takayasu arteritis (TA), an autoimmune systemic arteritis, by genome-wide association study (GWAS). Recently, we reported that SNP of *IL12B* plays an important role in mediating the development of TA through modulating the level of IL-12p40-related cytokines, including IL-12 and IL-23. However, it remains unknown whether *MLX* mutation is associated with the pathogenesis of TA. Here, we elucidate that whether SNP of the *MLX* (rs665268), a missense mutation of MLX that alters the 139th glutamine to arginine (Q139R), upregulates the factor(s) associated with the development of TA. Pull-down assays with recombinant proteins demonstrated that mutation of Q139R on MLX protein enhanced the heterodimer formation of MLX with MondoA, a binding partner of MLX that promotes thioredoxin-interacting protein (TXNIP) expression. As Gln is a neutral amino acid, we speculated that alteration from Gln^{139} to Arg^{139}, which confers a positive charge to the DNA binding site of MLX, should enhance formation of the MLX-MondoA-DNA complex. Consistent with our hypothesis, luciferase reporter gene assays showed that the TXNIP promoter activity of human aortic smooth muscle cells (hASMCs) co-transfected with TXNIP promoter-containing reporter, MondoA and MLX-Q139R plasmids was significantly higher than those with the wild type MLX (MLX-WT) (MLX-Q139R: 63138 ± 2557 RLU* vs. MLX-WT: 31263 ± 3433 RLU, *p < 0.05). The protein levels of both NLRP3 and procaspase-1, major components of inflammasome upregulated by TXNIP, was significantly increased in hASMCs transfected with MLX-Q139R than compared to those in transfected with MLX-WT. Immunohistochemical analyses of the human aortas revealed that NLRP3 in the SMCs accumulated more prominently in the aortas of TA than those in normal ones.
In conclusion, Q139R mutation on MLX plays a crucial role in the pathogenesis of TA through facilitating NLRP3 accumulation, which in turn facilitating inflammasome formation in the aorta.

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The P2Y2 Nucleotide Receptor Regulates Vascular Calcification

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OBJECTIVE: Vascular calcification is widespread in individuals with atherosclerosis, and is associated with inflammatory changes and expression of osteoblast-like cell phenotypes. Recent studies identified extracellular nucleotides and P2Y receptor cascade as important regulators of bone remodeling. We investigated the potential role of the P2Y2 receptor (P2Y2R) in vascular calcification.
METHODS AND RESULTS: P2Y2R-null mice were crossed with ApoE-null mice to generate P2Y2R/ApoE double knock-out mice. When fed a standard mouse chow diet for 16 weeks, P2Y2R−/−/ApoE−/− mice showed significant higher intimal calcification as compared to their APOE−/− counterparts. Smooth muscle cells (SMCs) isolated from aortas of P2Y2R +/+ and P2Y2R -/- mice were identical in morphology and stained positively for SM lineage proteins including, desmin, smooth muscle and SM22alpha. When cultured in medium containing high concentrations of inorganic phosphate, an inducer of vascular calcification, a remarkably higher calcification was observed in P2Y2R -/- SMCs compared to P2Y2R +/+ SMCs. Furthermore, retroviral transduction of mouse P2Y2RcDNA into P2Y2R -/- SMCs rescued the calcification phenotype of the cells.
CONCLUSION: These results demonstrate that inactivation of the P2Y2R gene regulates vascular calcification both in vivo and in vitro, suggesting that

drugs targeting this receptor could prevent complications associated with vascular calcification
C. Seye: None. **M. Sheldon:** None. **S. Qian:** None.

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Lack of Lipid Phosphate Phosphatase 3 Promotes Cardiac Dysfunction and Early Mortality in Mice
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Lysophosphatidic acid (LPA) is a naturally occurring glycerophospholipid and has been reported to increase heart rate and left ventricular pressure in the heart *in vivo*. The primary route of circulating LPA production involves hydrolysis of lysophosphatidylcholine by the secreted enzyme autotaxin (ATX). Lipid phosphate phosphatase-3 (LPP3) is a plasma membrane enzyme that regulates the availability of LPA by dephosphorylation. We made the novel discovery that tissue-specific deficiency of *Ppap2b* (gene that encodes LPP3) leads to embryonic lethality (endothelial LPP3), exaggerates vascular inflammation, increases heart rate, enhances endothelial permeability, and promotes the development of the neointima after vascular injury. Based on our earlier reports, we have generated mice that specifically lack LPP3 in cardiomyocytes. These mice showed early mortality ~8 months due to cardiac dysfunction. Whereas lack of LPP1 or LPP2 (global knockouts) didn't had any obvious phenotypic effect. Lack of LPP3 accounts for less than 10 percent activity in cardiomyocytes purified from the Myh6-Ppap2b$^{\Delta}$, which augments our previous finding that the other two LPP isoforms have a lesser role in the cardiovascular system. Blood pressure was similar in Ppap2b$^{fl/fl}$ (96 ± 9 mmHg; n = 19) and Myh6-Ppap2b$^{\Delta}$ mice (92 ± 7 mmHg; n = 19), although heart rates were significantly higher in Myh6-Ppap2b$^{\Delta}$ 3-month old mice (642 ± 21 bpm, compared to Ppap2b$^{fl/fl}$ with 600± 17 bpm; P<0.001). Knockdown of LPP3 enhanced cardiomyocyte hypertrophy induced by LPA based on analysis of sarcomere organization, cell surface area, levels of fetal genes ANP and BNP, and ANF release from nuclei, which are hallmarks of cardiomyocyte hypertrophy, indicating that LPP3 negatively regulates cardiac dysfunction caused by LPA. We observed an increase in ATX levels accompanied by a decrease in LPP3 expression following infarction in the myocardium of Ppap2b$^{fl/fl}$ mice. Infarction induced expression of IL-6 and KC, were 3 ± 0.5-fold and 2 ± 0.6-fold higher, respectively, in Myh6-Ppap2b$^{\Delta}$ mice. Analysis of plasma by cytokine antibody array confirmed the elevation in IL-6 and KC, whereas G-CSF and sICAM-1 appeared lower than in Ppap2b$^{fl/fl}$.
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Bayesian Selection of Modifier Genes in Hypertrophic Cardiomyopathy Through Whole Genome Sequencing
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Background. Technological advances have greatly reduced the cost of whole genome sequencing. For single individuals clinical application is apparent, while exome sequencing in tens of thousands of people has allowed a more global view of genetic variation that can inform interpretation of specific variants in individuals. We hypothesized that genome sequencing of patients with monogenic cardiomyopathy would facilitate discovery of genetic modifiers of phenotype. Methods and Results. We identified 48 individuals diagnosed with cardiomyopathy and with putative mutations in MYH7, the gene encoding beta myosin heavy chain. We carried out whole genome sequencing and applied a newly developed analytical pipeline optimized for discovery of genes modifying severity of clinical presentation and outcomes. Using a combination of external priors and rare variant burden tests we scored genes as potential modifiers. There were 96 genes that reached a modifier score of 6 out of 12 or better (9=2, 8=8, 7=17, 6=69). We identified NCKAP1, a gene that regulates actin filament dynamics, and CAMSAP1, a calmodulin regulate gene that regulates microtubule dynamics, as top scoring modifiers of hypertrophic cardiomyopathy phenotypes (score=9) while LDB2, RYR2, FBN1 and ATP1A2 had modifier scores of 8. Of the top scoring genes, 21 out of 96 were identified as candidates a priori. Our candidate prioritization scheme identified the previously described modifiers of cardiomyopathy phenotype, FHOD3 and MYBPC3, as top scoring genes. We identified structural variants in 21 clinically sequenced cardiomyopathy associated genes, 13 of which were at less than 10% frequency. Copy number variants in ILK and CSRP3 were nominally associated with ejection fraction (p=0.03), while 8 genes showed copy gains (GLA, FKTN, SGCD, TTN, SOS1, ANKRD1, VCL and NEBL). Structural variants were found in CSRP3, MYL3 and TNNC1, all of which have been implicated as causative for HCM.
Conclusion. Evaluation of the whole genome sequence, even in the case of putatively monogenic disease, leads to important diagnostic and scientific insights not revealed by panel-based sequencing.
M. Wheeler: 7. Ownership Interest; Modest; Personalis. **D. Waggott:** None. **M. Grove:** None. **F. Dewey:** 1. Employment; Significant; Regeneron Pharmaceuticals. 7. Ownership Interest; Significant; Personalis. **C. Pan:** None. **A. Pavlovic:** None. **R. Goldfeder:** None. **M. Puckelwartz:** None. **S. Day:** None. **E. McNally:** None. **G.W. Dorn:** None. **E. Ashley:** 7. Ownership Interest; Significant; Personalis.

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Finding the Achilles' heel of Muscle Giant---TALEN-mediated Gene-editing in Zebrafish Titin
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Background: TITIN (TTN) has more than 300 exons and encodes a gigantic protein that is crucial for heart and muscle development. Mutations in TTN caused a variety of human diseases including cardiomyopathy and muscular dystrophy. Recently, dilated cardiomyopathy-associated mutations on TTN have been found more frequently in exons encoding A-band domains but less in exons encoding the N-terminal Z-disc domains, suggesting that mutations in different exons of TTN cause distinct consequences. To elucidate the underlying mechanisms, we leveraged the Transcription Activator-Like Effects Nuclease (TALEN) technology in zebrafish to introduce truncating mutations in different exons of ttn, and then study their effects on heart and somites.
Results: We generated truncational mutations in different exons of zebrafish titins encoding Z-disc, N2B, Novex-3, and A domains, respectively. Because zebrafish contains two titin homologues, ttna and ttnb, we introduced mutations in both genes at the corresponding loci. While both Z-disc and A band mutations on ttna disrupted sarcomere assembly in heart and somites, Z-disc or A band mutations on ttnb only affect somites without affecting the heart.

Interestingly, a Z-disc mutation on ttna resulted in milder phenotypes than an A-band mutation, while a Z-disc mutation on ttnb generated severer phenotypes than an A-band mutation. No phenotype was observed in the homozygous fish in either ttna-novex-3 or ttnb-N2B mutant fish.
Conclusions: A spectrum of truncational mutations in ttna and ttnb has been generated in zebrafish using the TALEN technology. Mutations in different exons result in different phenotypes. Detailed characterization of these mutants and double mutants will be presented, which shall elicit distinct contribution of alternative splicing and exon skipping as two candidate mechanisms during pathogenesis of Titinopathies.
Y. Shih: None. **X. Xu:** None.

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Dramatic ATP Depletion Without Concomitant Declines in ATP Synthesis Rates Suggests Impaired Energy Metabolism Mechanisms in Adrenergic-Deficient Mouse Embryos
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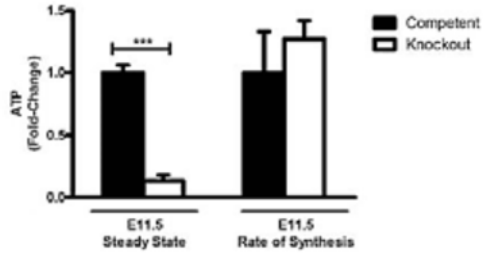
High cardiac energy demands increase during embryonic development, requiring oxidative phosphorylation converting ADP to ATP in mitochondria to meet these demands. We have recently shown that adrenergic hormones are required to maintain sufficient cardiac energy metabolism during embryonic development, but the specific mechanism(s) underlying this regulation are not known. Mouse embryos lacking adrenergic hormones, norepinephrine (NE) and epinephrine (EPI), due to targeted loss of the essential *dopamine β-hydroxylase* (*Dbh*) gene, have remarkably decreased steady-state ATP/ADP ratios. To determine if ATP synthesis was affected, we examined the rate of ATP formation in adrenergic-deficient and control embryonic hearts. Our rate data have shown that despite > 50-fold decrease of steady-state ATP concentrations in *Dbh*$^{-/-}$ embryos, the rate of ATP synthesis was not significantly different in adrenergic-competent and deficient embryos. This indicates that respiratory complexes in mitochondria are capable of producing ATP in adrenergic-deficient embryos, and suggest that ATP is either consumed at a faster rate than it is produced or its production is limited *in vivo* due to limited access to metabolic substrates ("starvation"). These findings reveal new mechanistic insights about how adrenergic hormones regulate energy metabolism during embryonic development.



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A Novel Interaction Between E2F and β-adrenergic Pathways Regulates Survival and Growth in Murine Myocardium
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The E2F/Pocket protein (Rb) pathway regulates cell growth, differentiation, and death by modulating gene expression. We previously examined this pathway in myocardium via manipulation of E2F6, which represses gene activity independently of Rb. Mice with cardiac specific expression of E2F6 develop dilated cardiomyopathy (DCM) without any signs of hypertrophic growth. We assessed the mechanisms of the apparent failure of compensatory growth as well as their response to the β-adrenergic agonist isoproterenol (iso). E2F6 transgenic (Tg) mice present with left ventricle dilation and significantly reduced ejection fraction as early as 2 weeks which persists into adulthood, but with no apparent increase in left ventricle weight: body weight (LVW:BW). E2F6-Tg mice treated with iso show double the increase in LVW: BW than their Wt counterparts (32% vs 16%, p-value: 0.007). Western blot revealed a specific activation of the β2-adrenergic pathway in Tg myocardium under basal conditions including a ~2-fold increase in β2-adrenergic receptors (β2-AR) (p-value: 8.9E-05), protein kinase A catalytic subunit (PKA-C) (p-value: 0.0176), activated c-SRC tyrosine-protein kinase (p-value: 0.0002), and an induction of the anti-apoptotic gene Bcl2. In contrast, a ~70% decrease in the cardiac growth regulator: AKT1 (p-value 0.0001) and a 4-fold increase in cyclic AMP dependent phosphodiesterase 4D (PDE4D), the negative regulator of PKA activity, was evident in Tg myocardium. The expression of E2F3 was de-regulated by E2F6, but was restored by iso while Rb expression was down-regulated. Thus deregulation of E2F/Rb pathway by E2F6 altered the β-adrenergic signaling pathway such that survival signaling was activated while hypertrophy was repressed resulting in the development of DCM without any increase in muscle mass. These data reveal a novel interplay between E2F and the β adrenergic pathway which regulate cardiac growth and fate.
J.L. Major: None. **M. Salih:** None. **B.S. Tuana:** None.

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Identification and Characterization of a miRNA Cohort Initiated Transitional Program That Controls Cell Cycle Arrest of the Perinatal Heart
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During fetal and early perinatal development the myocardium undergoes a period of hyperplastic growth, which results in an exponential increase in the number of cardiomyocytes (CM) that will constitute the adult heart. Soon after birth, CMs proceed through a final round of cell division in the absence cytokinesis that results in binucleation of >95% of adult CMs. Fetal heart genes are re-activated with the onset of pathological hypertrophic or dilated cardiomyopathies, yet there is no evidence of CM re-entry into the cell cycle. Despite the importance of this phenomenon, little is known about the molecular basis for the transition from hyperplastic to hypertrophic-based myocardial growth.
Hypothesis: A perinatal heart gene program is necessary for the normal transition from a fetal heart gene program to an adult heart gene program.
To identify the molecular mechanisms and pathways involved in CM differentiation during the perinatal transition, RNA was isolated from E18, and 1, 3, 5, 7, 10 and 35d old mouse hearts. CM gene expression and micro-RNA profiles (n=3 arrays/time point) were determined by oligonucleotide array analysis. The raw array data was normalized by Robust Multi-array analysis. Empirical Bayes estimation of gene-specific variances was performed between each of the time points in order to identify genes that are transiently and significantly changed at days 3 and 5 as compare to E18 and 10d post-birth. The analysis identified 2,799 genes (E18 v 5d) and 3,347 genes (5d v 10d) that were then clustered to determine significant pathway enrichment ($p<0.05$) with Ingenuity Pathway Analysis.
Our analysis confirmed previous observations of a down regulation of glucose oxidative metabolism (p=0.02) with an up-regulation of fatty acid metabolism (p=0.0001) between E18 and 5d post-birth. Also, 63

cell cycle genes are collectively down regulated (p=4.3x10-4) between 5d and 10d post-birth. We identified 131 genes that are transiently up regulated at 5d compared to E18 and 10d and this transition was proceeded by a specific cohort of miRNAs. The data generated from this study provide new insight into the molecular mechanisms by which CMs regulate and permanently exit from the cell cycle.
P.G. Burgon: None. **J.J. Weldrick:** None.

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p38γ and δ Promote Heart Hypertrophy by Targeting the Mtor-inhibitory Protein Deptor for Degradation
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Disrupted organ growth underlies the development of several diseases. Whereas the fetal heart grows mainly through proliferation, the postnatal heart grows through hypertrophy. Hypertrophy is also activated in the adult heart as a compensatory response to stress, but chronic ventricular hypertrophy leads to heart failure. The molecular mechanisms underlying ventricular hypertrophy are unknown. We show that cardiac expression and activation of p38γ and p38δ increases during postnatal development and these kinases are activated by the hypertrophy-inducing stress stimuli angiotensin II and phenylephrine. p38γ and p38δ promote cardiac hypertrophy by phosphorylating the mTORC1 and mTORC2 inhibitor DEPTOR, promoting its degradation and thereby activating mTORC1 and mTORC2. Hearts from mice lacking one or both kinases were below-normal size, had high levels of DEPTOR and consequently low activity of the mTOR pathway, and reduced protein synthesis. The mTOR inhibitor, rapamycin, equalized heart weight in wild-type and p38γ/δ-/- mice. Moreover, p38γ/δ-/- mice were protected against pathological ventricular hypertrophy, establishing p38γ/δ as key regulators of this process.

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Atorvastatin Attenuates Pressure Overload-induced Cardiac Hypertrophy and Dysfunction Through Enhanced Autophagy
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Aims: Cardiac hypertrophy is a maladaptive change in response to pressure overload and is also an important risk for developing heart failure. We previously demonstrated that atorvastatin inhibits cardiac hypertrophy and remodeling in a mouse model of transverse aorta constriction (TAC). This study was designed to determine the regulation of atorvastatin on cardiac autophagy and its association with the development of cardiac hypertrophy and dysfunction in the mice TAC model.
Methods and results: TAC or sham operations were performed in male C57/L6 mice at 8 weeks of age. Atorvastatin (50 mg/kg/day) or vehicle (normal saline) were administered daily by oral gavage to TAC mice (n=10 per group). Echocardiography and real-time PCR data showed that chronic atorvastatin treatment for four weeks significantly attenuated pressure overload-induced cardiac hypertrophy and dysfunction, as well as cardiac mRNA level of atrial natriuretic factor (ANF), a biomarker of cardiac hypertrophy and heart failure. After 4 weeks of TAC, results from electron microscopy and Western blot showed that cardiac autophagy was activated, evidenced by the increased expression of microtubule-associated protein-1 light chain 3-II (LC3-II), Beclin-1, caspase-3, and the formation of autophagosomes. Interestingly, cardiac autophagy was further increased by the treatment of atorvastatin for 4 weeks. Western blot analysis showed phosphorylated Akt and mammalian target of rapamycin (p-mTor) decreased in the heart of TAC versus sham mice, which were further decreased by atorvastatin treatment.
Conclusions: These findings suggest that atorvastatin attenuates cardiac hypertrophy and dysfunction in TAC mice probably through its regulation on cardiac autophagy via Akt/mTor pathways.
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Late-life Activation of GPR30 by G1 Reverses Diastolic Dysfunction in Senescent Fischer344 x Brown Norway Female Rats
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Introduction: The aged heart is characterized by impaired lusitropy and increased stiffness, and no proven pharmacologic interventions exist that limit these manifestations of left ventricular diastolic dysfunction (LVDD). G1, a selective agonist to the estrogen receptor GPR30, attenuated the effects of estrogen (E2) loss on LVDD in a hypertensive model. To extend prior studies, we determined the cardioprotective potential of chronic GPR30 activation in the female Fischer344 x Brown Norway (F344BN) rat, a normotensive aging model.
Methods and Results: Bilateral oophorectomy (OVX) or sham-operations were performed in middle- (Mid) (18 months) and Old-aged rats (27 months). At 28 months of age, Old rats were randomized to daily G1 (100 μg/kg/day, s.c.) or vehicle (VEH; soybean oil) injections (n=7 rats/group). Following 8 weeks of treatment, left ventricle (LV) mass was greater in Old vs. Mid-aged rats, independent of E2 or G1, while systolic function and LV wall thicknesses were similar among groups. Myocardial relaxation (e') was reduced (P<0.001) and LV filling pressure (E/e') was increased (P<0.01) in Old vs. Mid-aged rats, and this was exacerbated by OVX (P<0.01) and reversed by late-life G1 treatment. Systolic blood pressure (SBP) increased by 20% after mid-life OVX, whereas late-life E2 loss had no effect on SBP, irrespective of G1. Cardiac chymase expression was increased in E2 deficient hearts determined by Western blot and G1 modestly limited this E2/age effect.
Conclusion: Our data indicate that late-life G1 treatment reverses the detrimental effects of

advanced age and E2 deficiency on diastolic function, possibly via interactions with the noncanonical cardiac renin angiotensin system.
A. Alencar: None. **H. Wang:** None. **J. de Silva:** None. **M. Lin:** None. **X. Sun:** None. **L. Groban:** 2. Research Grant; Significant; NIH AG-042758 (LG), NIH AG-033727 (LG).

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Dual Specificity Phosphatase 8 (dusp8) Regulates Cardiac Remodeling and Function by Affecting Erk1/2 Phosphorylation
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Rationale: Mitogen-activated protein kinases (MAPKs) are activated in the heart by various stress-inducing stimuli, where they participate in cardiac hypertrophy, contractility, and cell death. A family of dual-specificity phosphatases (DUSPs) directly inactivates each of the MAPK terminal effectors, thus providing a feedback mechanism to regulate the activity and recycling of MAPKs. How DUSPs regulate MAPK signaling to influence cardiac function is not well understood. **Objective**: To determine the role of DUSP8 in regulating MAPK signaling and the effect on cardiac disease. **Methods and Results**: We generated DUSP8 null (KO) and inducible cardiac specific DUSP8 transgenic mice to assess the effect on cardiac structure-function at baseline and with stress-responsiveness. Loss of DUSP8 did not alter cardiac structure-function or MAPK phosphorylation at baseline. However, with pressure overload or myocardial infarction injury, DUSP8 KO mice developed concentric hypertrophy with preserved cardiac function compared to wild type controls, suggesting a cardioprotective role for loss of DUSP8. DUSP8 transgenic mice developed cardiac hypertrophy at baseline as evidenced by elevated expression of hypertrophic marker genes as well as increased heart weights and cross sectional area of the myocytes. DUSP8 transgenic mice also had mild interstitial fibrosis and reduced fractional shortening. Biochemical analysis of MAPK phosphorylation demonstrated increased ERK phosphorylation in KO mice upon stress, suggesting a molecular mechanism underlying the increased concentric growth of DUSP8 KO cardiomyocytes. **Conclusions:** Taken together, these data demonstrate that DUSP8 modulates ERK phosphorylation to influence cardiomyocyte growth and consequent cardiac function with injury. Further analysis of MAPK phosphorylation in DUSP8 transgenic mice will provide more insight into DUSP8-ERK signaling in the heart.
R. Liu: None. **J. Molkentin:** None.

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Mast Cell Inhibition Attenuates Cardiac Remodeling and Diastolic Dysfunction in Ovariectomized Female Fischer344×Brown Norway Rats
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Background: The incidence of left ventricular hypertrophy (LVH) and diastolic dysfunction (LVDD) increases in postmenopausal women, but no proven pharmacologic interventions exist. Mast cells have an important role in the pathological processes of a variety of cardiac diseases. We determined the cardioprotective effects of chronic mast cell inhibition by the mast cell stabilizer, cromolyn sodium, in middle-aged, female Fischer344×Brown Norway (F344BN) rats after estrogen (E2) loss.
Methods and Results: Bilateral ovariectomy (OVX) or sham-operations were performed in 18-month-old female F344BN rats. Four weeks after surgery, cromolyn (30 mg/kg/day), or vehicle (saline) was administered s.c. by osmotic minipump (n=7/group). Four weeks after treatment, systolic blood pressure (SBP) increased by 20% in OVX vs. sham rats, and cromolyn attenuated this effect. Myocardial relaxation (e') was reduced (P<0.01), left ventricle (LV) filling pressures (E/e') were increased (P<0.01), and LV mass, wall thicknesses, and percent interstitial fibrosis were increased in OVX vs. sham rats. All of these adverse effects of E2 loss on LV function and structure were mitigated by cromolyn treatment. While no differences in plasma angiotensin (Ang) II levels were observed between OVX and sham, Ang II levels were reduced in cromolyn-treated OVX rats (P<0.05). Cardiac chymase protein expression was increased after E2 loss, irrespective of cromolyn. Yet, treatment with cromolyn prevented the increase in cardiac Ang II type 1 receptor (AT1aR) mRNA expression with OVX. Conclusions: Mast cell inhibition with cromolyn attenuates adverse LV remodeling and LVDD in OVX-F344BN rats possibly through a chymase/Ang II-mediated mechanism. Cardiac mast cells could be a potential therapeutic target for cardiovascular diseases in postmenopausal women.
H. Wang: None. **J. da Silva:** None. **A. Alencar:** None. **M. Lin:** None. **X. Sun:** None. **L. Groban:** 2. Research Grant; Significant; NIH AG-042758 (LG), NIH AG-033727 (LG).

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Sirt1 Negatively Regulates Cardiac Inflammation Induced by Metabolic Stress
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Obesity is a major risk factor for heart disease. Metaflammation is a metabolically driven, chronic, low-grade inflammatory reaction that is seen in the heart under obese conditions. Cardiac myocytes are known to produce inflammatory cytokines such as interleukin-6 (IL-6). However, whether and how cardiac myocytes mediate metaflammation is unknown. Silent information regulator 1 (Sirt1), a histone deacetylase involved in numerous metabolic pathways, facilitates an anti-inflammatory function through inhibition and deacetylation of the NFkB p65 subunit. This project aims to examine whether Sirt1 plays a role in cardiac local metaflammation. Mice with cardiac specific Sirt1 gene deletion (c-Sirt1 KO), Sirt1 overexpression (Tg-Sirt1), and relevant controls (Flox/Flox and WT respectively) were each placed on high fat and regular chow diets. The mice were euthanized after 3 months and their hearts were extracted for genetic and molecular analyses. Sirt1 mRNA and protein levels were significantly decreased under HFD feeding conditions (50% reduction in HFD feeding compared to normal diet, P<0.05). Acetylation of p65 was significantly induced by HFD feeding, and was further enhanced in c-Sirt1 KO mice, but inhibited in Tg-Sirt1 mice. Cardiac inflammation was enhanced in the c-Sirt1 KO group as evidenced by a significant increase in mRNA levels of IL-6 in mice fed regular chow (3-fold increase in c-Sirt1 KO vs. Sirt1 Flox/Flox, p<0.05) and HFD (5-fold increase in c-Sirt1 KO vs. Sirt1 Flox/Flox, p<0.05). Intracellular IL-6 signaling steps, such as phosphorylation of STAT3, were enhanced in c-Sirt1 KO, but inhibited in Tg-Sirt1 mice under HFD feeding conditions. These results suggest that cardiac myocytes mediate metaflammation partly through downregulation of Sirt1.
K. Schesing: None. **J. Sadoshima:** None. **S. Oka:** None.

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Exercise Prevents Doxorubicin-induced Cardiotoxicity via Targeting Microrna-669a-5p in Mice
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Doxorubicin (Dox)-induced cardiotoxicity, usually associated with increased oxidative stress, myofibrillar deterioration, and impaired cardiac contractile function, is a serious complication of antitumor therapy which may not be detected for many years. Growing evidence indicates that the regulation of cardiac microRNA (miRNA, miR) in response to exercise is essentially involved in the protective effect of exercise in the treatment of cardiovascular diseases. However, it is largely unknown whether and how exercise could prevent Dox-induced cardiotoxicity via regulating miRNA biology. In the current study, C57BL/6 mice were either subjected to a 3-week swimming program or remained sedentary. Mice were then treated with Dox (ip. 4 mg/kg/week for 4 weeks) to induce cardiotoxicity. Our data demonstrated that Dox resulted in marked reduction of cardiac ejection fraction (EF, %) and fractional shortening (FS, %) as measured by echocardiography. Interestingly, exercise significantly improved cardiac EF (%) and FS (%) in Dox-treated mice, indicating the protective effect of exercise in Dox-induced cardiotoxicity. Then, we performed microarray analysis (Affymetrix 3.0) showing that miR-27a-5p, miR-34b-3p, miR-185-3p, miR-203-3p, miR-669a-5p, miR-872-3p, and let-7i-3p were significantly reduced, while miR-2137 was increased in the hearts of exercised Dox-treated mice versus sedentary Dox-treated mice (FC(abs)>1.5, $p<0.05$). Using qRT-PCR, we further verified that miR-669a-5p was reduced by exercise training in Dox-treated mice. These data reveal that miR-669a-5p might be a potential miRNA mimicking the benefit of exercise in Dox-induced cardiotoxicity. Further study is needed to clarify the functional effect of miR-669a-5p and to identify its downstream target gene that contributes to the prevention and treatment of Dox-induced cardiotoxicity.

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Angiotensin II as a Paracrine Factor in Heart Environment

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Besides its potent vasoconstrictor action, the Angiotensin II (Ang II) has other important biological roles as autocrine and paracrine actions, being fundamental in cardiovascular homeostasis. Changes in the levels of this substance in the pericardial fluid (PF) are associated with the development of cardiovascular diseases. The aim of this study was to evaluate the presence of members of the classical and alternative pathways of the Renin Angiotensin System (RAS), as well as one of its main effectors peptides, Ang II, in PF of patients with coronary artery disease and assess the role of Ang II as a factor able to promote the process of epithelial-mesenchymal transition epicardial mesothelial cells in genetically modified mice. Male and female patients with coronary artery disease and controls patients were involved in this study. The ELISA's results showed significant amounts of Ang II, Angiotensin Converting Enzyme (ACE) and chymase in PF coronary artery disease male patients. Chymase positive mast cells were identified by immunohistochemical and quantitative analysis demonstrated a greater density of mast cells in this group. Expression of AT1 and AT2 was detected by immunohistochemistry in patients of male and female groups with coronary artery disease and controls. The scaling of the parietal mesothelium is a process seen in all groups, although when quantified showed no significant differences between them. Ang II was not able to increase the epithelial-mesenchymal transition of the epicardial cells in infarcted mice genetically modified. The presence of constituents of the classical and alternative pathways of RAS in the PF suggest an important role for this fluid as a modulator of cardiac functions. Specifically in relation to Ang II, their biological roles in the cardiovascular system still need to be further investigated. The identification in the cardiovascular microenvironment of paracrine factors and your action mechanisms, which stimulate and promote cardiac repair, may allow a better understanding of heart disease and the development of new therapies.

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Manganese Superoxide Dismutase: a Novel Mediator of Heart Failure Development and Progression

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Manganese Superoxide Dismutase (MnSOD), an antioxidant enzyme that catalyzes the conversion of superoxide radicals (O_2•-) in mitochondria. Constitutive activation mitochondrial reactive oxygen species (ROS) has been implicated in both the pathogenesis and the progression of cardiovascular disease. Absence of *SOD2* (gene that encodes MnSOD) is found to be embryonic lethal in animal models due to impairment of mitochondrial function, most noticeably in the heart. In our earlier investigation, we have shown that the MnSOD mimetic, MnTnBuOE-2-PyP^{5+} distributes 3-fold more in mitochondria than in cytosol. The exceptional ability of MnTnBuOE-2-PyP^{5+} to dismute O_2•- parallels its ability to reduce ONOO– and CO3–. Based on our earlier reports, we have generated mice that specifically lack MnSOD in cardiomyocytes (Mhy6-SOD2$^{\Delta}$). These mice showed early mortality ~4 months due to cardiac mitochondrial dysfunction. Oxidative phosphorylation (OXPHOS) in mitochondria is the predominant mode for O_2 consumption in cells, and the mitochondria are the primary source of ROS in cells due to leaked electrons. FACS analyses using Mito-Tracker Green indicated that the mass of mitochondria per cell was slightly decreased in the Mhy6-SOD2$^{\Delta}$ to the wild type. We then examined OXPHOS levels in Mhy6-SOD2$^{\Delta}$ v.s. wild type using a Seahorse XF analyzer. The rate of oxygen consumption per cells was significantly lower in Mhy6-SOD2$^{\Delta}$ cardiomyocytes than that in wild type. The most noticeable difference in the O_2 consumption was found in the presence of FCCP (H+ ionophore / uncoupler). FCCP is an inner membrane pore opener which resets the proton gradient between the mitochondrial matrix and the interspace, resulting in continuous transport of protons and consuming O_2 at the maximum potential. Remarkably, while the FCCP treatment increased O_2 consumption in wild type, the treatment showed no effect on the O_2 consumption in the Mhy6-SOD2$^{\Delta}$ cardiomyocytes. The result indicated that the low basal OXPHOS activity in Mhy6-SOD2$^{\Delta}$ was due to unusually low OXPHOS potential. We examined glycolysis in these cells by measuring extracellular acidification (ECAR) and the pattern exactly opposite to that of oxygen consumption rate (OCR) was observed for glycolysis rates between Mhy6-SOD2$^{\Delta}$ and wild type.

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Pacemaker Induced Transient Asynchrony (PITA) Restores Contractile Reserve in Synchronous Heart Failure

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Heart failure (HF) with dyssynchrony treated with biventricular pacing (CRT) displays enhanced global and cellular function even compared to always synchronous HF. This suggests while HF is worsened by sustained dyssynchrony, it may paradoxically be improved by brief periods of Pacemaker Induced Transient Asynchrony (PITA). We tested this hypothesis in dogs tachypaced for 6 wks to induce HF. The HF group received atrial pacing and was compared to PITA (atrial pacing during day; right ventricular pacing, producing dyssynchrony, from 0000-0600). PITA blunted dilation (end diastolic and end systolic volumes reduced by 11 and 19%, respectively), reduced end-diastolic pressures from 22 to 13 mmHg, and improved the contractile response to dobutamine by 29%. Myocyte sarcomere shortening and calcium transient amplitude were depressed in HF and little improved by β adrenergic (βA) stimulation. PITA improved baseline function slightly, but virtually restored βA stimulated reserve. Membrane βA receptor density increased with PITA by 36% as well. Another contributor to the change in functional reserve was found in myofilament maximal calcium activated force (Fmax) normalized to cell cross sectional area (CSA). This declined ~40% in HF vs. Control, but was fully restored by PITA. However, as CSA was greater in HF and normalized by PITA, raw Fmax was similar despite hypertrophy in HF, suggesting HF myocytes had dysfunctional myofilaments, which PITA prevented. Electron microscopy confirmed normal myofilament structure in Control and PITA, whereas 40% of HF sarcomeres displayed deteriorated z-disks and loss of normal registration of the thick and thin filaments. In HF, 39% of HF isolated myofibrils produced virtually zero maximal force, whereas Control and PITA fibers functioned normally. Thus, there are two populations of myofibrils within HF hypertrophied cells, with ~40% structurally and functionally disrupted. PITA reverses this to restore force-calcium activation and with improved βA receptor signaling, restores functional reserve, suppressing chronic maladaptive remodeling. This surprising finding indicates PITA can ameliorate HF pathobiology and improve reserve function. Further studies are needed to test if such benefits translate to humans.

J.A. Kirk: None. **K. Chakir:** None. **K. Lee:** None. **G. Pironti:** None. **M.J. Ranek:** None. **R.S. Tunin:** None. **P. de Tombe:** None. **S.K. Shenoy:** None. **H.A. Rockman:** None. **R. Craig:** None. **D.A. Kass:** None.

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Tripartite Motif Protein 58 (trim58) is a Novel Regulator of Membrane Repair With Increased Expression in Failing Cardiac Muscle

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In recent years, members of the tripartite motif-containing (TRIM) family of E3 ubiquitin ligases have been shown to be both positively and negatively regulated in various disease pathologies. TRIM72 (MG53) has been directly linked with regulation of sarcolemmal membrane repair in striated muscle cells, including cardiomyocytes. Recently, we were first to identify that a novel tripartite motif family member, TRIM58, is a negative regulator of the cell membrane repair process in striated muscle cells. Overexpression of TRIM58 decreases the membrane repair capacity of cultured myoblasts as measured by dye occlusion following laser-mediated disruption of the sarcolemmal membrane. We also find that TRIM58 can directly interact with TRIM72/MG53 and alter signaling through phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). Protein profiling experiments show that TRIM58 expression increases during the development of heart failure suggesting that TRIM58 may be relevant in the development of cardiac failure. Western blot and histological examination in both human (Fig.1) and mouse transverse aortic constriction (TAC) heart failure samples clearly show an increased expression of TRIM58 in cardiac tissue. Our results suggest that TRIM58 levels might serve as a potential prognostic marker and that TRIM58 may be a therapeutic target for the management of cardiovascular disease.

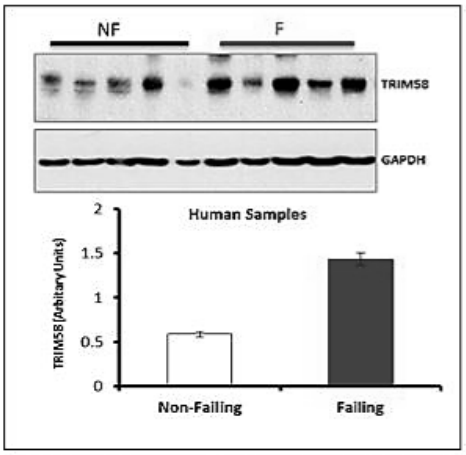

Fig. 1. **Western blot analysis showing increased expression of TRIM58 in failing human hearts.** Western blot analysis of TRIM58 in human non-failing (NF) hearts vs failing (F) hearts to determine the level of expression. (n=5 from each group).

K. Krishnamurthy: None. **J. Alloush:** None. **Z. Xu:** None. **E.X. Beck:** None. **P.J. Mohler:** None. **P. Janssen:** None. **N. Weisleder:** None.

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Differential Analysis of Thrombospondin Proteins in Cardiac Disease

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Thrombospondin (Thbs) proteins are multidomain, matricellular proteins comprised of 5 genes that each share similar domains and have been largely ascribed the same functional characteristics The Thbs protein family is divided in 2 subgroups based on multimerization as trimers, (group A: Thbs1 and 2), or as pentamers (group B: Thbs3, 4 and 5). Thbs proteins modulate aspects of cell- matrix interactions, and more

recently we have shown that they can also serve an intracellular chaperone function along the secretory pathway in response to ER stress. Thbs1 and 2 can also alter angiogenesis and modulate MMP activity as well as TGFb activity. The overall aim of this study is to functionally compare the two Thbs subgroups and elucidate their involvement in cardiac homeostasis and disease. Hence we analyzed Thbs1 as a representative of group A and Thbs3 for group B. Cardiomyocyte specific overexpression of either Thbs1 or Thbs3 results in robust activation of an ATF6- dependent ER stress response, similar to our previous working published with Thbs4. However, Thbs1 overexpressing mice suffer from cardiomyopathy and die within 12 weeks. In contrast, Thbs3 overexpressing mice are completely normal at baseline and Thbs3 overexpression even increased survival rates after MI similar to Thbs4 from our previous work. However, Thbs3 hearts also showed increased chamber dilation after MI and lower heart function suggesting that Thbs3 also has maladaptive effects. In contrast to loss of Thbs4, which die within the first week of TAC mediated pressure overload, Thbs3 KO mice are protected from TAC induced hypertrophy. Moreover, the combination of Thbs3/4 dKO more closely phenocopies the effect of Thbs3 deletion, as these mice show a significant reduction in hypertrophy and lung congestion after long term TAC. This is the first study comparing the function of different Thbs isoforms in the heart, which suggests a novel role of Thbs3.

T.G. Schips: None. **N.R. Correll:** None. **M.A. Sargent:** None. **J.D. Molkentin:** None.

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Loss of Cardiac Retinoic Acid Receptor Alpha Promotes Diastolic Heart Failure With Preserved Ejection Fraction

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Objectives:We have previously demonstrated that the expression/activation of retinoic acid receptor (RAR) is inhibited in diabetic hearts, and that activation of RARα prevents diabetes-induced diastolic heart failure, suggesting that impairment of RARα signaling may be a critical mechanism in heart failure.
Methods and Results:Cardiac RARα gene deletion (KO) was achieved by tamoxifen injection at 6-weeks old RARαfl/fl α-MHC-MerCreMer mice, RARαfl/fl mice were used as control (WT). Heart function was monitored by echocardiograph for 64 wks. Mice were sacrificed at 20 or 64 wks post gene deletion, respectively. A significant decrease in E/A ratio and TDI E' and increase in IVRT (isovolumic relaxation time) and DT (deceleration time) suggested diastolic dysfunction after 16 wks of gene deletion in KO mice, which was confirmed by cardiac catheterization (decreased dP/dtmin and increased tau). Concentric hypertrophy developed in KO mice after 56 wks of gene deletion, as confirmed by increased thickness of left ventricular wall and interventricular septum and elevated heart weight/tibia length ratio. However, no significant difference was observed in LVEF (LV ejection fraction), FS (fraction shortening) and dP/dtmax between KO and WT mice. Significantly increased gene expression of NOX2 (NADPH oxidase 2) and NOX4, decreased SOD1 and SOD2 levels and increased intracellular reactive oxygen species (ROS) were observed in KO mouse hearts, along with a significantly decreased protein expression of SERCA2a and CaMKIIδ, decreased phosphorylation of PLB, Akt and CaMKIIδ. Overexpression of RARα in cardiomyocytes rescued RARα deletion-induced changes in SERCA2a, PLB, Akt and CaMKIIδ. Deletion of RARα in cardiomyocytes impaired intracellular calcium reuptake into SR and cardiomyocyte relaxation. Inhibition of ROS by N-acetyl cysteine abolished RARα deletion-induced calcium mishandling and cardiomyocyte relaxation.
Conclusion: Cardiac specific deletion of RARα induces diastolic heart failure with preserved ejection fraction (HFpEF), by promoting intracellular ROS and disrupting SERCA2a-mediated calcium reuptake and cardiomyocyte relaxation. Our study suggests that deficiency in RARα signaling is a novel mechanism leading to HFpEF.

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Cardioprotective Effects of Adiponectin in Volume Overload Induced Electrophysiological Remodeling

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The electrophysiological hallmark of the failing heart is the prolongation of action potential duration that induces arrhythmia and sudden death. Depressed outward potassium currents (Ito) has been implicated as the major cause of altered action potential during ventricular remodeling. The molecular mechanism underlying depressed Ito in the diseased heart is still poorly understood. Recent studies have demonstrated that adiponectin (APN) has a cardio-protective effect in response to various pathological stimuli; however, little information is available regarding the potential effects of APN on electrophysiological remodeling under pathological conditions. The present study were to determine the effect of adiponectin treatment on ventricular potassium channel function in a rat model of volume overload induced heart failure. Volume-overload was induced by surgical creation of an infrarenal aorta-vena cava fistula. Rats were administrated with or without adenovirus-mediated overexpression of adiponectin (Ad-APN) at 2-, 5- and 8- weeks post-fistula. In vivo ECGs were used to evaluate changes in QT interval in rats at 10 weeks post-fistula. Ventricular myocytes were isolated at 10 weeks post-fistula. Western blots were used to measure cytoplasmic and membrane protein expression of potassium channels Kv4.2 and Kv 4.3, as well as, KChIP2. Whole cell patch clamp was used to evaluate action potential and Ito currents. Results showed that adiponectin levels in serum and myocytes were significantly reduced following fistula. The duration of action potential was prolonged in ventricular myocytes following 10-week fistula, which was correlated with the in vivo QT interval prolongation, as well as a depression of functional Ito and decreased protein expression of Ito channel subunits in ventricular myocytes. In vivo supplementation of Ad-APN increased the protein levels of Ito channel subunits and prevented Ito depression in ventricular myocytes following 10-week fistula. This further restored the duration of action potential and the QT interval on the ECG back to the normal. These results indicate that adiponectin was able to prevent volume overload-induced ventricular electrophysiological remodeling.

J. Zhong: None. **L. Wang:** None. **D. Miller:** None. **D. Schwartz:** None. **R. Amin:** None. **R. Judd:** None.

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Recovery of Exercise Intolerance and Ventricular Dysfunction of Infarcted Spontaneously Hypertensive Rats After Oral Treatment with an Agonist of Adenosine Receptor

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Severe ventricular dysfunction is observed in spontaneously hypertensive rats (SHR) submitted to myocardial infarction (MI). The present work evaluates the cardioprotective effects of oral administration of a novel agonist of adenosine A2A receptor, LASSBio-294, in infarcted SHR. Methods: Male SHR (12-14 wks old) were randomly divided into groups: sham and infarcted (MI) which were either treated orally with vehicle or LASSBio-294 (10 mg/kg) for 28 days. Before and after the animals were treated with LASSBio-294, cardiac function and exercise capacity were evaluated through the echocardiography and treadmill test. Mean blood pressure (MBP), left ventricular end diastolic pressure (LVEDP) and negative dP/dt were also determined. Fibrosis in heart sections were detected using H&E staining. Immunohistochemical staining for TNF-alpha and SERCA2a in LV tissues were observed. Results: MI in SHR reduced the running distance from 257.9 ± 13.2 to 39.0 ± 4.4 m which normalized to 296.0 ± 26.4 m after treatment with LASSBio-294. Reduced anterior wall thickness was observed after MI (0.51 ± 0.14 mm) which was prevented with treatment (1.65 ± 0.21 mm). Ratio of early and late transmitral filling velocity was reduced from 1.48 ± 0.09 to 0.99 ± 0.04 and recovered to 1.35 ± 0.07 after treatment. MBP was reduced from 169.0 ± 5.6 to 120.4 ± 7.4 mmHg in SHR-IM treated with LASSBio-294. Increased LVEDP of 25.6 ± 3.2 observed in SHR-IM was reduced to 7.3 ± 1.0 mmHg after treatment. The -dP/dt was reduced in SHR-MI to -5698 ± 408.1 mmHg/s and returned to -7894 ± 631.6 mmHg/s after LASSBio-294 treatment. There was an increase in collagen deposition after MI (from 14.5 ± 3.5 to 59.8 ± 5.4 %) which was prevented with LASSBio-294 treatment (29.5 ± 2.2 %). Increase of positive staining for TNF-α was observed in SHR-MI (from 9.5 ± 1.0 to 32.3 ± 2.1%) which recovered in SHR-MI treated group (14.4 ± 1.3%). Also, the expression of SERCA2a was reduced in ventricular muscle from SHR-IM (from 68.7 ± 5.1 to 21.4 ± 2.3 %) which partially recovered to 40.6 ± 1.19% with LASSBio-294 treatment.
Conclusion: LASSBio-294 reduced exercise intolerance, prevented cardiac remodeling and diastolic dysfunction in infarcted SHR.

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Pathological Effects of Trimethylamine N-oxide (TMAO) on Pressure Overload-induced Heart Failure

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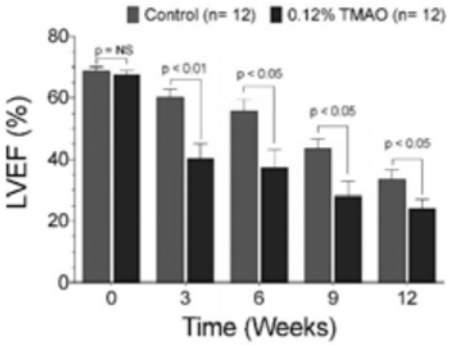
Rationale: Trimethylamine N-oxide (TMAO), a metabolite formed in the metabolism of dietary phosphatidylcholine, is elevated in the circulation of patients at increased risk for heart attack and adverse prognosis during heart failure.
Objective: We investigated the effects of dietary choline and TMAO on the severity of heart failure following transverse aortic constriction (TAC).
Methods and Results: Male C57Blk/6J mice were fed either control diet, a diet containing choline (1.2%) or a diet containing TMAO (0.12%) at 3 weeks prior to surgical TAC and were studied for 12 weeks. Left ventricular (LV) structure and function were monitored at 3 week intervals and myocardial tissue was collected at 12 weeks. Plasma TMAO levels were significantly ($p < 0.01$) increased in the choline (28.64 ± 2.30 μM) and TMAO (28.18 ± 4.27 μM) compared to the control group (1.87 ± 0.26 μM). Left ventricular ejection fraction (LVEF) was significantly ($p < 0.05$) worse in mice fed TMAO compared to control diet. LV end-diastolic and end-systolic diameters were significantly ($p < 0.05$) increased in the TMAO group compared to control diet. Myocardial fibrosis as measured with Picrosirius Red staining was also significantly greater ($p < 0.01$) in the TMAO and choline groups. Circulating BNP levels were significantly ($p < 0.05$) increased in the TMAO and choline groups.
Conclusions: These data demonstrate that heart failure severity is significantly enhanced in mice fed diets containing either TMAO or choline. Our results suggest that consumption of food high in dietary nutrients that increase TMAO levels such as phosphatidylcholine may increase heart failure severity.



C.L. Organ: None. **H. Otsuka:** None. **J. Bradley:** None. **S. Bhushan:** None. **R. Trivedi:** None. **W. Tang:** None. **X. Li:** None. **S. Hazen:** None. **D.J. Lefer:** None.

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Domain-specific Roles for GRK2 in Cardiac Hypertrophy and Heart Failure

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During heart failure (HF), cardiac levels and activity of the G protein-coupled receptor (GPCR) kinase (GRK) GRK2 are elevated and contribute to adverse remodeling and contractile dysfunction, while inhibition via a carboxyl-terminal peptide, βARKct, enhances heart function and can prevent HF development. Mounting evidence supports the idea of a dynamic "interactome" in which GRK2 can uncouple GPCRs via novel protein-protein interactions. Several GRK2 interacting partners are important for adaptive and maladaptive myocyte growth; therefore, an understanding of domain-specific interactions with signaling and regulatory molecules could lead to novel targets for HF therapy. For instance, GRK2 contains a putative amino-terminal Regulator of G protein Signaling (RGS) domain (βARK-RGS) that directly interacts with Gq and appears to inhibit signaling without altering Gq enzymatic activity. Previously, our lab investigated cardiac-specific transgenic (Tg) expression of a fragment of this RGS domain (βARKnt). This fragment did not alter acute hypertrophy after pressure overload or demonstrate RGS activity in vivo against Gq-mediated signaling. In contrast, βARKnt induced hypertrophy and elevated β-adrenergic receptor (βAR) density without altering agonist-induced contractility or adenylyl cyclase activity, due to a compensatory increase in GRK2 activity. Importantly, though, βAR downregulation in response to chronic agonist administration was attenuated by βARKnt expression, indicating a novel regulation of βAR receptor density. Given these findings we have recently investigated the effect of βARKnt expression during chronic pressure overload post trans-aortic constriction (TAC). Echocardiographic analysis revealed increased posterior wall thickness and left-

ventricular mass 4 weeks post-TAC compared to non-transgenic littermate controls (NLC). Importantly, despite enhanced hypertrophy, the progression to HF was inhibited in βARKnt mice 14 weeks post-TAC (%LV Ejection Fraction of 36.1 ± 0.2 in NLC versus 56.6 ± 0.9 in Tg mice). While mechanistic characterization is underway, these data indicate that βARKnt-mediated regulation of βAR density may provide a novel means of cardioprotection during pressure-overload induced HF.
S.M. Schumacher: None. **E. Gao:** None. **J. Chuprun:** None. **W.J. Koch:** None.

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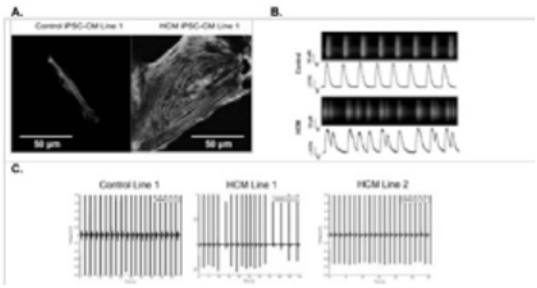
Disease Phenotype Assessment Across a Library of iPSC-Derived Cardiomyocytes From Patient Cohorts Carrying Distinct Mutations for Familial Hypertrophic Cardiomyopathy
Jason Tsai, Jason Lam, Veronica Sanchez-Freire, Rishali Gadkari, Maya Agarwal, Jing Bian, Guanyi Huang, Ashita Magal, Feng Lan, Andrew S. Lee, Stem Cell Theranostics, Menlo Park, CA

Familial hypertrophic cardiomyopathy (HCM) is the leading cause of sudden cardiac death in the young, and is the most common inherited heart defect affecting 1 in 500 individuals worldwide. Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) have been demonstrated to model aspects of HCM, but only one iPSC model has been reported for a single HCM mutation in one gene. Here we compare disease phenotypes across a library of patient-specific HCM iPSC-CMs carrying distinct mutations to assess the range of phenotypes that may present in iPSC-CMs derived from different patient cohorts. iPSCs were generated from three patient cohorts carrying known hereditary mutations for HCM in TNNI3, TNNT2, and MYH7 and family-matched controls. Disease phenotypes in patient-specific iPSC-CMs were modeled using immunostaining, Ca2+ imaging, multielectrode array, and video analysis of contractile motion. HCM iPSC-CMs displayed a range of disease phenotypes as assessed by cell size, Ca2+ homeostasis, electrophysiology, and contractile arrhythmia. Different HCM mutations resulted in distinct disease phenotype presentation. Importantly, identical mutations demonstrated similar readouts across multiple lines and clones whereas distinct mutations exhibited differential disease phenotypes. These findings indicate disease-specific iPSC-CMs present with a range of phenotypes for HCM that vary by specific mutation and that iPSC libraries are important for cellular characterization of diseases such as HCM.

Figure 1. Derivation and disease phenotype modeling of iPSC-CMs generated from patients carrying distinct familial HCM mutations and family-matched controls.



J. Tsai: None. **J. Lam:** None. **V. Sanchez-Freire:** None. **R. Gadkari:** None. **M. Agarwal:** None. **J. Bian:** None. **G. Huang:** None. **A. Magal:** None. **F. Lan:** None. **A.S. Lee:** None.

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Lysyl Oxidase Expression by Cardiac Fibroblasts is Regulated by Integrin-mediated Signaling
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Cardiac fibrosis following myocardial infarction (MI) leads to reduced cardiac function, and contributes to heart failure and mortality. Recent studies shown the extent of adverse remodeling may be mitigated by therapeutic strategies which regulate cardiac fibroblast mediated-remodeling. Since cross-linking by lysyl oxidase (LOX) increases following MI and alters the mechanical properties of the infarct, it is critical to characterize how its expression is regulated by CFs post-MI. While LOX expression is attributable to TGF-β1 signaling, we hypothesize that changes in the stiffness and composition of the ECM can also alter LOX expression via integrin-mediated signaling. To investigate this, we isolated CFs from healthy left ventricle (LV) and infarcted cardiac fibroblasts (ICFs) from 1 week post-MI LV and cultured them on tissue culture plastic (TCP) and collagen I-coated plates (COL) in serum-free media for 48 hours to assess the expression of genes associated with LOX signaling, fibrosis, and myofibroblast activation. Our results show an upregulation of LOX gene expression in both CFs and ICFs when cultured on COL and this is further emphasized with the presence of TGF-β1 (Fig. 1A). Gene expression of col1α1, integrin β1 subunit and αSMA (Fig. 1B-D) also exhibit similar upregulation. Ongoing studies will investigate how altered substrate stiffness and composition affect gene expression of LOX and other genes associated with fibrosis. By understanding the effect of the physical microenvironment on the expression of fibrotic genes including LOX, we aim to develop novel therapeutic strategies to attenuate cardiac fibrosis and thus improve cardiac recovery following MI.

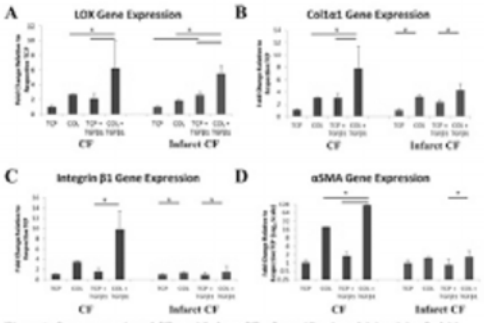

Figure 1. Gene expression of CFs and Infarct CFs. Quantification of delta-delta Ct fold change, with respect to GAPDH and TCP within respective cell types, of (A) LOX, (B) col1α1, (C) Integrin β1 subunit, (D) αSMA (log scale). *p<0.05 by ANOVA with Tukey's Post-Hoc.

A. Gao: None. **L.D. Black:** None.

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Biomechanical Properties of Scar ECM: from the Acute to Chronic Stages of Myocardial Infarction
Bryn Brazile, J. Ryan Butler, Sourav Patnaik, Mississippi State Univ, Mississippi State, MS; Yanyi Xu, Ohio State Univ, Columbus, OH; Andrew Claude, Raj Prabhu, Lakiesha Williams, Mississippi State Univ, Mississippi State, MS; Jianjun Guan, Ohio State Univ, Columbus, OH; Jun Liao, Mississippi State Univ, Mississippi State, MS

Introduction: Myocardial infarction (MI) affects more than 8 million Americans, causing massive heart cell death and heart function decrease. To better understand the scar biomechanics, we characterized the mechanical properties of pure scar ECM, obtained by decellularizing the MI tissues.
Materials and Methods: Infarcted rat hearts were generated by a permanent left coronary artery ligation (PLCAL) and harvested at 15 min, 1, 2, and 4 weeks (per acute to chronic stages of MI)(N = 6 each). Scar

ABSTRACTS

ECM were obtained by decellularizing the infarcted hearts in 0.1% sodium dodecyl sulfate (SDS) solution for 3 weeks. Scar ECM specimens were trimmed into square shape, and then subjected to biaxial testing with one edge aligned with the circumferential direction and the other edge aligned with the longitudinal direction of the rat heart. After 10 cycle preconditioning, an equibiaxial tension protocol of T_{circ}:T_{rad} = 30:30 N/m was performed to capture the tissue biaxial behavior.
Results and Discussion: Scar ECM 15 minutes through 4 weeks post infarction showed a stiffening biaxial behavior along with the time (Fig.1). The decrease of extensibility along longitudinal direction was more noticeable than circumferential direction, which led to a decrease in degree of anisotropy.
Conclusions: Scar ECM biomechanics showed a stiffening behavior with a marked reduction in extensibility (longitudinal) with time. This change in biomechanical properties can be correlated to the collagen structure changes with progression of MI. Knowledge of the structural-mechanical relationship of scar ECM will help us understand MI progression and help formulate regenerative therapies.

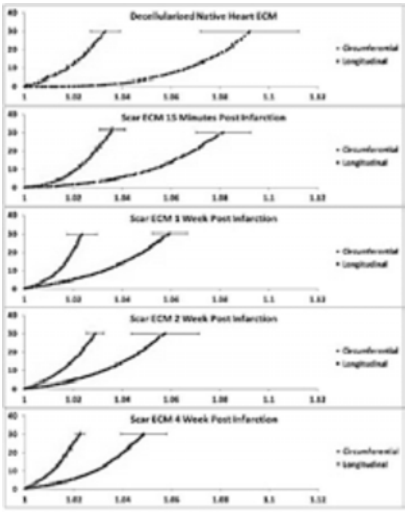

Figure 1: *Equibiaxial behavior of native and scar ECM: (a) Decellularized native heart ECM; (b) scar ECM 15 minutes post infarction; (c) scar ECM 1 week post infarction; (d) scar ECM 2 weeks post infarction; and (e) scar ECM 4 weeks post infarction.*

B. Brazile: None. **J.R. Butler:** None. **S. Patnaik:** None. **Y. Xu:** None. **A. Claude:** None. **R. Prabhu:** None. **L. Williams:** None. **J. Guan:** None. **J. Liao:** None.

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MicroRNA-19b Contributes to Cardiac Fibrosis
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Fibrosis is one of the most important characteristics of cardiac remodeling during heart failure. The accumulation of extracellular matrix (ECM) within myocardium is the major feature of cardiac fibrosis. microRNA (miR)-19b, a key functional member of miR-19-72 cluster family, has been suggested to be involved in aging-induced heart failure through regulating ECM-related proteins, such as connective tissue growth factor (CTGF), thrombospondin-1 (TSP-1), collagen-1A1, and collagen-3A1. In the current study, we aimed to investigate the role of miR-19b in cardiac fibroblast function and ECM production using neonatal rat cardiac fibroblasts in primary culture. We found that overexpression of miR-19b increased, while inhibition of miR-19b decreased the proliferation and migration of cardiac fibroblasts, using Cell Counting Kit-8 (CCK-8) (0.660±0.019 vs 0.720±0.014 in nc-mimic and miR-19b mimic, 0.506±0.009 vs 0.454±0.008 in nc-inhibitor and miR-19b inhibitor, respectively), EdU incorporation assay (0.059±0.002 vs 0.096±0.006 in nc-mimic and miR-19b mimic, 0.059±0.006 vs 0.040±0.003 in nc-inhibitor and miR-19b inhibitor, respectively), and wound healing assay (0.528±0.024 vs 0.896±0.027 in nc-mimic and miR-19b mimic,0.520±0.028 vs 0.174±0.019 in nc-inhibitor and miR-19b inhibitor, respectively), respectively. Meanwhile, the inhibition of miR-19b downregulated the mRNA levels of α-SMA (0.556±0.048 vs 1.038±0.137 in nc-inhibitor and miR-19b inhibitor, respectively) and collagen-1 (1.023±0.116 vs 0.551±0.033 in nc-inhibitor and miR-19b inhibitor, respectively) in cardiac fibroblasts, indicating a reduction in fibroblast activation and ECM production via miR-19b inhibition. Furthermore, we found that PTEN was negatively regulated by miR-19b in cardiac fibroblasts using western blot analysis. PTEN, a well-known tumor-suppressor gene, has been known to inhibit cell proliferation and migration. However, it remains to be further clarified whether PTEN could mediate the effect of miR-19b in the proliferation, migration and activation of fibroblasts. These data might provide important evidence suggesting that miR-19b could be a potential therapeutic target for cardiac fibrosis.
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Down Regulation of ECM Genes Laminin and Collagen IV Lead to Preserved Cardiac Function With Age and Increased Lifespan in Drosophila
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Increased deposition of extracellular matrix (ECM) is observed in all advanced age heart failure patients, but current model systems are complex and slow to age. To investigate the effect of extracellular remodeling on mechanical function in genetically tractable, rapidly aging, and simple model organisms, we employed Drosophila melanogaster, which has a simple trilayered heart tube.
We found that two common wildtype strains of Drosophila, i.e. yellow-white (yw) and white-1118 (w1118), exhibit different cytoskeletal and ECM remodeling with age. Using a recently developed nanoindentation method to measure cardiomyocyte stiffness and high speed optical imaging to assess contractility of intact Drosophila hearts, we found that yw flies had stiffer intercalated discs (ICD) and exhibited diastolic dysfunction with age. On the other hand, w1118 flies had a shorter lifespan compared to yw, did not exhibit ICD stiffening, had a less severe diastolic dysfunction, and showed an increase in ECM layer thickness between ventral muscle (VM) and cardiomyocyte (CM) layers of the heart tube. To modulate ECM and assess its effect in the aged w1118 flies, we knocked-down ECM genes LamininA and Viking (homologous to Collagen IV). Both ECM KD genotypes exhibited diastolic dilation with increased fractional shortening at adult (1wk) and aged (5wk) time points. The LamininA KD resulted in decreased cardiomyocyte stiffness correlating with increased relaxation velocities in adult flies and preservation of shortening and relaxation velocities in aged flies over controls. However, both the LamininA and Collagen IV KD flies experienced a basal increase in the decoupling of their cardiomyocytes as determined by heart period variance and % fibrillar heart-beats. These conductance issues

were not enough to counteract the increased cardiac output and performance with age, and the Collagen IV KD outlived controls by 1.5 weeks median survival and the LamininA KD by 3 weeks. This suggests that the cell-ECM contacts in the basement membrane are intimately tied not only to the coupling of the cardiomyocytes of the Drosophila heart tube but also to cytoskeletal remodeling, but perhaps different ECM proteins have different mechanisms for interacting with the cardiomyocyte cytoskeleton.
A.O. Sessions: None.

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Regulation of Ski and Scleraxis in the Infarcted Rat Heart
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Introduction: Coronary heart disease is causal to myocardial infarction (MI) and cardiac fibrosis. Upon ischemic myocardial injury, resident cardiac fibroblasts phenoconvert to myofibroblasts and synthesize large amounts of fibrillar collagens to produce scar tissue. Although the myofibroblast numbers are reduced in the infarct scar following the completion of wound healing, a sub-population of cells persist in the wounded area, leading to maladaptive chronic remodeling of the scar area and eventually the non-infarcted myocardium. Ski has been identified as a repressor of the TGF-β1 signaling pathway, attenuating the myofibroblast phenotype and its functional properties. Scleraxis has been implicated in canonical TGF-β1 signaling to promote collagen1α2 expression. We investigated how Ski and Scleraxis contribute to physiological and pathological wound healing in vivo.
Methods: The study was carried out using 64 male Sprague-Dawley rats. The left anterior descending (LAD) coronary artery was ligated to induce a myocardial infarction. Control (sham) operated animals underwent surgery without ligation of the LAD artery. Animals were sacrificed at 2, 4, and 8 weeks post-MI and tissue collected for Western blot and qPCR studies.
Results: Scleraxis mRNA expression remained at baseline at 2 and 8 weeks post-MI, but was significantly increased 4 weeks post-MI. Scleraxis protein expression was down-regulated within the scar area of infarcted hearts when compared to control samples 2 and 4 weeks post-MI. Ski mRNA expression was up-regulated within the scar area of infarcted hearts 2, 4 and 8 weeks after infarction.
Conclusions: Scleraxis protein is down-regulated in myofibroblasts of the infarct scar in the chronic stages of myocardial infarction, corresponding to the maturation of the scar. At these stages of wound healing, we have previously published that Ski is up-regulated in the cytosol of these same cells. We suggest reciprocal feedback in the expression of these two proteins exists in myofibroblasts in the infarct scar. We hope to learn more about the Ski/Scleraxis feedback loop in pathological wound healing to identify novel therapeutic targets.
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Spatial Expression Analysis of the Ischemic Heart Identifies Novel Factors Involved in Cardiac Remodeling and Disease
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Cardiac ischemic injury is associated with expressional changes in a broad range of coding and non-coding genes. While genome-wide expression techniques like RNA sequencing, give valuable information about the transcriptional activity of genes, spatial clues are lost. Here, we use a novel method (TomoSEQ) based on traditional histological techniques with low-input RNA sequencing to generate a spatial gene expression atlas for the infarcted heart. Transcriptome profiling of 50 consecutive sections from hearts exposed to ischemia reperfusion or sham surgery, allowed us to trace differential gene regulation throughout the infarct, borderzone and remote area. Fourteen days post-injury, we clearly observed differential expression curves specific for genes known to be relevant for cardiac function and disease, like *Nppa* (remodeling/hypertrophy), *Col3a1* (fibrosis) and *Atp2a2* (calcium handling), with the first two being more abundantly expressed in the infarct, and the latter more dominant towards the remote. Interestingly, the exact spatial information on these 3 reference genes enabled us to identify a set of genes that show an identical regulation, potentially linking these transcripts to the aforementioned biological function of the reference genes. The validity of using this method to identify genes that are expressionally linked was underscored by the observation that the correlation between the expression level of the *Nppa*, *Col3a1*, *Atp2a2* and the newly identified genes could be confirmed in human cardiac tissue samples from patients suffering from ischemic heart disease. We next used an *in silico* approach to identify common transcription factors among the co-regulated genes. TomoSEQ analysis revealed a strong spatial correlation between these identified transcription factors and their target genes. This provides further support for our methodology, but also provides an opportunity to identify novel transcription factors relevant for cardiac function and disease.
Our study shows the power of spatial transcriptome profiling throughout the infarcted heart by giving local clues on changes in gene expression and allowing for the identification of novel genes and key transcription factors that could be relevant for cardiac repair upon injury.
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Cardiac Fibroblasts are Essential During Pathological Stress
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Cardiac fibroblasts reportedly play a role in normal heart function and are responsible for adverse remodeling after heart injury. Although fibroblast activities can be detrimental during disease, a basal level of cardiac fibroblast activity is required to maintain structural integrity and prevent rupture. We use transgenic mouse models to demonstrate that reduced fibroblasts numbers have serious consequences during pathological stress. At baseline, we observe a dramatic loss of the cardiac fibroblast lineage, which results in vasculature alterations, such as capillary dilation and decreased capillary density. Baseline changes in basement membrane (laminin) and cardiomyocyte structure have also been identified in fibroblast deficient hearts. These phenotypic changes become exacerbated after surgery indicating that fibroblasts are necessary for crosstalk between other cell types in the heart. In addition, we predict that the loss of fibroblasts will cause enhanced deterioration in cardiac function after injury due to reduced structural integrity of the heart. We will use transverse aortic constriction (TAC) as a pressure-overload model. After 5 weeks of TAC, we observe a ~50% (Baseline 71.99±6.06, TAC5wks 22.09±1.89) decrease in ejection fraction in fibroblast deficient hearts compared

to a ~16% (Baseline 76.21±7.08, TAC5wks 59.59) decrease in control hearts. Our data suggest that a specific level of cardiac fibroblast activity is required to maintain normal heart function. Our goal is to identify both deleterious and beneficial roles of fibroblasts in the response of the heart to the types of pathological stress commonly encountered in patients.
J.T. Kuwabara: None.

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Mechanotransduction via Titin's N2B Element Contributes to Cardiac Remodeling
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Pathological remodeling is responsible for the functional deficits characteristic of heart failure patients. Understanding mechanotransduction is limited, but holds potential to provide novel therapeutic targets to treat patients with heart failure, especially those with diastolic dysfunction and preserved ejection fraction (HFpEF). Titin is the largest known protein and is abundant in muscle. It is the main contributor of passive stiffness in the heart and functions as a molecular mechano-sensor for stress and strain in the myocyte. Titin is composed of four distinct regions, (N-terminal Z-line, I-band, A-band, and C-terminal M-line), and acts as a molecular spring that is responsible for the assembly and maintenance of ultrastructure in the sarcomere. The elastic N2B element found in titin's I-band region has been proposed as a mechano-sensor and signaling "hot spot" in the sarcomere. This study investigates the role of titin's cardiac specific N2B element as sensor for stress and strain induced remodeling in the heart. The previously published N2B knock out (KO) mouse was subjected to a variety of stressors including transverse aortic constriction (TAC), aorto-caval fistula (ACF), chronic swimming, voluntary running and isoproterenol injections. Through chronic pathologic stress, pressure overload (TAC) and chronic volume overload (ACF), we found that the N2B element is necessary for the response to volume overload but not pressure overload as determined by changes in cardiac remodeling. Furthermore, the response to exercise either by chronic swimming or voluntary running was reduced in the N2B KO mouse. Finally, unlike the wild-type (WT) mouse, the N2B KO mouse did not respond to isoproterenol injections with hypertrophic remodeling. Ongoing work to elucidate the molecular pathways involving the N2B element and response to stress, is focused on its binding protein Four-and-a-half-LIM domains 2 (FHL2) and the mitogen activated protein kinase (MAPK) pathway. Taken together our data suggest that the N2B element contributes significantly to mechanotransduction in the heart.
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Amino Terminal C0-C1f Region of Cardiac Myosin Binding Protein-C is Essential for Normal Cardiac Function
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Rationale: Cardiac myosin binding protein-C (cMyBP-C) is a trans-filament protein that has been shown to regulate cardiac function via its amino terminal (N') regions. However, it is unknown whether the first 271 residues (C0-C1f region) are necessary to regulate contractile function in vivo.
Hypothesis: The N'-region of cMyBP-C is critical for proper cardiac function in vivo.
Methods and Results: Transgenic mice with approximately 80% expression of mutant truncated cMyBP-C missing C0-C1f (cMyBP-C^{110kDa}), compared to endogenous cMyBP-C, were generated and characterized at 3-months of age. cMyBP-C^{110kDa} hearts had significantly elevated heart weight/body weight ratio, fibrosis, nuclear area and collagen content compared to hearts from non-transgenic (NTG) littermates. Electron microscopic analysis revealed normal sarcomere structure in cMyBP-C^{110kDa} hearts but with apparently weaker cMyBP-C stripes. Furthermore, the ability of cMyBP-C to slow actin-filament sliding within the C-zone of native thick filaments isolated from NTG hearts was lost on thick filaments from cMyBP-C^{110kDa} hearts. Short axis M-mode echocardiography revealed a significant increase in left ventricular (LV) internal diameter during diastole in cMyBP-C^{110kDa} hearts. Importantly, cMyBP-C^{110kDa} hearts displayed a significant reduction in fractional shortening compared to hearts from NTG littermates. We further observed a decrease in the thickness of the LV interventricular septum and free wall during systole in cMyBP-C^{110kDa} hearts. Strain analysis using images acquired from ECG-Gated Kilohertz Visualization identified a significant deficit in global longitudinal strain in cMyBP-C^{110kDa} hearts compared to NTG hearts.
Conclusion: The N'-region of cMyBP-C is indispensable for maintaining normal cardiac morphology and function and loss of this region promotes contractile dysfunction both at the molecular and tissue levels.
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Endothelial Cell Hypoxia-inducible Factor (HIF) Activity is Necessary for Right Ventricular Remodeling in a Murine Model of Chronic-hypoxic Pulmonary Hypertension
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Introduction: Pulmonary hypertension induced myocardial
remodeling is required to minimize RV wall stress as pulmonary arterial pressures increase. Myocardial angiogenesis is presumed to play an integral role in early RV remodeling, though molecular regulators remain undefined. Endothelial cell (EC) expression of HIF-1 is necessary for adaptive LV remodeling during chronic pressure overload, with loss of EC-specific HIF-1 activity associated with reduced LV capillary density. We hypothesized that EC HIF activity may also be necessary for adaptive RV remodeling and angiogenesis in a model of chronic RV pressure overload.
Methods: We used a murine model of chronic hypoxic pulmonary hypertension (CH-PH) to induce RV remodeling. Conditional transgenic mice lacking expression of the obligate HIF-1α/HIF-2α co-factor ARNT (aryl hydrocarbon receptor nuclear translocator) in Tie2+ lineage cells ($ARNT^{fl/fl}$;$Tie2\text{-}Cre^{+/-}$) and $ARNT^{fl/fl}$;$Tie2\text{-}Cre^{-/-}$ controls were exposed to normobaric hypoxia (10% FiO_2) for 1-5 weeks. We measured RV

hypertrophy (RVH), RV systolic pressure (RVSP), and myocardial EC proliferation (flow cytometry) as a marker for sprouting angiogenesis.
Results: After three weeks of CH-PH, RVH was observed in *ARNT$^{fl/fl}$;Tie2-Cre$^{-/-}$* control mice, as anticipated (0.332±0.02 vs. 0.231±0.02; $P < 0.05$). CH-PH induced RVH in *ARNT$^{fl/fl}$;Tie2-Cre$^{-/-}$* mice was associated with an early (1 week) increase in RV EC proliferation (vs. LV, 4.5±1.3% vs.1.8±0.5%; $P < 0.05$). Conversely, *ARNT$^{fl/fl}$;Tie2-Cre$^{+/-}$* mice did not develop RVH after 3 weeks of CH (0.222±0.01 vs. 0.241±0.02; P = NS), and early RV EC proliferation was attenuated (vs. LV, 2.9±1.2% vs. 1.5±0.3%; P = NS). Despite similar increases in CH-PH induced RVSP after 3 weeks, after 5 weeks RVSP normalized in *ARNT$^{fl/fl}$;Tie2-Cre$^{+/-}$* mice, but remained elevated in *ARNT$^{fl/fl}$;Tie2-Cre$^{-/-}$* controls.
Conclusions: These preliminary findings confirm that EC-specific HIF
activity is necessary for CH-PH induced RVH, and suggest that reduced EC proliferation (and potentially angiogenesis) may contribute to abrogated RV remodeling in *ARNT$^{fl/fl}$;Tie2-Cre$^{+/-}$* mice. We hypothesize that late normalization of RVSP in these mice may be indicative of premature RV failure.
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Inhibition of Bcl-x Phosphorylation Prevents Cardiomyocyte Death, Cardiac Remodeling and Heart Failure After Myocardial Infarction
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Cardiac remodeling promotes heart failure (HF). Cardiomyocyte (CM) death is one of the mechanisms to develop cardiac remodeling. We recently reported that Mst1 phosphorylates Bcl-xL at Ser14, which promotes apoptosis by inducing dissociation of Bcl-xL from Bax and consequent activation of Bax in CMs. Its phosphorylation is increased in response to ischemia-reperfusion (IR) in an Mst1-dependent manner. However, the functional significance of endogenous Bcl-xL phosphorylation remains unclear in vivo. To address this question, knock-in (KI) mice with alanine mutation at Ser14 in Bcl-x were generated. At baseline, cardiac function was similar between wild-type (WT) and heterozygous KI (HKI) mice (EF 76% and 79%, respectively). HKI mice exhibited smaller % infarct area (30%) than WT (43%) (p=0.016) upon IR, suggesting that phosphorylation of endogenous Bcl-xL at Ser14 plays an essential role in mediating IR injury. In order to test the role of Bcl-xL phosphorylation in the development of HF, HKI and WT mice were subjected to permanent ligation of LAD for 4 weeks. During progression of cardiac remodeling, Mst1 was activated in both WT and HKI mice. Phosphorylation of Bcl-xL and Bcl-xS, an alternative transcriptional variant of Bcl-x, both at Ser14, were increased in WT mice, which were abrogated in HKI mice. The infarct area evaluated with TTC staining at Day 1 was similar in WT and HKI mice (59.1% and 61.2%, p=0.65). Four weeks after myocardial infarction (MI), WT mice exhibited lower cardiac contraction (EF 46.5%) and higher LVEDP (10.8mmHg) than those in HKI mice (EF 68.9% and LVEDP 7.0mmHg) (both p<0.05). Scar area and TUNEL-positive CMs were greater in WT (49.0% and 1.6%, respectively) than in HKI mice (29.2% and 0.4%, respectively) (both p<0.05). Cleaved caspase 3 and 9 were significantly increased (3.2- and 5.7-fold, respectively) in WT but not in HKI mice. In vitro experiments with overexpression of phospho-mimicking mutant (Bcl-xS-S14D) showed 13% reduction in cell viability compared with that of phospho-resistant mutant (Bcl-xS-S14A) (p=0.01%). Our results suggest that phosphorylation of Bcl-xL and Bcl-xS at Ser14 contributes to CM death in response to IR and chronic MI in vivo, thereby promoting cardiac remodeling and HF.
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Resident Cardiac Fibroblasts Give Rise to Periostin+ Myofibroblasts Which are the Primary Mediators of Cardiac Fibrosis
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Resident cardiac fibroblasts (CFs) are potential therapeutic targets in treating or preventing heart failure since they play a critical role in cardiac remodeling and fibrosis after injury or with prolonged stress stimulation. Heterogeneity among activated fibroblasts within the heart has been noted by a number of previous studies in the literature. In addition to resident CFs, many cell types such as endothelial, perivascular and bone marrow cells have been suggested to go through a mesenchymal transition and acquire a myofibroblast-like phenotype during disease conditions. Hence, the cellular origin of the activated myofibroblast within the heart remains uncertain, in part because of a lack in reliable genetic strategies to define cellular lineage. Recent studies suggest that epicardial precursor cells expressing transcription factor 21 (Tcf21) give rise to resident CFs in the adult heart. In addition, the secreted matricellular protein periostin (Postn), appears to be expressed only within activated fibroblasts (myofibroblasts) within the heart. Here we used Tcf21-MerCreMer (Tcf21MCM) knockin mice and Postn-MerCreMer (PostnMCM) knock-in (KI) mice to lineage trace resident CFs and myofibroblasts with injury stimulation. To account for other potential cellular lineages giving rise to fibroblasts in the heart we also performed lineage tracing with the mouse genetic models including LysM-Cre (macrophage), ckit-Cre (bone marrow), Tie2CreERT2 (endothelial) and Myh11-CreERT2 (smooth muscle) in conjunction ROSA26 (R26) locus based loxP inactivated reporter alleles. Results of this study indicate that the Tcf21+ resident CFs are the predominant source for the activated periostin+ MFs which are the key mediators of extracellular matrix (ECM) production and ECM stability in heart whereas the contribution of other lineages to MFs are minimal. Additionally, we have performed single cell RNA sequencing on TCF21+ and Postn+ isolated CFs pre and post myocardial injury in order to define the fibroblast lineage itself at greater molecular depth.
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Prolonged Renal Failure Leads to Reduced Number of Active Cardiac Mitochondria in a Rat Model for Long Term Chronic Kidney Disease
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Objectives - Cardiorenal syndrome type 4 is characterized by primary chronic kidney disease (CKD) leading to an impairment of cardiac function. We

recently showed a reduced expression of several cardiac mitochondrial genes in short-term CKD rat model. We aimed to evaluate whether cardiac mitochondrial structure and function is modified in long-term CKD and if so, to characterize the potential associated mechanisms.
Methods- Lewis rats underwent 5/6 nephrectomy for induction of CKD. Upon necroscopy, eight months later, cardiac sections were analyzed by histology and electron microscopy (EM). Mitochondrial DNA content was determined by the mitochondrial gene, cytochrome B. Mitochondrial content was assessed by citrate synthase (CS) activity in tissue homogenate and respiratory chain function was determined by the activity of complexes I-IV in isolated mitochondria. The levels of PGC1a, a transcription factor for mitochondrial biogenesis, Angiotensin II type 1 receptor and cytosolic cytochrome C were assayed by western blot. Cytokine serum profile was determined by microarray.
Results- Long-term CKD leads to cardiac hypertrophy and increased interstitial fibrosis. EM analysis revealed a massive spatial disarrangement accompanied by a considerably increased volume of swollen-damaged mitochondria in CKD hearts (32±3%, n=5, 48±6%, n=4; respectively; $p<0.05$). Total mitochondrial DNA content was decreased in cardiac tissue of CKD rats. Concomitantly, active mitochondrial content was significantly reduced. Conversely, no differences were observed in respiratory chain enzymes' functions (complexes I-IV) in isolated active mitochondria. Moreover, inflammatory response and activation of Renin-Angiotensin-Aldosterone-System (RAAS) were detected in the CKD setting.
Conclusion- CKD results in a marked reduction of active
mitochondria in the heart. Inflammatory cytokines and RAAS, may set a deleterious environment to cardiac mitochondria, as suggested in non-CKD models. The data may represent a significant milestone in the personalized medicine strategy for treating CKD patients who present with normal cardiac function accompanied by positive biomarkers for cardiac mitochondria damage.
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Reduced Mitochondrial Function and Metabolic Dysregulation in Aneurysmal Fibulin-4 Mice.
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Thoracic aortic aneurysms are a life-threatening condition often diagnosed too late. The underlying mechanism is largely unknown. An accurate and early predictive biomarker for aneurysm formation has not yet been identified, although some molecular processes, such as disturbed TGF-β signalling, have been implicated. To discover novel robust biomarkers, we aimed to better understand the molecular mechanisms involved in aneurysm initiation and progression.
In Fibulin-$4^{R/R}$ mice, the extracellular matrix protein Fibulin-4 is 4-fold reduced, resulting in progressive ascending aneurysm formation and early death around 3 months of age. We performed LC-MS/MS proteomics and transcriptomics analyses on the aortas of Fibulin-$4^{R/R}$ and Fibulin-$4^{+/+}$ mice. Protein and gene data sets were separately analysed for genotype specific differences with Ingenuity Pathway analysis tools. Intriguingly, we observed alterations in mitochondrial composition in aortas from Fibulin-$4^{R/R}$ mice. Consistently, functional studies in Fibulin-$4^{R/R}$ vascular smooth muscle cells (VSMCs) revealed lower oxygen consumption rates, but increased acidification rates compared to Fibulin-$4^{+/+}$. The mitochondria in VSMCs of Fibulin-$4^{R/R}$ mice were reduced in size and had increased complex I-IV levels. Furthermore, aortas of aneurysmal Fibulin-$4^{R/R}$ mice displayed increased levels of ROS. Consistent with these findings, gene expression analyses revealed the dysregulation of metabolic pathways. In accordance, ketone levels in the blood of Fibulin-$4^{R/R}$ mice were reduced and liver fatty acids were decreased, while liver glycogen was increased. As predicted by these findings, activity of PGC1α, a key regulator between mitochondrial function and organismal metabolism, was downregulated in Fibulin-$4^{R/R}$ VSMCs.
In conclusion, our data indicate altered mitochondrial function and metabolic dysregulation, leading to increased ROS levels and altered energy production, as a novel mechanism, which may contribute to thoracic aortic aneurysm formation. This discovery will not only provide new biomarkers that can be validated in human aortas, but they will also provide the rational for new interventions such as alterations in diet to prevent aneurysm formation.
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17 beta-Estradiol Enhances Mitochondrial Function of Cerebral Arteries From Ovariectomized Female Rats
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Previous studies have provided indirect evidence that circulating sex hormones alter the function of the cerebral circulation perhaps via effects on mitochondrial dynamics. However, effects of estrogen on mitochondrial respiration have never been directly examined. We have previously observed a difference in the mitochondrial function of cerebral arteries from male and female rats but the exact mechanisms are not clear.
We tested the hypothesis that mitochondrial respiration in isolated cerebral arteries from ovariectomized (OVX) Sprague Dawley rats treated with a 21 d release, 0.5 mg of 17 β-estradiol pellet (OVX+E) was enhanced compared with arteries from placebo treated OVX rats. The Seahorse Bioscience XFe24 system was used to measure mitochondrial oxygen consumption rate (pM/min/μg protein) in cerebral arteries. Western blot was used to investigate the arterial expression of proteins. Radioimmunoassay was used to measure serum estradiol level.
Treatment with 17 β-estradiol resulted in a higher serum estradiol level (146.9±18.16 pg/ml) and uterus weight (0.15±0.0058 g) in the OVX+E compared with the OVX (14.7±1.2 pg/ml, 0.07±0.003, respectively; $p<0.05$). The components of mitochondrial respiration in pM/min/μg protein including basal respiration (147±9), ATP production (44±4), proton leak (102±7), and maximal respiration (212±13) were elevated in OVX+E compared with OVX (105±13, 21±4, 50±6, 138±10, respectively; $p<0.05$).

Expression of the mitochondrial DNA encoded Complexes I and III, the nuclear DNA encoded Complexes II, IV, V, and the voltage-dependent anion channel protein were similar in all groups. However, the ratios of phosphorylated endothelial and neuronal nitric oxide (NO) synthase normalized to total protein were significantly ($p<0.05$) elevated in the OVX+E (1.43±0.06, 1.15±0.2, respectively) compared with OVX group (0.92±0.06, 0.53±0.19, respectively). Our findings provide direct evidence for sex-specific differences in mitochondrial function on freshly isolated cerebral arteries. Thus, estradiol replacement enhances the efficacy of the oxidative phosphorylation resulting in an increased mitochondrial respiration which is not due to increased mitochondrial protein expression but may be due to enhanced NO.

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Leucine, Serine and Methionine Differentially Affect Protein Synthesis in Rat Heart

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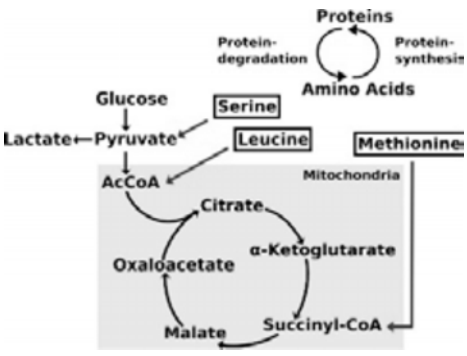
Alternating flux through metabolic pathways enables the heart to rapidly adapt to stress. As a consequence, compensatory mechanisms are often associated with changes in mitochondrial function and cellular redox state. The collective importance of these metabolic changes to energy provision and protein turnover remains unknown. We therefore integrated radioactive tracer studies with a mathematical model of cardiac metabolism - CardioNet. We assessed through flux rate analysis the impact of altered amino acid supply on mitochondrial metabolism. In combination with leucine, both serine and methionine resulted in a two- to threefold increased variance of mitochondrial flux rates. Next we used the isolated working rat heart to determine whether supply of glucose and leucine, in combination with either serine or methionine at different concentrations, affects cardiac protein turnover and energy provision. While glucose remained the major energy source, oxidation of leucine contributed to ATP production. Methionine, but not serine, markedly increased both myocardial oxygen consumption and cardiac power at normal physiologic concentrations. Protein synthesis rates (units given in µmol/min/g dry wt) did not significantly differ from leucine alone (0.037±0.001) or leucine in combination with serine (0.038±0.003) or methione (0.045±0.007). However, with increased serine supply, protein synthesis significantly increased (0.0907±0.009) compared to methionine (0.059±0.006, $p=0.024$) or leucine only (0.015±0.002, $p=0.031$). The results suggest that myocardial protein synthesis is differentially regulated by specific amino acids.



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385

D-2-hydroxyglutaric acid Acutely Impairs Oxidative Decarboxylation in the Heart

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Mutations in isocitrate dehydrogenase 1 and 2 (IDH1, IDH2) have been described in low-grade glioma and in acute myeloid leukemia. Accumulation of the oncometabolite D-2-hydroxyglutaric acid (2HG) and its release into the blood is associated with dilated cardiomyopathy. The mechanisms leading to changes in cardiac metabolism and contractile function are unknown. We studied in the isolated working rat heart preparation metabolic consequences of increased 2HG supply and its impact on cardiac energy provision. In combination with physiological levels of glucose (5mM) and lactate (0.5mM), hearts were perfused at different concentrations with 2HG (0.5mM, 1.0mM). We confirmed the uptake and enrichment of 2HG in the tissue through liquid chromatography followed by mass spectrometry (LC/MS). 2HG markedly decreased cardiac power and cardiac efficiency in a concentration dependent manner. At the same time glucose oxidation increased significantly (1.4±0.1 µmol/min/g dry wt, $p<0.05$) at higher workloads. The alpha-Ketoglutarate Dehydrogenase activity was reduced by two-fold (2.84±0.56 µmol/min/g dry wt, $p<0.01$) and production of reactive oxygen species (e.g. H2O2) increased by three-fold (1.06±0.07 µmol/min/g dry wt, $p<0.01$) in presence of 2HG. Consistent with this reduction in oxidative decarboxylation, the cytosolic NAD+/NADH redox state increased (3.1±0.1, $p<0.001$), while the cellular energy charge declined (AMP:ATP ratio, 0.15±0.1, $p<0.001$). Together, our results demonstrate a direct impairment of cardiac energy substrate metabolism with a resulting decline in contractile function. The data suggests that 2HG directly suppresses cardiac function by specific metabolic alterations in a range of pathologies.

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The Role of GAPDH in the Cardio-metabolic Response to Ischemia

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Fundamental changes in nutrient metabolism underlie many disease processes, including obesity, diabetes, and cancer. Metabolic derangements are also a hallmark of heart disease, both in the context of failure and ischemia, and likely contributors to its etiology. For example, while the healthy heart favors fats as a fuel, failing hearts will consume more glucose; but it is unclear if this is pathological or protective. It is very likely that manipulation of metabolic pathways can affect, and possibly augment and protect, heart function. Yet no drug therapy exists that targets cardiac metabolism. Staples of ischemic disease management largely focus on anti-platelet, cholesterol-lowering, and beta-adrenergic blocking regimens. Heart failure medicines focus on maladaptive neuro-hormonal responses, a strategy several decades old. Our goal here is to delineate exactly how critical aspects of metabolism are altered in cardiac ischemia, and to identify new targets of pharmacological intervention. Metabolism largely remains unexplored because of its intricate network of regulation, compounded by the

fact that variations in this network will often escape traditional methods of gene expression profiling. But by using high throughput metabolomic and proteomic platforms, we have striking evidence that the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is instrumental in the heart's response to ischemia. Dynamic, real-time metabolomic flux using heavy carbon isotope labeling points toward a partial shift of glycolytic intermediates toward lipid synthesis during ischemic stress in cardiomyocytes, precisely at the step catalyzed by GAPDH. Expansive phospho-proteomic analyses from Langendorff ex vivo hanging heart experiments show that this shift in metabolism may be triggered by an inhibitory phosphorylation of GAPDH. Our hypothesis that glycolysis is in part rerouted toward triglyceride synthesis is a revolutionary departure from decades-old perceptions of energy management. Modulating GAPDH function, either directly or indirectly via partner proteins, may offer insight into untapped areas of therapy. Our early attempts with chemicals that inhibit GAPDH already show heart cells with improved survival under ischemia.
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Role of Glycolysis in Cardiac Adaptation to Exercise
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Cardiac stressors affect the function and structure of the heart with prominent effects on metabolism. While the heart typically derives most (~80%) of its energy from fatty acids, the exercised heart is associated with an enhanced capacity to utilize a variety of carbon sources to sustain energy demands that would be advantageous during increased workloads and ischemic events. Due to the central role of glycolysis in glucose metabolism, it is likely that changes in the glycolytic rate regulate cell growth, homeostasis and stress responses. In this study, we assessed how exercise affects glucose metabolism in the myocardium and whether genetically decreasing glycolysis in the heart - using a validated, cardiac-specific, dominant negative form of phosphofructokinase 2 (kd-PFK2) - affects cardiac adaptation to exercise. Adult male FVB/NJ mice were subjected to forced treadmill running for 30 days. Exercise performance was measured and echocardiographic analysis was used to assess changes in cardiac function. Immunoblotting was performed to quantify the abundance of key enzymes involved in glucose metabolism. Exercise training increased exercise capacity by 70% ($p < 0.01$) and work performed by 69% ($p < 0.01$). Exercise training also induced cardiac hypertrophy (15%; $p < 0.01$) associated with a 1.4 fold increase in mitochondrial content ($p < 0.05$). Cardiac function remained largely unchanged with a significant reduction of 5% in ejection fraction ($p < 0.01$) and an increase in the left ventricle chamber dimensions ($p < 0.05$). Relative myocardial abundance of thirteen glycolytic and glucose metabolism enzymes remained unchanged. The kd-PFK2 mice showed that low levels of glycolysis in the heart promote cardiac hypertrophy (15%; $p < 0.01$) and prevent physiological hypertrophy due to exercise. In comparison with WT mice, the kd-PFK2 mice had a 20% lower exercise capacity ($p < 0.05$). These findings suggest that the glycolytic rate regulates cardiac adaptations to exercise and may mediate some of the beneficial effects of exercise training on exercise capacity.
A. Gibb: None. **A. Bhatnagar:** None. **B. Hill:** None.

388
High-throughput Single Cell Tracking of Mitochondrial Function in Cardiomyocytes
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Mitochondria play a significant role in the regulation of multiple functions in the heart, ranging from metabolism to cell death. Most mitochondrial assays require either isolating the organelle, thus disrupting the intracellular signaling or using intact cells with average measurements for the entire population neglecting the ability to distinguish cell heterogeneity. Here we describe a novel method for tracking single cell mitochondrial function in cell populations over time. Adult mouse myocytes were exposed to the mitochondrial uncoupler FCCP and hydrogen peroxide to induce changes in membrane potential and oxidative stress, respectively. TMRM and mitosox fluorescence were used to quantify mitochondrial membrane potential and ROS production, Fluo-4 fluorescence was used to assess intracellular calcium. Tracking of single cells was performed using Matlab and Imaris software. After FCCP exposure TMRM signal intensity decreased before there was a significant change in myocyte length that led to hypercontracture and cell death within 10 minutes. To better understand the dynamics of mitochondrial function and its relationship with cell death a dose response curve was established for hydrogen peroxide at 100, 500 and 1000 µM. The higher doses of hydrogen peroxide induced hypercontracture faster than lower doses. For further studies 100 µM was used to assess how homogenous the response to hydrogen peroxide was in cell populations. We found 3 distinct populations of cells responding at different times, a population of cells hypercontracted between 7-10 min, another between 10-15 min and a third population only after 15 min. Changes in membrane potential, oxidative stress and intracellular calcium were simultaneously assessed for every single cell during these time points. In addition, given the organized structure of the mitochondria in the myocyte we have adapted our technique to track individual mitochondria to study heterogeneous responses at the single mitochondrion level. This method provides a unique tool to simultaneously assess multiple parameters of mitochondrial function in single cells over time. In doing so, we have unmasked a complex heterogeneity of single cell behavior that is lost in methods than only average cell populations.
G. Fajardo: None. **K. Bezold:** None. **T. Meyer:** None. **D. Mochly-Rosen:** None. **D. Bernstein:** None.

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Alterations of Mitochondrial Calcium Handling in Heart Failure
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Heart failure (HF) and sudden cardiac death (SCD) are major public health concerns that are increasing in incidence, yet the mechanisms underlying SCD in patients with HF are poorly understood. In a novel guinea pig model of HF/SCD, we showed that in vivo treatment with a mitochondrial Na+/Ca2+ exchanger (mNCE) inhibitor attenuates cardiac remodeling, preserves cardiac contractile function, and improves survival, supporting a critical role for altered mitochondrial Ca2+ dynamics in the pathophysiology. Here, we investigate whether the intrinsic mitochondrial Ca2+ transport rates are altered in this HF model. Methods: Ascending aortic constriction, combined with daily i.p. injection of isoproterenol (ISO), were used to induce HF (ACi) with acquired long QT. This group was compared with animals subjected to aortic constriction alone (AC), or sham-operated animals with (SHAMi) or without (SHAM) ISO treatment. Ca2+ Green-5N was used to measure total mitochondrial Ca2+ uptake and to quantify mitochondrial Ca2+ influx and efflux rates in isolated cardiac mitochondria.

Results: Both the total mitochondrial Ca2+ load and the Ca2+ capacity prior to triggering permeability transition pore (mPTP) opening were reduced in HF mitochondria (5mM NaCl present). Mitochondrial Ca2+ fluxes, individually measured with sequential additions of 15μM free Ca2+, 10nM Ru360 and 5mM NaCl, showed that initial Ca2+ uptake rate through the mitochondrial Ca2+ uniporter (mCU: 0.55 nmol/sec/mg) was not significantly changed in HF; however, the Ca2+ extrusion rate through mNCE was larger in HF (AC:0.022 nmol/sec/mg; SHAM:0.018; ACi:0.013; SHAMi:0.009), but with a lower affinity for Na+. Interestingly, Na+-independent efflux via mPTP increased in HF (AC:0.0040 nmol/sec/mg; SHAM:0.0022; ACi:0.0013; SHAMi:0.012). Mitochondria from failing hearts also showed decreased respiration and increased ROS emission.
Conclusions: The data indicate that an increase of intrinsic Ca2+ efflux and the increase in cytoplasmic Na+ in HF could both contribute to blunted mitochondrial Ca2+ in HF, which will affect cardiac energetics and ROS balance. Inhibitors of mNCE or mPTP are thus proposed to be therapeutic interventions that would improve mitochondrial Ca2+ balance and function in HF.

A. Wei: None. **T. Liu:** None. **B. O'Rourke:** None.

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End-stage Human Heart Failure is Characterized by a Deficit in Energetic Lipids in Both Diabetic and Non-diabetic Patients

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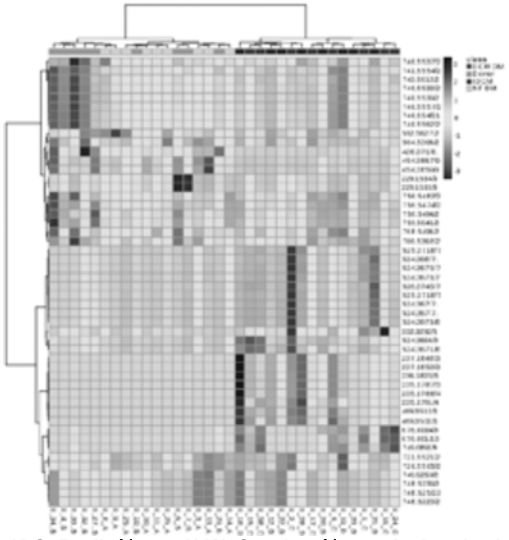
Introduction. Animal models and human studies have identified increased intramyocardial lipid accumulation and a lipotoxicity hypothesis has been emerging as a mechanism of myocardial dysfunction in diabetes. We have identified a significant *decrease* in energetic lipids in chronic advanced *non-diabetic* heart failure. We hypothesized that intramyocardial lipid species would be increased in diabetic as compared to non-diabetic end-stage heart failure patients.
Methods Left ventricular samples procured at the time of orthotopic heart transplantation from non-diabetic (IDCM n=8) and diabetic (DCMDM n=8) patients as well as organ donors with (NFDM) and without a history of diabetes (Donor) were quantitated for lipids with high-resolution mass spectrometry . Stable isotope labeled essential nutrient in cell culture internal standards for acyl-CoAs were generated using [$^{13}C_3$ $^{15}N_1$]-pantotheonate in Hepa1c1c7 cells.
Results The lipidomic signature of end-stage failing myocardium marked by a significant decrease in energetic lipids, a decreased myocardial Succinyl CoA (p-value 0.0079 for failing versus non-failing diabetic subjects) and a decreased ratio of [Succinyl CoA]/[Acetyl Coa] consistent with deficient TCA cycling, is indistinguishable in diabetic and non-diabetic patients (Figure 1).
Discussion Despite the known bioenergetic deficits in insulin-resistant diabetes, we have not identified any significant differences between diabetic and non-diabetic subjects in the lipidomic signature of end-stae failing myocardium. Future studies are needed with focused metabolomics to elucidate differences in the diabetic phenotype of human heart failure.



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Endogenous Drp1 Modulates Cardiac Respiration Through mPTP and Independent of Fission

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BACKGROUND: The cardiac mitochondria exhibit a stable morphology with a rather low level of dynamic changes. However, fission and fusion proteins, such as dynamin-related protein 1 (DRP1) are abundant in the heart. Whether these proteins bear other functions in the heart than mitochondrial dynamics regulation are largely unknown. We hypothesize that endogenous DRP1 in the heart regulates mitochondrial respiration independent of fission.
METHODS: Mitochondrial respiration was determined by measuring the OCR with Seahorse assay or Clark type electrode in adult rat cardiomyocytes or mitochondria isolated from adult mouse heart. Confocal imaging was used to quantify mitochondrial morphology in adult cardiomyocytes and H9C2 myoblasts. To evaluate the role of mitochondrial permeability transition pore (mPTP), we monitored superoxide flashes (SOF) and laser-induced mPTP openings, and used cyclophilin D knockout mice (CypD KO). Mitochondrial ROS and Ca2+ were also monitored.
RESULTS: Inhibiting the DRP1 GTPase activity by Mdivi-1 or overexpression of the dominant-negative mutant (DRP1-K38A) induced mild mitochondrial morphological changes in adult cardiomyocytes, and inhibited mitochondrial respiration. Modulation of fission/fusion by overexpressing DRP1 or treating cells with S3, a compound facilitates fusion, exhibited significant morphological changes, but failed to influence respiration. Therefore, endogenous DRP1 activity may regulate respiration in the heart and this effect is dissociated with morphological changes. Further, inhibiting DRP1 activity attenuated the frequency of SOF, indicating decreased transient mPTP openings, delayed laser-induced permanent mPTP opening, and increased mitochondrial Ca2+. Inhibiting DRP1 activity decreased mitochondrial ROS levels. The role of DRP1 inhibition on respiration absents in CypD KO myocytes, suggesting the involvement of mPTP in the modulation of respiration by endogenous DRP1.
CONCLUSION: These results suggest that endogenous DRP1 positively regulates respiration in the heart. This

effect is likely independent of its role in mitochondrial fission. DRP1 regulation of respiration may involve transient opening of mPTP and contribute to mitochondrial Ca2+ and ROS signaling.
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Metabolic Signaling Mechanisms Governing Heart Regenerative Capacity
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Energy metabolism and metabolic signaling circuits orchestrate cell commitment to self-renewal, lineage specification, differentiation and regeneration. When energy resources are plenty, cell can grow, proliferate and regenerate, however when energy is low - augmented adenylate kinase (AK)-mediated AMP signaling turns on AMPK which silences p53/p21/cyclin cell cycle metabolic checkpoint and halts cell division. Using mouse neonatal hearts, with high and low regenerative capacity, we have determined metabolomic profiles and dynamics of phosphotransfer circuits using 18O-phosphoryl labeling mass-spectrometric and 18O-assisted 31P NMR techniques. We demonstrate that loss of heart regenerative capacity after birth is associated with marked changes in heart AK-catalyzed phosphotransfer flux and AMP signaling along with changes in expression levels of p21, cyclin A and E and thymidine kinase. It appears, that in adult heart increased expression of AK isoforms (AK1, AK2 and AK1β) and augmented high energy phosphoryl and AMP signal dynamics is misread by AMPK-sensor as "low energy" state inducing blockade of cell cycle metabolic checkpoint and cardiomyocyte proliferation and renewal. Using AK-GFP constructs and immunocytochemistry we further demonstrate the distribution of AK1, AK1β, AK2 and AMPK between cytosol and nucleus and association with mitotic spindle and cytokinetic apparatus during cell division cycle. AK1 translocation to the nucleus, however, doesn't occur in adult cardiomyocytes deficient in cytokinesis. Protein knockdown using siRNA indicates that AK2 is critical for cardiomyocyte mitochondrial biogenesis and network formation. Furthermore, we have discovered that deficiency of the AK2 isoform, which is localized in mitochondria intermembrane-intracristae space, arrests developmental programming and is embryonically lethal in mice. The uncovered shift in metabolic signaling mechanisms opens new avenues for targeted regulation of heart regenerative potential critical for repair of injured hearts.
S. Zhang: None. **P. Dzeja:** None.

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MCL-1 Promotes Survival and Influences Mitochondrial Dynamics in Cardiac Myocytes
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The BCL-2 family proteins are important regulators of mitochondrial structure and integrity. MCL-1 is an anti-apoptotic BCL-2 protein that is highly expressed in the myocardium compared to the other anti-apoptotic proteins BCL-2 and BCL-X_L. Recently, we reported that MCL-1 is essential for myocardial homeostasis. Cardiac-specific deletion of MCL-1 in mice led to rapid mitochondrial dysfunction, hypertrophy, and lethal cardiomyopathy. Surprisingly, MCL-1 deficient myocytes did not undergo apoptotic cell death. Instead, the cells displayed signs of mitochondrial deterioration and necrotic cell death, suggesting that MCL-1 has an additional role in maintaining mitochondrial function in cardiac myocytes. Similarly, deletion of MCL-1 in fibroblasts caused rapid mitochondrial fragmentation followed by cell death at 72 hours. Interestingly, the MCL-1 deficient fibroblasts retained cytochrome *c* in the mitochondria, confirming that the cells were not undergoing apoptotic cell death. We have also identified that MCL-1 localizes to the mitochondrial outer membrane (OM) and the matrix in the myocardium and that the two forms respond differently to stress. MCL-1_{OM} was rapidly degraded after myocardial infarction or fasting, whereas MCL-1_{Matrix} levels were maintained. Similarly, starvation of MEFs resulted in rapid degradation of MCL-1_{OM}, whereas MCL-1_{Matrix} showed delayed degradation. Treatment with the mitochondrial uncoupler FCCP led to rapid degradation of both forms. This suggests that the susceptibility to degradation is dependent on its localization and the nature of the stress. Our data also suggests that these two forms perform distinct functions in regulating mitochondrial morphology and survival. Overexpression of MCL-1_{Matrix} promoted mitochondrial fusion in fibroblasts under baseline conditions and protected cells against FCCP-mediated mitochondrial fission and clearance by autophagosomes. Thus, our data suggest that MCL-1 exists in two separate locations where it performs different functions. MCL-1_{Matrix} promotes mitochondrial fusion, which protects cells against excessive mitochondrial clearance during unfavorable conditions.
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Transient Bone Morphogenic Protein Antagonism Directs Differentiation of iPSCs into the Cardiac Neural Crest and cKit+ Myocardial Progenitor Lineages
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INTRODUCTION: The capability of cKit+ cardiac progenitor cells (CPCs) to participate in cardiomyocyte regeneration remains controversial, despite basic and clinical studies supporting such a role. HYPOTHESIS: A non-permissive cardiac milieu minimizes the generation of cardiomyocytes from CPCs. METHODS: We lineage-traced CPCs using novel dual-recombinase responsive indicator mice (cKitCreERT2;Wnt1::Flpe;RC::Fela) and iPSCs derived from cKitCreERT2;IRG (iPSCKit) mice. RESULTS: Intersectional genetic fate-mapping of cKitCreERT2;Wnt1:: Flpe;RC::Fela embryos supported that cKit marks Wnt1-expressing cardiac neural crest (CNC) progenitors, emerging at ~E9.5 and contributing a limited number of cardiomyocytes. To decipher the mechanisms underlying cardiomyocyte differentiation of CPCs, we lineage-traced CPCs during stage-specific cardiogenic differentiation of iPSCKit. Ascorbate treatment promoted differentiation of cKit+ iPSC-derived embryoid bodies (EBs) into Nkx2.5+ myocardium, 45.5%±6.7% of which co-expressed the Cre-reporter EGFP (n=154 EBs; 12 preparations), suggesting that CPCs encompass fully competent cardiomyogenic progenitors. Noggin (or Dorsomorphin), a BMP antagonist transiently expressed in the heart at E7.5-E8.5 but not during CNC invasion, directed the differentiation of iPSCkit-EBs into Mesp1+/Isl1+/Nkx2.5+ cardiac mesoderm progenitors ($p \leq 0.0001$). Remarkably, the same signaling pathway subsequently directed EBs into the cKit+/Wnt1+/Pax3+/Mitf-H+/Isl1+/Nkx2.5+ CNC lineage ($p \leq 0.0001$), while suppressing the generation

of WT1+/Tbx18+ epicardium (p<0.05). Stage-specific induction of Cre-recombination delineated that iPSCkit-derived CPCs encompass Mesp1−/cKit+/Nkx2.5+ CNC progenitors which contributed EGFP+ CNC derivatives, including Nkx2-5+ cardiomyocytes, to 60.7%±7.3% of spontaneously beating EBs (n=147 EBs; 12 preparations). CONCLUSIONS: Collectively, our data show that CPCkit are fully competent CNC-derived cardiomyogenic progenitors, whose differentiation to cardiomyocytes is minimized by a latent Noggin-mediated signaling pathway. Therefore exploiting CPCkit therapeutically, provides an important strategy for maximizing myocardial regeneration.

K.E. Hatzistergos: 7. Ownership Interest; Modest; Vestion Inc.. 8. Consultant/Advisory Board; Modest; Vestion Inc.. **L.M. Takeuchi:** None. **D. Saur:** None. **B. Seidler:** None. **S.M. Dymecki:** None. **J. Mai:** None. **R. Kanashiro-Takeuchi:** None. **W. Balkan:** None. **J.M. Hare:** 7. Ownership Interest; Modest; Vestion Inc. 8. Consultant/Advisory Board; Modest; Vestion Inc.

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Transient Inactivation of Retinoblastoma Induces De-Novo Cardiomyogenesis From Human Myocardial Progenitors but Not Pre-Existing Cardiomyocyte Replication

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INTRODUCTION: Activation of cardiac cell cycle re-entry is considered the primary therapeutic strategy for cardiomyocyte (CM) regeneration. However, the role of cardiac cell-cycle control in cardiomyogenesis remains elusive. Here, we combined RNA interference and stem cell modeling to investigate the role of Retinoblastoma (RB) in human cardiomyogenesis. HYPOTHESIS: RB regulates proliferation and differentiation of cardiac progenitors (CPCs) but not CM replication. METHODS: H9 human embryonic stem cells (hESCs) stably expressing tetracycline (tet)-inducible shRNAs against RB (hESCshRB) or hemagglutinin-tagged RB (hESCHA-RB) were tet-induced at selected time-points during or after CM differentiation. RESULTS: Analysis of ser-608 illustrated stage-specific differences in the degree of RB inactivation during normal hESCs-cardiogenesis. Transient shRB knockdown in hESCshRB-derived embryoid bodies (EBs) during the CPC-stage (EB-days 5-8), significantly upregulated GATA4, ISL1, CTNNI, and cKit transcription (p<0.05), while increasing the yield of beating EBs by 2.4-fold (n=6/group, p<0.0001 vs. vehicle). Gene-expression arrays of 22 RB-related genes, illustrated that shRB-knockdown upregulated CCND1, CCND2, CCND3, and CDK4, CDK6 (p<0.05), followed by a 3.6-fold increase in E2F3 (p<0.05) expression. Moreover, expression of p107 and p130, p27, p57, ARF and CDKN3 were also significantly increased (p<0.05), whereas TP53 and MDM2 remained unchanged. Ectopic HA-RB in CPCs did not significantly affect cardiogenesis (n=18). Conversely, shRB knockdown in EB-day 60-derived CMs (n=15) did not stimulate cell cycle re-entry, as assessed by analysis of EdU incorporation and Aurora-B kinase (AurB). Remarkably, co-culture of hESCHA-RB-derived CMs with adult cardiac (CSCs) and/or mesenchymal (MSCs) stem cells (n=15/group), increased cell-cycle re-entry ~2.8-fold, assessed by ser-10 Histone H3 (p=0.0002) and AurB (p<0.0001). CONCLUSIONS: These findings suggest that RB regulates proliferation and differentiation of human CPCs in a cell-autonomous manner, via a CCND-CDK4/6-E2F3 mechanism. Conversely, CM replication may be enhanced via cell-cell interactions with MSCs and/or CSCs, but not cell-autonomously via RB inactivation.

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Uncontrolled Endothelial Migration In Hereditary Hemorrhagic Telangiectasia: Disease Modeling With Ips Cell

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Introduction: Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant heritable disease caused by mutation of Activin like receptor-1 (ALK1), an endothelial specific TGF-β family receptor. The histological hallmark in HHT is abnormal atriovenous communication, caused by abnormal migration of endothelial cells (ECs). These findings suggest that the enhanced migration of ECs would have a role in the development of HHT. However, how EC migration is regulated in HHT remains unclear. Hence, the aim of this study is to develop an in vitro HHT model with ECs derived from patient-specific induced pluripotent (iPS) cells.

Methods: We generated iPS cells from a HHT patient with ALK1 mutation (ALK1mt) and two control subjects (WT). ECs were derived from these iPS cells with prespecified differentiation method. We compared the effects of BMP-9, a selective agonist of ALK1, between ALK1mt- and WT-ECs. Also, microarray analysis comparing transcriptome of ALK1mt- and WT ECs with or without BMP-9 stimulation was conducted.

Results: Migration capacity was significantly reduced with BMP-9 in WT-ECs, but not in ALK1mt-ECs. With BMP-9 stimulation, elevation of genes associated with vascular stabilization and maturation was observed in WT-ECs but not in ALK1mt-ECs.

Conclusion: With ECs derived from iPS cells, modeling of HHT phenotype, uncontrolled endothelial migration, was achieved.

Clinical implications:

By elucidating the biological process with which BMP-9 controls the migration of endothelial cells, new drug targets for HHT may be found. Also, these result may lead to the development of a new experimental drug screening system for HHT.

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Generation of Raf1 Mutant and Crispr-cas9 Corrected Isogenic iPSC-derived Cardiomyocytes to Model Hypertrophic Cardiomyopathy in Noonan Syndrome

Fabrice Jaffré, Beth Israel Deaconess Medical Ctr, Boston, MA; Gang Wang, Amy Roberts, William Pu, Boston Children's Hosp, Boston, MA; Andreas Hahn, Univ Hosp Giessen, Giessen, Germany; Maria Kontaridis, Beth Israel Deaconess Medical Ctr, Boston, MA

Background: Hypertrophic cardiomyopathy (HCM) is a major cause of death in infants and children. Noonan Syndrome (NS), an autosomal dominant RASopathy disorder, is characterized by multiple defects, including short stature, facial dysmorphia, and congenital heart defects that include HCM. RASopathies are caused by germ-line mutations that affect the canonical RAS-MAPK pathway. Indeed, 95% of NS patients with a mutation in *Raf1*, a gene that plays an integral role in

this signaling cascade, exhibit HCM. However, the molecular mechanisms that elicit HCM in these patients remain poorly understood.
Objective: To generate human NS *Raf1* induced-pluripotent stem cells (iPSCs), correct the mutation by genome editing and subsequently differentiate isogenic iPSC lines into cardiomyocytes to characterize the molecular and genetic basis of HCM in NS patients.
Results: We generated iPSCs from skin fibroblasts obtained from a NS pediatric patient with a single point mutation in the *Raf1* gene. Using electroporation of four episomal vectors containing the Yamanaka factors, we obtained several iPSC clones with normal karyotypes and strong expression of pluripotent markers (Nanog, Oct4, Lin28, Sox2) as detected by RT-qPCR and immunofluorescence. We next corrected the mutation in the NS *Raf1* iPSCs using genome editing CRISPR-Cas9 nickase technology. Correction of the *Raf1* mutation was determined at the clonal level by PCR followed by Restriction Fragment Length Polymorphism and confirmed by Sanger sequencing. In addition, by inducing a Cas9 nickase-dependent frame shift mutation we also generated an isogenic iPSC line where *Raf1* gene was knockout (KO), as demonstrated at the RNA level by RT-qPCR and at the protein level by Western Blot. We next differentiated these multiple iPSC lines (mutant, corrected and KO) into isogenic beating cardiomyocytes, with more >98% of the cells positive for specific cardiomyocyte markers (α-actinin and cardiac TroponinT).
Conclusion: We have successfully generated human NS *Raf1* isogenic iPSC lines and corresponding cardiomyocytes. Currently, we are in progress of characterizing these cardiac cells to determine the molecular basis of NS-dependent HCM. Ultimately, our work should reveal new targets to treat HCM in NS patients.
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Reactive Oxygen Species in Myocardial Ischemic Injury and Protection
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Myocardial injury following ischemia/reperfusion (I/R) is a common clinical scenario in patients suffering from ischemic heart disease. An excessive production of reactive oxygen species (ROS) during the early phase of reperfusion following myocardial ischemia has been proposed to contribute to reperfusion injury. Paradoxically, the ROS has also been recognized as a trigger of pro-survival signaling pathways mediating cardioprotection at a low level. However, the precise mechanisms for the dual role of ROS have not yet been fully clarified. To address this question, using intermittent hypobaric hypoxia (IHH) as a cardioprotective model, combining with H2O2 pre- and post-conditioning, we studied the level and roles of ROS during early reperfusion following ischemia in I/R injury and protection and explored the regulatory mechanisms underlying. Our results reveal that the elevated ROS generated during early reperfusion are injurious but insufficient to reach the threshold to trigger protective signaling pathways. The moderate level of ROS, higher than that elevated by I/R, during early reperfusion is critical for triggering cardioprotection against I/R injury via alleviating intracellular Ca2+ overload and preserving mitochondrial function through the efficient activation of Akt, PKCε and JAK2/STAT3 pathways. We then identified the downstream target of ROS-JAK2/STAT3 signals that interacts with the sarcoendoplasmic reticulum Ca2+-ATPase 2 (SERCA2) to improve the activation of SERCA2 and subsequently regulate intracellular Ca2+ homeostasis. When the heart is exposed to excessive ROS, the activation of protective mechanisms reaches a plateau and fails to counteract the severe nonspecific oxidative stress. These findings provide a new angle to interpret the controversial roles of ROS in myocardial I/R and demonstrate that the differential effects of ROS in myocardial I/R are derived from a quantity-dependent wrestling between its detrimental and signaling roles.
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Regulated Levels of Sarcolemmal Membrane Protein Isoform 3 Impact Cardiac Function in Mice
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The sarcolemmal membrane-associated proteins (SLMAPs) are a family of tail-anchored membrane proteins generated by alternative splicing of the SLMAP gene. A ubiquitously expressed SLMAP isoform 3 encompasses an N-terminal FHA domain with extended coiled-coil structure and has been implicated in cell cycle control. Heart function in transgenic mice with cardiac-specific overexpression of SLMAP3 cDNA driven by α myosin heavy chain promoter was evaluated by echocardiography. qPCR and western blot were used to analyze gene and protein expression respectively. Structure and fibrosis was analyzed by H&E and Masson's Trichrome staining. Function analysis showed a 15% ($p<0.05$) decrease in ejection fraction and 19% ($p<0.05$) decrease in fractional shortening in transgenic mice as early as 5 weeks and persisted into old age at 44 weeks. Transgenic mice presented a mild systolic dysfunction and a trend towards dilated cardiomyopathy without any premature death. Natriuretic peptide ANP and BNP levels were not changed and there was no difference in left ventricular mass or activation of the hypertrophic factor Akt1 in SLMAP3 expressing myocardium. However, significant changes in calcium handling proteins with a significant decrease in phosphorylation of phospholamban ser16 ($p<0.05$) along with a down-regulation of sarco-endoplasmic reticulum Ca2+ ATPase protein and increased ryanodine receptor 2 phosphorylation ser2808 ($p<0.05$) were noted at 5 weeks of age in transgenic hearts. These data indicate that increased SLMAP3 levels did not influence cardiac remodeling or hypertrophic growth but did impact membrane biology of calcium transport systems in myocardium leading to depressed contractility. Thus regulated levels of SLMAP3 are important to support normal heart function.
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Shortening of Action Potential Duration With Increased Work in Contracting Rabbit Heart
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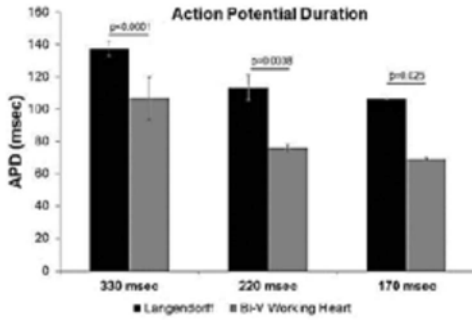
Studying heart function in contracting hearts at higher workloads provides crucial knowledge of cardiac performance in high-stress situations. Ratiometric optical mapping of fully loaded hearts is a novel method to study electrical activity while replicating in vivo energy consumption.
We predict that an imbalance between energy supply

and demand during increased work and hypoxia will manifest in shortened action potential duration (APD), especially in the more physiologically-relevant bi-ventricular (BiV) working heart.
Rabbit hearts were perfused with oxygenated Krebs-Henseleit solution in unloaded Langendorff and fully-loaded BiV mode. Epicardial action potentials (APs) were measured using optical mapping of Di-4-ANEPPS. Excitation ratiometry using 450 and 505 nm illumination on alternate camera frames together with motion tracking removed motion artifact. Aortic pressure, left atrial preload, and LVDP was measured. A range of workloads were studied by pacing at 330, 220, and 170 ms cycle length (CL). Gradual hypoxia was induced by bubbling with N2 gas.
In Langendorff mode, the APD was 137.67±4.29 ms, 113.44±7.89 ms, and 106.44±0.44 ms at CL of 330ms, 220ms, and 170ms, respectively, while in BiV mode, the APD was 106.56±13.03 ms, 78.00±2.33 ms, and 69.33±0.77 ms. Aortic pressure dropped from NSR in BiV hearts by 1.26% at 330ms, 4.11% at 220ms, and 11.65% at 170ms CL.
Shortening of APD, independent of restitution, with increasing HR indicates an imbalance of energy supply and demand with greater workload. KATP channels are implicated. This novel method of optical mapping reveals important implications of electrophysiological changes during high-stress situations.



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Selective Protein Secretion Upon Endoplasmic Reticulum Stress Protects Cardiac Myocytes
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Protein secretion is important for proper cardiac myocyte function. Many secreted proteins are synthesized and folded in the sarco- endo-plasmic reticulum (SR/ER). A number of diseases, including heart disease, alter the ER in ways that impair ER protein folding, causing ER stress, which can result in cardiac myocyte dysfunction and decreased viability. In studies aimed at assessing the effects of ER stress on cardiac myocyte viability, heart disease-related ER stress was mimicked by treating neonatal rat ventricular myocytes (NRVM) with either tunicamycin (TM) or thapsigargin (TG), which inhibit SR/ER protein glycosylation or decrease SR/ER calcium, respectively. When treated in high culture media volumes, both TM and TG caused cardiac myocyte death; however, in low culture media volumes, while TM still caused death, remarkably, TG was protective, suggesting that potentially protective factors were secreted in response to TG but not TM. To characterize these factors, the identities of proteins in control-, TM-, and TG-conditioned medium from NRVM were determined by proteomic approaches using high performance liquid chromatography and mass spectrometry. Twenty-four different proteins, known to be synthesized in the ER, were identified in control-conditioned medium. The levels of eighteen of these proteins, including extracellular matrix proteins, hormones, and growth factors were decreased in TM- and TG-conditioned medium. However, the levels of three SR/ER-resident, calcium-binding chaperones, glucose regulated protein 78 (GRP78), glucose regulated protein 94 (GRP94), and calreticulin were increased in TG-conditioned medium but not in TM-conditioned medium. Furthermore, we found that ischemia/reperfusion, which decreases SR/ER calcium, upregulated secretion of the proteins selectively secreted in response to TG. Thus, while ER stress mediated by TM or TG decreases the movement of most proteins through the secretory pathway, TG, which mimics the effects of heart disease on SR/ER calcium in cardiac myocytes, selectively enhances the secretion of a subset of proteins, which confer protection. Therefore, proteins once thought to be permanent residents of the SR/ER may have novel, extracellular, protective roles in the diseased heart.
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Mitochondrial Complex II is a Source of the Reserve Respiratory Capacity that is Regulated by Metabolic Sensors via Sirtuin 3
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A cell's survivability depends on its ability to meet its energy requirements. We hypothesized that the cells' mitochondrial reserve respiratory capacity (RRC) is a critical component of its bioenergetics that can be utilized during an increase in energy demand, thereby, enhancing viability. Our goal was to identify the elements that regulate and contribute to the development of RRC and its involvement in cell survival. Our results show that development of RRC is dependent on metabolic substrate availability in a cell type-dependent manner. While the neonatal rat cardiac myocytes (NRCM) utilize glucose as the main substrate, developing a RRC [1.4-2.5 fold higher than basal oxygen consumption rate (OCR)] required fatty acids in addition to glucose. Accordingly, inhibition of either glucose or fatty acid oxidation separately, completely abrogated RRC, while having little impact on basal OCR, which is sustainable with either substrate or glutamate in the medium. Conversely, RRC was enhanced (1.4-1.8 fold) through increasing glucose oxidation via inhibiting pyruvate dehydrogenase kinase with dichloroacetate, or through increasing fatty acid oxidation via activation of AMP-activated kinase (AMPK). The latter was partly mediated through peroxisome proliferator-activated receptor alpha. These results suggested that RRC is an independently regulated entity of the cells' bioenergetics. An electron flow activity assay revealed that the increase in RRC correlated with a specific increase in complex II (cII) activity. Inhibiting or disassembling holo cII completely abolished RRC, accompanied by a slight decrease in basal OCR (0.82-0.9 fold), thus confirming it as the source of RRC. Moreover, the development of RRC required Sirtuin (Sirt)3. Functionally, we show that enhancing RRC via fatty acid oxidation with 5-Aminoimidazole-4-carboxamide 1-β-Dribofuranoside in NRCM results in a burst of cII-dependent oxidative phosphorylation accompanied by reduced superoxide production and enhanced cell survival post-energy deprivation conditions. Thus, for the first time, we show that metabolic sensors increase the cells' RRC via activating

cII in a Sirt3-dependent manner, and that this mechanism can be exploited for increasing cell survival after hypoxia.
J. Pfleger: None. **M. He:** None. **M. Abdellatif:** None.

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High-throughput Screening Reveals the Mitochondrial Complex I Inhibitor Nornicotine is Cardioprotective in Ischemia-reperfusion Injury When Delivered at Reperfusion
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Background: To date, there are no FDA-approved therapies for the reduction of infarct size in acute myocardial infarction. Previously, we developed a cell-based phenotypic assay of ischemia-reperfusion (IR) injury, which was used to identify novel cytoprotective agents delivered prior to ischemia. Herein, we sought to identify cytoprotective agents in a more clinically relevant model: drug delivery at reperfusion, and to investigate possible underlying mechanisms of protection.
Methods: Primary adult mouse cardiomyocytes were subjected to simulated IR injury using a modified Seahorse XF24 apparatus with drug addition at the onset of reperfusion. Cell death was estimated using LDH release. Drugs which protected cardiomyocytes in vitro were tested in a Langendorff model of IR injury, measuring functional recovery and infarct size. In separate experiments, metabolites extracted from perfused hearts were resolved by HPLC.
Results: Nornicotine was identified as a cardioprotective agent in the screen. In perfused hearts, 10 nM nornicotine injected at the onset of reperfusion improved functional recovery and decreased in infarct size (13.1% ± 2.4 vs 49.2% ± 2.5 in non-treated hearts, $p<0.05$, n=16-20). Nornicotine also exhibited profound inhibitory effects on mitochondrial complex I activity.
Succinate is known to accumulate in ischemia, and its rapid consumption during early reperfusion exacerbates reperfusion injury via ROS generation from electron backflow through complex I [PMID: 25383517]. In non-treated hearts, we confirmed that high post ischemic levels of succinate rapidly declined during the first 2 min of reperfusion. In contrast, nornicotine slowed post-ischemic succinate consumption, suggesting that electron backflow through complex I is the major pathway driving succinate consumption.
Conclusions: Herein, we demonstrated that nornicotine was cardioprotective when delivered at early reperfusion in vitro and ex vivo. The mechanism of cardioprotection may be due to inhibition of rapid succinate consumption during early reperfusion via reverse electron flow back through complex I.
J. Zhang: None. **M.K. Karcz:** None. **S.M. Nadtochiy:** None. **P.S. Brookes:** None.

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Inhibition of Class I Histone Deacetylases at Reperfusion Attenuates Ischemia-reperfusion Injury and Modifies Mitochondrial Acetylation
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Although rapid reperfusion of ischemic tissue is the treatment of choice for myocardial infarction, much of the resultant damage occurs as a consequence of reperfusion itself. Previously, we have shown that pretreatment with MS-275, a selective class I histone deacetylase (HDAC) inhibitor, preserves left-ventricular (LV) function and substantially reduces the area of infarcted tissue in isolated rat hearts subjected to ischemia-reperfusion (IR) injury. Here, we tested the hypothesis that MS-275 treatment at reperfusion reduces LV tissue damage and improves post-ischemic LV contractile function. To do this, hearts from male Sprague-Dawley rats were isolated and perfused ex vivo on a Langendorff perfusion apparatus. A saline-filled balloon was inserted into the left ventricle of the heart to monitor ventricular pressure development throughout the experiment. Hearts were subjected to 30 minutes of ischemia, followed by 60 minutes of reperfusion. MS-275 was administered during the entire reperfusion phase, and resultant functional data were compared to untreated hearts. There was no difference in any metric of pre-ischemic contractile function between groups. 10nM MS-275 administered at reperfusion significantly improved multiple measures of LV function, including dP/dtmax, -dP/dtmax, developed pressure and rate pressure product. We also observed a significant reduction in infarct area of treated hearts compared to control, as measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Unexpectedly, mass spectrometry analysis revealed significant changes in acetylation state of multiple mitochondrial enzymes. Administration of MS-275 during the reperfusion phase of IR is sufficient to partially rescue LV function from reperfusion-induced damage. This study emphasizes the importance of exploring class I HDAC inhibitors for protection against ischemia-reperfusion.
D.J. Herr: None. **S.E. Aune:** None. **D.R. Menick:** None.

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Interaction of T Cells From Non-ischemic Heart Failure Patients With the Activated Vascular Endothelium is Dependent on Intercellular Adhesion Molecule-1
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Background: Increasing evidence supports a role for inflammation in the pathogenesis of chronic heart failure (HF). Our previous studies demonstrate that T cell recruitment to the heart contributes to the progression of non-ischemic pressure overload induced HF. However, clinical data describing T cell dependent mechanisms contributing to the etiology of this disorder remains unknown. We hypothesized that non-ischemic HF activates human T cells resulting in increased adhesion to the vascular endothelium and recruitment to the heart through mechanisms involving specific endothelial adhesion molecules.
Methods and Results: We used T cells from non-ischemic HF patients and non-HF controls as well as left ventricular (LV) tissue from end stage HF after LV assisted device (LVAD) support. Using FACS analysis we found that systemic T cells are significantly elevated in HF patients compared to controls ($p<0.05$), including Th1, Th17, and Treg cells. Immunohistochemistry analysis revealed that CD3+ and CD4+ T cells infiltrate the LV of HF subjects which were not observed in control. To evaluate the mechanisms through which T cells interact with the vasculature and potentially infiltrate the heart, we used in vitro real time video microscopy under shear flow conditions and found that T cells from HF patients firmly adhered to TNF[[Unsupported Character - Symbol Font ]] activated HUVECS. Further analysis indicated that HF T cells adhered to ICAM-1, but not to VCAM-1 in higher numbers than control T cells. The surface expression levels of the integrin ligands for VCAM-1 and ICAM-1 (VLA-4 and LFA-1 respectively) were similar between both groups, however, HF T cells exhibited a highly polarized phenotype on ICAM-1 ($p<0.005$ vs control), suggesting LFA-1 is in its high affinity conformation in HF T cells.
Conclusions: Our findings suggest that T cells are activated in non-ischemic HF and have high affinity for the activated endothelium through mechanisms involving ICAM-1- LFA-1 adhesion. Future studies will evaluate the mechanisms regulating LFA-1 activation in HF T cells and their contribution to cardiac remodeling.

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Modulation of Delta-Like Ligand-4 Signaling Improves Myocardial Revascularization Following Coronary Ischemia

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Delta-like ligand-4 (DLL4) is an arterial-specific Notch ligand and balanced signaling by DLL4 is required for functional neovascularization. We hypothesized that modest inhibition of DLL4 signaling improves myocardial revascularization following coronary ischemia. Myocardial infarction was induced by ligation of mid-left anterior descending (LAD) artery via rapid left thoracotomy in 6-8 week-old male mice with inducible endothelial-specific knockout or overexpression of DLL4. DLL4 silencing in homozygous mice on the day of or 5 days prior to LAD ligation resulted in significant reduction in revascularization. At four weeks, explanted hearts showed 1.8-fold fewer vessels in LAD territory, 3-fold greater myocardial hypoxia, 1.7-fold larger fibrotic scar and 17% increased perfusion defect in the left ventricle (LV) by scintigraphy. CT angiography confirmed fewer collateral vessels. Echocardiography showed increased LV dilation (37% higher end-diastolic volume) and 12% greater reduction in ejection fraction (EF) compared to baseline (all $p<0.001$). Overexpression of DLL4 showed a similarly worse outcome. Conversely, partial DLL4 knockout in heterozygous animals resulted in improved outcomes in all parameters. For translational application, animals were systemically administered 1.5mg/kg or 3mg/kg DLL4-Fc intraperitoneally three times a week beginning the day of ligation. RT-PCR analysis of downstream molecules confirmed that systemic DLL4-Fc partially inhibits Notch signaling in endothelial cells in the ischemic LV. When LAD was ligated very proximally, DLL4-Fc improved survival. With mid-LAD ligation, DLL4-Fc induced a dose-dependent increase in number of CD 31-positive vessels and by CT angiography. There was a dose-dependent reduction in hypoxic myocardial area, scar burden, and scintigraphic perfusion defect. DLL4-Fc treated mice had lower end-diastolic LV volume and preserved or improved EF. In an ischemia-reperfusion model, DLL4-Fc increased the number of vessels in the ischemic zone. Our data suggests that balanced DLL4 signaling is crucially required for myocardial angiogenic recovery following coronary ischemia. Modulation of DLL4 signaling has translational therapeutic potential.

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Myocardial Knockdown of Mir-375 Attenuates Post-mi Inflammatory Response and Left Ventricular Dysfunction via Pdk-1-akt Signaling Axis

Venkata N Garikipati, Suresh K Verma, Mohsin Khan, Anna M Gumpert, Jibin Zhou, Zhongjian Cheng, Cindy Benedict, Emily Nickoloff, Jennifer Johnson, Ancai Yuan, Erhe Gao, Raj Kishore, Temple Sch of Med, Philadelphia, PA

MicroRNAs are known to be dysregulated in the ischemic heart disease and have emerged as potential therapeutic targets for treatment of myocardial infarction (MI). Recently MicroRNA-375 has been shown to be up-regulated in humans with MI. In this study, we assessed whether inhibition of the miR-375 using an i.v.-delivered locked nucleic acid (LNA)-modified anti-miR (LNA-antimiR-375) can provide therapeutic benefit in mice with pre-existing pathological cardiac remodeling and dysfunction due to myocardial infarction (MI).After the induction of acute myocardial infarction, mice were treated with either control or LNA based miR-375 inhibitor, and inflammatory response, cardiomyocyte apoptosis and LV functional and structural remodeling changes were evaluated. Anti-miR-375 therapy significantly suppressed infiltration of inflammatory cells, expression of proinflammatory cytokines in the myocardium and cardiomyocyte apoptosis. These changes were associated with miR-375 mediated activation of 3-phosphoinositide-dependent protein kinase 1 (PDK-1) and downstream AKT phosphorylation on Thr-308. LNA anti-miR-375 therapy significantly improved LV functions, reduced infarct size, and attenuated infarct wall thinning. Moreover, LNA based miR-375 therapy significantly increased capillary density in the infarcted myocardium. Further, our in vitro studies demonstrated that miR-375 negatively regulates the expression of PDK-1 by directly targeting the 3'UTR of the PDK-1 transcript. Taken together, our studies demonstrate that anti miR-375 therapy suppresses inflammatory response, cardiomyocyte death and contributes to improved LV function and enhanced angiogenesis via activation of PDK-1/AKT signaling.

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Role of the Mitochondrial AAA+ Lon Protease in Cardioprotection

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Mitochondrial Lon is an ATP-powered proteolytic machine that selectively degrades regulatory proteins as well as misfolded, unassembled and damaged proteins. Lon is a key mediator of mitochondrial quality control, adapting mitochondrial metabolism to hypoxic, oxidative, and proteotoxic stresses. Although studies imply that up-regulating Lon in the failing or ageing heart is ameliorative, there is little detailed in vivo data about its importance in cardiovascular health and disease. To test the hypothesis that Lon protects the heart in vivo, we generated transgenic mice with cardiac-specific overexpression of Lon under the control of the alpha-MHC promoter (Tg-Lon). Lon was specifically overexpressed in heart with no change in the levels of endogenous substrates TFAM or mitochondrial aconitase, or the mitochondrial ClpXP protease. Baseline analysis of 2-11 month old mice showed no significant difference in body or heart weight or ventricular weight/tibial length. At 2-3 months, no significant difference was observed by echocardiography or histology. However, Tg-Lon mice showed significantly reduced cardiac injury in response to ischemia reperfusion (I/R)- ischemia (45 min)/reperfusion (24 hr). At 6-9 months Tg-Lon, infarct size/area at risk (AAR) was 32%±2.3 SEM versus NTg that was 51%±3.2 SEM (p-value<0.003, n=9, n=4). At 3-5 months the infarct size/AAR for Tg-Lon was 37%±2.9 SEM versus NTg 51%±1.6 SEM, p<0.009, n=4, n=4); and at 3 months, Tg-Lon was 37%±2.0 SEM versus NTg 47% ± 1.4 SEM, p<0.008, n=3, n=4). In all cases, there was no change in

AAR/left ventricle (AAR/LV), and no difference in LV/body weight (LV/BW). At baseline, mitochondria isolated from Tg-Lon hearts showed lower oxygen consumption rates (OCR, pmoles O2/min) as compared to NTg. Average OCR for Tg-Lon was 12,997±708 SD AUC (1 µg), by contrast to NTg that was 32,042±320 (1 µg) ($p<0.001$, n=5, n=5). Associated with lower OCR of Tg-Lon, was a significant reduction of Complex I subunit NDUFB8 of NADH dehydrogenase, which was reduced 2.2 fold ($p<0.001$) as compared to NTg in LV extracts as well as in isolated heart mitochondria. These data demonstrate that Lon overexpression promotes cardioprotection, and that this effect may be linked to a reduction in basal mitochondrial oxygen consumption.

C.K. Suzuki: None.

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Interleukin-21 Promotes Pulmonary Arterial Hypertension Through M2 Macrophage Polarization

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Interleukin-6 (IL-6) is a multifunctional proinflammatory cytokine that is elevated in the serum of pulmonary arterial hypertension (PAH) patients and can predict the survival of idiopathic (I)PAH patients. Previous animal experiments and clinical human studies indicate that IL-6 is important in PAH; however, the molecular mechanisms of IL-6-mediated pathogenesis of PAH have been elusive. Here we identified IL-21 as a novel downstream target of IL-6-signaling in PAH. First, we found that IL-6 blockade by the monoclonal anti-IL-6 receptor antibody, MR16-1, ameliorated hypoxia-induced pulmonary hypertension (HPH) and prevented the hypoxia-induced accumulation of Th17 cells and M2 macrophages in the lungs. Furthermore, the hypoxia-induced upregulation of IL-17 and IL-21, which are primarily produced by Th17 cells, was also ameliorated by IL-6 blockade in mice. Whereas IL-17 blockade with an anti-IL-17 neutralizing antibody had no effect on HPH, IL-21 receptor-deficient mice were resistant to HPH and exhibited no significant accumulation of M2 macrophages in the lungs. Consistently, IL-21 indeed promoted the polarization of primary alveolar macrophages toward the M2 phenotype. Moreover, significantly enhanced expressions of IL-21 and M2 macrophage markers were detected in the lungs of IPAH patients who underwent lung transplantation. Collectively, these findings suggest that IL-21 promotes PAH through M2 macrophage polarization, downstream of IL-6-signaling. IL-6/Th17/IL-21-signaling axis may be a novel potential target for treating PAH.

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Discovery and Cardioprotective Effects of the First Non-peptide Agonists of Prokineticin Receptor-1

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Prokineticins are angiogenic hormones that activate two G protein-coupled receptors: PKR1 and PKR2. PKR1 has emerged as a critical mediator of cardiovascular homeostasis and cardioprotection. Identification of non-peptide PKR1 agonists that contribute to myocardial repair and collateral vessel growth hold promises for treatment of heart diseases. Through a combination of in silico studies, medicinal chemistry, and pharmacological profiling approaches, we designed, synthesized, and characterized the first PKR1 agonists, demonstrating their cardioprotective activity against myocardial infarction (MI) in mice. Based on high throughput docking protocol, 250,000 compounds were computationally screened for putative PKR1 agonistic activity, using a homology model, and 10 virtual hits were pharmacologically evaluated. One hit internalizes PKR1, increases calcium release and activates ERK and Akt kinases. Among the 30 derivatives of the hit compound, the most potent derivative, IS20, was confirmed for its selectivity and specificity through genetic gain- and loss-of-function of PKR1. Importantly, IS20 prevented cardiac lesion formation and improved cardiac function after MI in mice, promoting proliferation of cardiac progenitor cells and neovasculogenesis. The preclinical investigation of the first PKR1 agonists provides a novel approach to promote cardiac neovasculogenesis after MI.

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Dissection of Thrombospondin-4 Domains Involved in Adaptive ER Stress Responsive Signaling and Secretory Pathway Functions

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Thrombospondins are a family of stress-inducible secreted glycoproteins with well-characterized roles in the extracellular matrix and tissue remodeling. We recently reported critical intracellular functions for thrombospondin-4 (Thbs4) by activation of an adaptive endoplasmic reticulum (ER) stress pathway in cardiomyocytes, in part by promoting activation of activating transcription factor 6α (Atf6α). Here, we dissect the domains of Thbs4 that mediate interactions with ER proteins and activation of this adaptive ER stress response, and determine the domains of Thbs4 involved in secretory pathway functions. We show that the N-terminal laminin G like domain (LamG) of Thbs4 exhibits intracellular localization almost exclusively at the Golgi apparatus and is robustly secreted in cultured cardiomyocytes, indicating rapid flux through the secretory pathway. We also generated a full length Thbs4 with mutations in calcium-binding motifs within the type III repeat (T3R) domain, including mutations homologous to human disease-causing mutations identified in THBS5/COMP. While wildtype Thbs4 localized to the ER and post-ER vesicles and was actively secreted in cardiomyocytes, the Thbs4 calcium-binding mutant localized predominately to the ER and was not secreted. We identified BiP (Grp78) as a novel binding partner of Thbs4 in cardiomyocytes in vitro and in vivo, and utilized adenoviruses overexpressing Thbs4 domain mutants in cardiomyocytes to map the TSP-C domain (L-type lectin domain) of Thbs4 as the BiP-interacting motif. Additionally, the TSP-C domain of Thbs4 bound Atf6α and overexpression of the TSP-C domain alone was sufficient to induce the adaptive ER stress pathway in cardiomyocytes, activate Atf6α, and protect against ER stress-induced cell death. These studies characterize the functional domains of Thbs4 and aid in parsing out the intracellular and extracellular roles of thrombospondins. Taken together, data indicate critical functions for the TSP-C and LamG domains of Thbs4 in ER stress-responsive and secretory pathway functions, respectively.

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Engineering Targeted Growth Factors for Selective Pro-survival Signaling in Apoptotic Cardiomyocytes Following Ischemic Injury

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Background: Despite the benefits of reperfusion therapy after myocardial infarction (MI), most patients still progress to heart failure. MI patients suffer irreversible loss of heart function due to cardiomyocyte (CM) death and tissue scarring. Growth factors (GFs) reduce scar size and limit CM apoptosis in preclinical studies, however native GFs have poor drug-like qualities and have not been successful in the clinic. Silver Creek Pharmaceuticals is developing a new class of targeted, growth factor-based therapeutics (Smart Growth Factors) that are engineered to have optimized pharmacokinetics, dynamics and safety profiles. Our first generation of SGFs use annexin-V (AnxV) to target IGF-1 to damaged CMs and selectively activate pro-survival signaling.

Methods and Results: We used biophysical simulation to design an IGF-1-based SGF that selectively activated the PI3K pathway in damaged cells. SGFs were engineered to target IGF-1 to damaged cells through the binding of AnxV to phosphatidylserine exposed on the surface of apoptotic cells. These bispecific proteins were constructed with half-life modulators and linkers, then screened for their ability to selectively increase pAKT levels in apoptotic iPSC-derived human CMs. We identified a subset of SGFs that were able to selectively increase pAKT levels in apoptotic cells as compared to healthy cells ($p<0.05$). Based on these data, we tested the ability of SGFs to activate pro-survival signaling and reduce infarct size in a rat ischemia/reperfusion model of acute MI (AMI). SGFs were able to selectively prolong pAKT in the infarcted region of the left ventricle without activating signaling in remote healthy tissue out to 2 hours post-reperfusion (n=3-6/group, $p<0.05$). For efficacy studies, rats were subjected to 60 minutes of ischemia followed by 72 hours of reperfusion. A single IV dose of SGF (600 pmol/kg) administered at time of reperfusion was able to significantly reduce infarct size relative to the area-at-risk (infarct/AAR%) as compared to controls ($p<0.05$; SGF 12.7±5% n=10; wt IGF-1 20.3±3% n=8 and vehicle 27.6±7% n=12).

Conclusions: This work demonstrates that SGFs selectively activate pro-survival signals in distressed CMs and lead to reduced infarct size in vivo without off-target effects.

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β2-Adrenergic Receptor-mediated Regulation of Chemokine Receptor 2 Expression in Hematopoietic Cells is Essential for Immune Cell Recruitment and Repair Following Myocardial Infarction

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β-Adrenergic receptors (βAR) are critical regulators of cardiac function normally and during heart failure (HF). The importance of βAR on cardiomyocyte contractility and survival is well defined however, following myocardial infarction (MI), inflammatory responses occur, which are critical for healing and scar formation. Catecholamines acting through βAR, particularly the β2AR subtype, are known to modulate immune responses, however, the influence of β2AR in regulating the inflammatory response following MI is unknown. To investigate the contribution of β2AR on immune cells following myocardial infarction (MI), wild-type (WT) mice were irradiated and then received β2ARKO or WT control bone marrow (BM) transplants to create immune cell specific knockout (KO) animals. Following BM reconstitution, mice were subjected to MI and cardiac function and survival were monitored. Cardiac function, as assessed by echocardiography, did not differ between WT and β2ARKO chimeric mice. However, mice lacking β2ARKO in their BM resulted in 100% mortality from cardiac rupture within two weeks of receiving MI in contrast to their WT counterparts that had ~20% death. Masson trichrome staining demonstrated infarct expansion in β2ARKO chimeric mice occurred more rapidly than their WT counterparts. While there was no change in total cell infiltration into the heart following MI, a retention of granulocytes occurred in the spleen of β2ARKO chimeric mice resulting in reductions in infiltrating monocyte/macrophage, neutrophil and mast cell populations. Additionally, alterations in chemokine receptor levels, particularly CCR2, on BM resulted in decreased cellular migration which could be rescued as well as CCR2 expression using a β2AR lentivirus to re-express the β2AR. Use of a CCR2 antagonist or CCR2KO BM transplant recapitulated a portion of the phenotype observed in β2ARKO chimeric mice. These results demonstrate the critical role of β2AR in the regulation of CCR2 expression on hematopoietic cells and its importance in mounting an immune response and promoting healing following MI.

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Cardioprotective Effects of Interleukin-15 in Immortalized Human Ventricular Cardiomyocytes

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Interleukin-15 (IL-15) is a pleotropic cytokine that has a profound effect on the proliferation, survival and differentiation of many distinct cell types. The IL-15 receptor complex has 3 subunits: the unique receptor chain IL-15 receptor alpha (IL-15Rα), and two receptor chains shared with interleukin-2 (IL-2) and/or other cytokines, referred to as IL-2 receptor beta (IL-2Rβ) and IL-2 receptor gamma/gamma common chain (IL-2Rγ/γc), respectively. To our knowledge, this is the first study to examine the effects of IL-15 in immortalized human cardiomyocytes. Data collected by RT-PCR shows mRNA expression of IL-15Rα, IL-2Rβ and IL-2 Rγ/γc in these cells. Additionally, western blotting for IL-15Rα, IL-2Rβ and IL-2 Rγ/γc confirms the presence of all three IL-15 receptors. Early experiments examining the effect of IL-15 on cardiomyocyte cell survival show a statistically significant protective effect of IL-15 on the survival of cells exposed to tunicamycin, a pharamacological endoplasmic reticulum (ER) stress inducing agent. These findings suggest that IL-15 signaling may be an important cardioprotective pathway that is involved in the cardiac ER stress response. As ER stress is a major component of

multiple different cardiac pathologies, such as myocardial infarction, heart failure and diabetes, uncovering the molecular mechanism by which IL-15 protects the heart will allow for deeper understanding of the cardiac ER stress response.
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B-Raf Loss Suppresses Extracellular-Regulated Kinase Activation and Cardiomyocyte Proliferation
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Objectives: The postnatal heart does not retain the proliferative capacity it had during fetal life. Unlike large mammals, murine cardiomyocytes (CM) continue to divide into the first week of life before terminal differentiation and binucleation. We hypothesized that B-Raf regulates ERK activation in newborn CM and that loss of B-Raf suppresses cyclin levels and reduced proliferation. To test this, we determined whether loss of B-Raf disrupts the **ERK** (extracellular-regulated kinase) cascade and impairs CM growth.
Methods: CM specific knockout (KO) of B-Raf was generated using CRE/lox (floxed B-Raf x αMHC CRE) resulting in a truncated, unstable B-Raf and a null phenotype. KO mice (α-MHC-CRE / B-Raf $^{lox/lox}$) were compared to CRE negative / B-Raf $^{lox/lox}$ mice (wild type; WT). Hearts from 3d and 8d old pups were harvested for molecular analysis of B-Raf signaling and cell cycle markers. Hearts from 3d old pups were harvested and CMs isolated for culture using a trypsin/DNAse digestion. The cells were treated with Isoproterenol (Iso;10uM), forskolin (20uM), and IGF-1 (1ng/ml) for 15 min to determine if the loss of B-Raf results in reduced activation of ERK.
Results: Heart weight to body weight (HW/BW) ratio was less in 3d KO versus 3d WT (n=50, $p<0.05$). HW/BW ratio became greater in 8d KO; there was no difference in 3d and 8d HW/BW in WT animals. Baseline B-Raf and phosphorylated ERK levels were reduced in KO hearts (*$p<0.05$). Cell cycle inhibitors p21 and p53 were increased in 3d KO hearts with decreased levels of all cyclins ($p<0.05$). In 8d KO hearts, increased p21, p27, and p53 expression was accompanied with increased cyclin levels ($p<0.05$). In vitro ERK activation was blunted in KO CMs by forskolin and Iso compared to IGF-1.
Conclusions: ERK activation was suppressed in KO hearts resulting in smaller newborn hearts but which exceeded normal HW/BW by 8d. This is may represent premature hypertrophy as the proliferative period of CM development had ended. Cell cycle analysis supports reduced CM mitotis among 8d CM. Such early disturbances in normal CM growth may increase susceptibility for reduced cardiac function in the face of increased postnatal load stress.
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Adenosine Diphosphate Ribosyl Cyclase via PI3K-Akt and FOXO3a Up-regulating MMP-9 to Enhance Vascular Smooth Muscle Cell Proliferation and Migration in Mice
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The current study was designed to explore the mechanisms of vascular smooth muscle cell (VSMC) proliferation and migration induced by adenosine diphosphate ribosyl cyclase(ADPRC). In this study, 32 Male ApoE-/- mice(6 weeks old, 18-22g)on a C57BL/6J background were divided into four groups, which received normal chow (n=8, NC group), high-fat Western-type diet (n=8, 0.25% cholesterol, 21% fat,HFD group), high-fat Western-type diet,infusion of 2,2′-dihydroxyazobenzene(DHAB, a ADPRC inhibitor, 2mg/kg/day, n=8, HFD-DHAB group) intraperitoneally or high-fat Western-type diet,infusion of LY294002(a Inhibitor of Akt, 5mg/kg/d, n=8, HFD-LY group) intraperitoneally, for 10 weeks. 8 male C57BL/6J mice served as control. After 10 weeks, mice were anesthetized with chloral hydrate, aorta was removed and immediately frozen in liquid nitrogen. Aortic atherosclerotic lesions, VSMC proliferation and migration were assessed by histomorphological observation, smooth muscle actin-α(α-SMA)and proliferating cell nuclear antigen (PCNA) examination. ADPRC expression and alterations of Akt, FOXO3a, phospho-FOXO3a and MMP-9 were determined by RT-PCR, Western Blot, Immunohistochemistry or Immunofluorescence. The results showed that, in aortic atherosclerotic lesions derived from atherosclerotic mice of HFD group, an increased VSMC proliferation and migration, reflected by the up-regulation of α-SMA and PCNA expression, were observed followed by increased expression of ADPRC, Akt, FOXO3a, phospho-FOXO3a and MMP-9. The enhanced expression of ADPRC and followed alterations of FOXO3a, phospho-FOXO3a, MMP-9 as well as α-SMA, PCNA, VSMC proliferation and migration were absent in NC group and C57BL/6J control mice. Treatment with DHAB or LY294002 reversed VSMC proliferation, migration and expression of Akt, FOXO3a, phospho-FOXO3a and MMP-9 in HFD-DHAB and HFD-LY group. These data shows that high-fat Western-type diet induced ADPRC may via PI3K-Akt to phosphorylate FOXO3a up-regulating MMP-9 to enhance vascular smooth muscle cell proliferation and migration in mice.
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Mdm2 E3 Ligase-mediated Ubiquitination of Histone Deacetylase 1 in Vascular Calcification
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Vascular calcification (VC) often associates with many cardiovascular and metabolic diseases. Although VC is the cause of high morbidity and mortality, molecular mechanisms have yet to be elucidated. Here we report that MDM2-induced ubiquitination of histone deacetylase 1 (HDAC1) mediates VC. Loss of HDAC1 activity enhanced VC *in vivo* and *in vitro*. HDAC1 protein was reduced in cell and animal calcification models and in human calcified coronary artery and this reduction preceded VC. Calcification stresses induced MDM2 E3 ligase, which resulted in HDAC1 K74 ubiquitination. Forced expression of MDM2 enhanced VC, whereas loss of MDM2 blunted it. A decoy peptide spanning HDAC1 K74 prevented VC. These results demonstrate a previously unknown ubiquitination pathway as well as the involvement of HDAC1 in VC. Our results suggest MDM2-mediated HDAC1 ubiquitination as a new therapeutic target in VC.
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Stim1 is Associated with Calcium Microdomains That are Required for Myofilament Remodeling and Signaling During Cardiac Hypertrophy

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Stromal Interaction Protein 1 (STIM1) is the intracellular component of the store operated calcium channels. It is a ubiquitous Ca2+ sensor, prevalently located in the sarcoplasmic reticulum. In non-excitable cells, STIM1 is a key element in the generation of Ca2+signals that lead to gene expression and cell proliferation. A growing body of literature now suggests that STIM1 is important for normal heart function and plays a key role in the development of pathological cardiac hypertrophy. However, the precise mechanisms involving STIM1 and the Ca2+ signaling in excitable cells are not clearly established. We show that in neonatal rat cardiomyocytes, the spatial properties of STIM1-dependent Ca2+ signals determine restricted Ca2+ microdomains that regulate myofilaments remodeling and spatially segregated activation of pro-hypertrophic factors. Indeed, in vivo data obtained from an inducible cardiac restricted STIM1 knockout mouse, exhibited left ventricular dilatation associated with reduced cardiac contractility, which was corroborated by impaired single cell contractility. Furthermore, mice lacking STIM1 showed less adverse structural remodeling in response to pathological pressure overload-induced cardiac hypertrophy (transverse aortic constriction, TAC). We further show that the Ca2+ pool associated with STIM1 is the ON switch for extracellular signal-regulated kinase (ERK1/2)-mediated cytoplasm to nucleus signaling. These results highlight how STIM1-dependent Ca2+ microdomains have a major impact on intracellular Ca2+ homeostasis, cytoskeletal remodeling, signaling and cardiac function, even when excitation-contraction coupling is present.

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Delineating the Caspase-dependent Targets and Signal Pathways That Promote Pathological Cardiac Hypertrophy

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Although cardiac hypertrophy is initially an adaptive response, chronic stress on the heart is a maladaptive process that inevitably leads to end-stage heart failure. Interestingly, this pathological process is also characterized by cell behaviors associated with apoptosis. We previously demonstrated the essential role of the intrinsic cell death pathway during cardiac hypertrophy; however, the caspase-dependent pathways and cleavage targets remain elusive. To this aim, we evaluated a myocyte enhancer factor 2 (MEF2) transcription factor inhibitor, histone deacetylase 3 (HDAC3), and gelsolin as potential caspase cleavage substrates involved in the induction and/or maintenance of cardiac hypertrophy. In vitro cleavage assays were completed with effector caspase and recombinant substrate protein which demonstrated caspase-dependent cleavage. HDAC3 cleavage was observed during early stages of hypertrophy and reduced in the presence of a caspase inhibitor. Luciferase assays demonstrated that the transcriptional activity of MEF2 is dependent on intact caspase function suggesting caspase-directed HDAC3 cleavage may serve as a novel regulatory mechanism to alleviate MEF2 suppression to engage the hypertrophy gene expression program. Unlike HDAC3, caspase mediated gelsolin cleavage occurs at latter stages and is coincident with the cytoskeletal alterations that occur during this process. As gelsolin is a potent actin capping/severing enzyme, we hypothesize that caspase-mediated gelsolin activation acts as a key regulatory step in the structural rearrangements that allow for hypertrophy to occur. We have generated adenoviral vectors containing caspase cleavage mutants and cleaved forms of HDAC3 and gelsolin and will discuss the impact of these modified substrates on the hypertrophy process in vitro and in vivo. Collectively, this work suggests that caspase signalling acts to engage both the transcriptional program and cytoskeletal accommodations that characterize cardiac hypertrophy. Importantly, these observations suggest that identification of inhibitors that suppress caspase activity and/or activity of its cognate substrates may offer novel therapeutic targets to limit the development of pathological hypertrophy.

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Nck Promotes Podosome Formation and Function in Vascular Endothelial Cells

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Podosomes are actin rich adhesion structures capable of extracellular matrix remodeling (ECM) and facilitate invasive cell migration. ECM remodeling and migration of endothelial cells (ECs) are prerequisites for angiogenesis, an important process in development and cardiovascular disease. In this study using a combination of molecular genetics and high resolution microscopy, we have demonstrated that adaptor proteins Nck1 and Nck2, that links signaling by tyrosine phosphorylation with actin dynamics, promote podosome formation and function. Expression of human Nck1-YFP or human Nck2-mCherry induces podosome formation in human umbilical vein endothelial cells. Silencing of Nck by retroviral expression of short hairpin RNA disrupted podosome formation in Src transformed endothelial cells. Both number of cells with podosomes and number of podosomes per cell decreased significantly ($P<0.05$) compared with control and rescued cells. Functionally, Nck silenced cells were deficient in fluorescent gelatin matrix degradation, an effect that could be almost completely reversed by expression of siRNA-resistant Nck2. Further, overexpression of Nck in Src transformed endothelial cells induced an even higher ($p<0.05$) index of matrix degradation when compared with Src transformed control cells. Overall, a high degree of colocalization between F-actin and areas of degradation was observed. However, areas of degradation without cellular/F-actin colocalization – presumably due to the highly motile nature of HUVEC – were also observed. Mechanistically we found that Nck promote podosome biogenesis through interaction with p62Dok. Collectively, these results provide strong support for a critical role of Nck adaptors in the invasive program of endothelial cells which is important in health and diseases.

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Activation of the p38 Branch of Mitogen Activated Protein Kinase Pathway Stimulates Proteasome Proteolytic Function

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Ubiquitin-proteasome system (UPS) dysfunction is associated with development of most heart diseases. Proteasomal functional insufficiency has been experimentally demonstrated to play an important role in cardiac pathogenesis. Hence, enhancing proteasome function is potentially a powerful new strategy for therapeutic exploration but this research area is hindered critically by poor understanding of the regulation of cellular proteasomes. Here, we sought to determine whether and how the p38 branch of mitogen activated protein kinase (MAPK) regulates the UPS. GFPu is a rationally modified green fluorescence protein (GFP) proven to be a UPS-specific substrate. In both an HEK293-GFPu/RFP stable cell line and cultured neonatal rat ventricular myocytes (NRVMs), serum withdrawal activated p38 and decreased GFPu/RFP protein ratios. The decrease was abolished by p38 inhibitor (SB230580). Similar effects were also observed when p38 was activated by anisomycin. Cycloheximide (CHX) chase assays showed that p38 activation by either anisomycin treatment or MKK3 overexpression significantly shortened the half-life of GFPu proteins. These compelling data demonstrate for the first time that p38 stimulates UPS performance. To pinpoint the step of the UPS pathway which p38 acts on, we examined the level of total ubiquitin conjugates and proteasomal peptidase activity. Activation of p38 via various means failed to increase the level of total ubiquitin conjugates but was able to increase significantly proteasomal chymotrypsin-like activity in cultured NRVMs and this increase was remarkably blunted by SB230580. The altered proteasome activity was not associated with discernible changes in the protein abundance of representative subunits of the 19S (Rpt6, Rpn2) and 20S proteasomes (α5, β5, β7); however, 2-dimensional PAGE followed by immunoblot showed that the isoelectric point (pI) of a fraction of β5, but not α2, subunits of the 20S proteasome was shifted to the acidic side, consistent with increased phosphorylation, by MKK3 overexpression in cultured NRVMs. It is thus demonstrated for the first time that p38 MAPK stimulates proteasome proteolytic function and it achieves such effect likely through post-translational modifications of the proteasome.

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Proteasome Priming by Protein Kinase G Protects Against Myocardial Ischemia-reperfusion Injury

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Cardiac proteasome functional insufficiency is implicated in a large subset of heart disease and has been experimentally demonstrated to play an essential role in cardiac proteotoxicity, including desmin-related cardiomyopathy and myocardial ischemia-reperfusion (I-R) injury. Pharmacological inhibition of phosphodiesterase 5 (PDE5) via sildenafil for example, which can stabilize cGMP and thereby increase cGMP-dependent protein kinase (PKG) activity, is consistently reported to protect against I-R injury; however, the underlying mechanism is not fully understood. We have recently discovered that PKG activation enhances proteasomal degradation of misfolded proteins (Ranek, et al. Circulation 2013), prompting us to hypothesize that proteasome-priming may contribute to cardioprotection-induced by PDE5 inhibition. Here we used a cardiomyocyte-restricted proteasome inhibition transgenic mouse line (Tg) and non-Tg (Ntg) littermates to interrogate the action of sildenafil on I-R injury created by left anterior descending artery (LAD) ligation (30 min) and release (24 hr). Sildenafil was administered 30 min before LAD ligation. Results showed that (1) the 26S proteasome activity of the Ntg I-R hearts was significantly elevated by sildenafil but this elevation was blocked in the Tg line; (2) the infarct size reduction by sildenafil treatment in Ntg mice was completely abolished in the Tg mice with the same treatment; and (3) systolic and diastolic function impairment after I/R was markedly attenuated in sildenafil-treated Ntg mice, but not in the sildenafil-treated Tg mice. Additionally, immunoprecipitation assays show that PKG interacted with the proteasome in cultured cardiomyocytes, and this interaction appeared to be augmented by sildenafil treatment. Moreover, in vitro incubation of active PKG with purified human 26S proteasomes increased proteasome peptidase activities and the phosphorylation at specific serine residues of a 19S proteasome subunit as revealed by “gel-free” nano-LC-MS/MS. We conclude that active PKG directly interacts with, phosphorylates, and increases the activities of, the proteasome and that proteasome priming mediates to cardioprotection of PDE5 inhibition against I-R injury.

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Activation of the S1P3 Receptors is Responsible for S1P-mediated RhoA Activation and Cardioprotection

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Sphingosine-1-phoshpate (S1P) is a bioactive lysophospholipid, generated and released at sites of tissue injury. S1P signals through a variety of G-protein coupled receptor subtypes and there are three major sub-types, $S1P_1$, $S1P_2$, and $S1P_3$, to mediate cardiovascular responses. $S1P_2$ and $S1P_3$ receptors couple to $G\alpha_i$, $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_q$ and we first examined the contribution of $S1P_2$ and $S1P_3$ to cardiac hypertrophy using $S1P_2$ and $S1P_3$ knockout (KO) mice and found that there is no difference in hypertrophy induced by pressure-overload. We previously showed that S1P provides cardioprotection against oxidative stress such as ischemia/reperfusion in which RhoA activation and its downstream effector PKD1 play an important role. It has not, however, been determined which S1P receptor subtype is responsible for S1P mediated cardioprotection. We knocked down the three major S1P receptors using siRNA in neonatal rat ventricular myocytes (NRVMs) and assessed RhoA and PKD1 activation induced by S1P. Knockdown of $S1P_3$ abolished RhoA activation and largely attenuated phosphorylation of PKD1 while knockdown of $S1P_1$ and $S1P_2$ did not. Using siRNA or pertussis toxin to inhibit different G-proteins, we further established that S1P regulates RhoA activation through $G\alpha_{13}$, but not $G\alpha_{12}$, $G\alpha_q$, or $G\alpha_i$. To investigate the role of $S1P_3$ receptors in the adult heart, hearts were isolated from wild-type or $S1P_3$ KO adult mice, perfused in the Langendorff mode and subjected to ex vivo ischemia/reperfusion. As previously reported, S1P perfusion significantly reduced infarct size induced by ischemia/reperfusion in WT hearts (by 50%), but this protection was abolished in the $S1P_3$ KO mouse heart. To further confirm the role of $S1P_3$ in cardioprotection we perfused WT mouse hearts with an $S1P_3$-specific agonist CYM-51736. We observed that CYM-51736 attenuated the infarct size

to a similar degree as that observed with S1P. Our findings reveal that activation of the $S1P_3$ receptor coupling to $G\alpha_{13}$ and subsequent RhoA activation is responsible for cardioprotection against ischemia/reperfusion. Accordingly specific drug targeting of $S1P_3$ receptors could provide therapeutic benefits in ischemic heart disease without the undesirable effects of global activation of other cardiac S1P receptors.

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Induced Pluripotent Stem Cell Derived Cardiomyocyte Tissue Engineered Scaffold Improves Left Ventricular Function and Electro-Mechanical Coupling in Rats with Heart Failure

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Background: Chronic Heart Failure (CHF) is the leading cause of hospital readmissions in the United States. It may result from systolic or diastolic dysfunction, which often coexists. Here we report the effects of delivering human induced pluripotent stem cells derived cardiomyocytes (hiPSC-CMs) via a bioengineered patch on left ventricular function in rats with CHF.
Methods: Adult male Sprague-Dawley rats underwent left coronary artery ligation and were randomized to Sham, CHF, and hiPSC-CM patch. High purity human hiPSC-CMs were obtained from Cellular Dynamics International, seeded and co-cultured onto a vicryl matrix embedded with human dermal fibroblasts. Echocardiography was performed at 3 and 6 weeks post-randomization. Hemodynamic pressure measurements were performed at 6 weeks post-ligation with Millar solid state micromanometer catheters. Open chest Electrophysiologic (EP) mapping was performed at 6 weeks post ligation.
Results: Patches constructed with hiPSC-CMs displayed synchronized and spontaneous contractions within 48hrs of culture which developed in robustness over time. At maximal robustness, contractions were visualized across the full thickness of the construct. Contractions were recorded at 36+5 beats BPM. Three weeks after implantation, the hiPSC-CM patch decreased LV EDP (45%), Tau (29%), E/e' (23%) and increased, EF (14%), e' (20%), and e'/a' (36%) versus CHF. EP studies show electro-mechanical coupling between the patch and the native myocardium with normal activation through the patch and increases ($P<0.05$) voltage amplitude in CHF versus hiPSC-CM patch treated rats (1 ± 0.5 mV vs 6 ± 1.5mV).
Conclusion: Cardiac patch implantation with human iPSC derived cardiomyocytes is an effective and feasible method of treating CHF with improvements in systolic function, diastolic function, and electro-mechanical coupling in rats with CHF.

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A Novel Method to Study Viral Cardiomyopathy

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Latent coxsackievirus B3 (CVB3) cardiac infections are implicated in the development of DCM with persistent enteroviral RNA found in up to 66% (30/45) of DCM patient biopsies who are at a 6-fold higher risk of fatality. Current treatments aim at delaying heart failure but none address the viral etiology of the disease. Viral protein presence is thought to disrupt normal cellular signaling leading to tissue dysfunction. The downstream effects of viral perturbations are complex and wide-ranging; especially in proinflammatory contexts seen clinically. Thus, comprehensively understanding the molecular mechanisms of CVB3-mediated disease is key in developing treatments for viral DCM patients. In this study we built a novel in vitro model of chronic CVB3 infection to facilitate high-throughput, systems-level studies of viral cardiomyopathy.
Current methods of studying CVB3 rely on animals or animal derived cardiac cells, making large-scale intracellular signaling studies difficult, time intensive, and expensive. To facilitate high-throughput studies we used immortalized human cardiomyocytes to engineer single-cell derived cell lines that express a maturation deficient CVB3 genome. CVB3 RNA expression was validated in each line by two independent methods: gene-specific nested qRT-PCR and single molecule RNA fluorescence in situ hydridization (smFISH). Plaque-assay verified that viral RNA expression did not result in live virus release.
Microarray analysis shows that CVB3 expressing cell lines have altered immune cytokine, extracellular matrix protein, and stress-signaling protein expression. Further, differences between CVB3 expressing lines suggest differential responses to viral RNA expression which may identify a set of beneficial adaptations. Future studies will include high-throughput signaling protein activity assays we have developed specifically for phosphatase and kinase activity quantification with the aim of linking immune cytokine signaling dysregulation to pathogenic gene expression. These studies will deepen our knowledge of CVB3-mediated DCM and identify proteins whose targeted modulation could offer new treatment strategies to patients whose current options are either palliative care or heart transplantation.

M. Shah: None. **C. Borgman:** None. **K. Janes:** None.

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Micrornas Have Differential Degree of Expression in Pathological Cardiac Hypertrophy Compared to Physiological Cardiac Hypertrophy

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Physiological and pathological left ventricular hypertrophies (LVH) are distinct processes that have differential pattern of gene expression. Based on initial stimuli, miRs expression levels can fluctuate and then cause a variance on their targets culminating in diverse cellular pathway activation. AIM: Here we compared miRs expression between pathological cardiac hypertrophy induced by transverse aortic constriction (TAC) and physiological cardiac hypertrophy induced by voluntary exercise in running wheels (EXE).
METHODS: Adult male Balb/c mice (12-14 weeks old) mice were subjected to TAC or EXE protocol and data were evaluated at 7 (TAC-7D; EXE-7D) and 35 (TAC-35D; EXE-35D) days. Hypertrophy was measured by normalizing left ventricular weight to body weight (LVW/BW). We evaluated left ventricular expression levels of miRs: -26b, 27a, -143, -150, -195 and -499 by qRT-PCR in TAC and EXE groups. Comparisons between groups were performed by ANOVA with Bonferroni correction. Results are shown as mean±SEM.
Results: Sedentary and Sham groups were similar

among all variables tested. Animals subjected to TAC surgery demonstrated a greater hypertrophy than EXE animals at both time points (7D: 16% vs. 7%; 35D 26% vs 12%, p<0.05 for both). MiR-26b had increased levels in TAC group at both time points (7D: 1.14±0.1 vs 0.6±0.01; 35D: 4.8±1.4 vs 1.17±0.12; p<0.01 for both). We only detected an increase in miR-27a levels in TAC-7D compared to EXE-7D (2.7±1.0 vs 0.78±0.1, p <0.05). We identified an augmentation in miR-143 levels in TAC group at both time points (7D: 1.1±0.1 vs 0.75±0.1; 35D: 1.42±0.2 vs 0.9±0.1; p<0.05 for both). We detected an increase in miR-499 levels at both time points in TAC group (7D: 4.1±0.5 vs 0.67±0.2, p<0.001; 35D: 2.2±0.4 vs 0.9±0.2, p<0.01). We found an increase in miR-195 levels only in TAC-35D group compared to EXE-35D (2.6±0.3 vs 0.9±0.1, p<0.05). We did not notice any change in miR-150 levels neither at 7 days nor at 35 days.
Conclusions: These preliminary data demonstrate a differential degree of miR expression between physiological and pathological hypertrophy. Further studies comparing physiological and pathological cardiac hypertrophy are necessary to find out the turning point that deviates heart from adaptive to maladaptive growth.
N.C. Martinelli: None. **C. Cohen:** None. **D. Silvello:** None. **A. Biolo:** None. **M. Andrades:** None. **K. dos Santos:** None. **N. Clausell:** None. **L.E. Rohde:** None. **U. Matte:** None.

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DNA Methylation of ASC is Associated with Decreased ASC and IL-1β Expression in Heart Failure
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Introduction: Heart failure (HF) is associated with formation and activation of inflammasome, a complex of intracellular interaction proteins that trigger maturation of inflammatory cytokines to initiate inflammatory response. ASC, a vital component of the inflammasome, is controlled through epigenetic modification via methylation of CpG islands surrounding exon 1.
Methods: To assess the relationships between DNA methylation of ASC, ASC expression, and inflammatory cytokines IL-1β and IL-18 in HF, stored samples from 155 chronic HF patients (age 56.9±12.0 yr, 64% male, 47% black, and ejection fraction 29.9±14.9) were analyzed. DNA extracted from PMBCs were analyzed by pyrosequencing for percent methylation of seven CpG sites in the intron region preceding exon 1 of the ASC gene. ASC mRNA was quantified via real-time PCR and analyzed as the ratio ASC:GAPDH. Serum ASC, IL-1β, and IL-18 were measured by ELISA.
Results: Higher ASC methylation was associated with lower ASC mRNA (r=0-.328, p<0.001) and protein (r=-.464, p<0.001) expression. Lower ASC mRNA expression was associated with lower ASC protein expression (r=0.494, p<0.001). Decreased IL-1β expression was associated with higher ASC methylation (r=-.424, p=0.005) and lower ASC mRNA (r=.619, p<0.001) and ASC protein (r=.433, p<0.001). IL-18 expression was not significantly associated with ASC methylation or expression.
Conclusions: Increased ASC methylation was associated with lower IL-1β, likely via decreased ASC gene expression. As ASC is required for inflammasome activation of IL-1β, this study implicates the inflammasome pathway as a driver of inflammation in HF, proving a potential target for novel interventions.
B. Butts: None. **J. Butler:** None.

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MicroRNA Control of the Pacemaker Channel and Training-induced Bradycardia
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Background: Athletes are prone to bradyarrythmias and have a higher-than-normal incidence of pacemaker implantation but the underlying mechanisms are poorly understood. In previous work we demonstrated a training-induced electrical remodelling of the sinoatrial node (SAN), including a down regulation of the key pacemaker channel HCN4 that carries the pacemaker current I_f. Here we investigated post-transcriptional regulation of HCN4 by microRNAs (miRNAs) in the trained SN.
Methods and Results: Mice subjected to 60-min swimming twice daily for 28 days (TM) were compared to parallel sedentary mice (SM).TM were bradycardic (cycle length *in vivo*: SM, 81±1.3 ms; TM, 102±3 ms; n=6, p<0.05). The cycle length of the isolated, denervated SAN (intrinsic heart rate) was also prolonged by exercise training (SM, 110±3 ms; TM, 150±5 ms; n=7, p<0.05). Deep sequencing for miRNAs on SAN punch biopsies revealed 10 differentially expressed miRNAs in TM *vs* SM (FDR adjusted P<0.05); 8 miRNAs were upregulated, 2 were downregulated. These findings were further confirmed by real-time PCR. We used the computation prediction tools *Targetscan* and *Ingenuity Pathway Analysis* to identify miR-423-5p and miR-486-3p as putative miRNAs participating in the post-transcriptional repression of HCN4 in the trained SAN. Both miRNAs had conserved seed sequences in the HCN4 3'UTR. To validate whether these miRNAs directly recognise the 3'UTR of HCN4, they were co-transfected with a construct containing HCN4 3'UTR fused downstream to a luciferase coding sequence in H9c2 cells. Transient transfection of
precursor miR-423-5p and miR-486-3p decreased HCN4 3'UTR luciferase reporter activity in H9C2 cells by 47% and 22% respectively, compared with unaltered luciferase activity on co-transfection with control miR or miR-27a.
Conclusions: This is the first report of miRNA dysregulation in
the trained SAN. We describe a novel molecular mechanism whereby an altered miRNA profile in the trained SAN promotes HCN4 downregulation and altered sinus automaticity. Data suggest miR-423-5p as a potential therapeutic target for bradyarrythmias in the athlete.
A. D'Souza: None. **E. Gill:** None. **C. Cox:** None. **H. Dobrzynski:** None. **E. Cartwright:** None. **D. Oceandy:** None. **M.R. Boyett:** None.

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Circadian Control of Heart Rate
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Background: Bradyarrhythmias occur more frequently at night. On the basis of heart rate variability this is attributed to high vagal tone. Here we tested the alternative hypothesis that an intrinsic circadian clock-driven remodelling of pacemaking ion channels underlies fluctuations in heart rate (HR). **Methods and results**: The occurrence of a circadian rhythm in HR was tested by placing nocturnal C57BL6/J mice under a strict 12/12h light-dark cycle and telemetry-based ECG intervals measured every 2 h for 48 h. Under these conditions, the R-R interval was rhythmic (n=10). To test whether this is caused by circadian rhythms in the expression of ion channels controlling HR, sinus node

(SAN) biopsies were collected at time points corresponding to the minima (ZT0, subjective day) and maxima (ZT12, subjective night) of HR, as determined by ECG recordings. Real-time PCR normalised to *28s* demonstrated an elevated expression of the key pacemaking ion channel HCN4 that carries the pacemaker current I_f and genes encoding the Ca^{2+}-handling proteins SERCA2a and RYR2 at ZT12 ($P<0.05$, n=10). Presence of clock machinery (essential transcription factors involved in setting intrinsic circadian rhythms) as potential regulators of ion channel oscillation were investigated in the SAN of *mPer1*^{Luc} mice which carry the 5' upstream region of the *mPer1* gene (a key core clock component) fused to a luciferase gene. *mPer1-luc* bioluminescence was recorded in the isolated SAN using a light-tight photomultiplier tube assembly to reveal a circadian rhythm with a periodicity of 24 h (n=3). Disruption of the molecular clock by global knockout of core clock components *Cry1 and Cry2* abrogated the circadian cycling of *mPer1-luc* in the SN of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$double knockout mice (n=3). Examination of 10 kb of the *Hcn4* promoter revealed a conserved consensus binding site for CLOCK and its heterodimer BMAL1, other essential transcription factors involved in setting intrinsic circadian rhythms. **Conclusions:** This is the first demonstration of a peripheral circadian clock in the cardiac pacemaker and circadian oscillations in key pacemaker mechanisms. Data reveal a novel regulator of SN function and the occurrence of bradyarrythmias at night.

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ScaRNAs Regulate Cardiac Differentiation and Development by Fine Tuning the Spliceosome

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Alternative splicing (AS) of mRNA adds diversity to the proteome and precise regulation of AS is essential for proper development. We recently showed that multiple genes critical for heart development are irregularly spliced in the right ventricle of babies with tetralogy of Fallot (TOF). We also observed reduced expression of several noncoding small cajal body associated RNAs (scaRNAs). scaRNAs direct the biochemical modification of spliceosomal RNAs and are essential for the stability and function of the spliceosome. Our results provide compelling evidence for a direct role of scaRNAs in regulating splicing of genes that are critical for heart development. To further explore this novel paradigm of developmental regulation, we analyzed the transcriptome of stem cells as they differentiated to beating cardiomyocytes. We observed significant alternative splicing with respect to timepoint (with Bonferroni correction and fdr of 5%) in 3,165 of 20,301 (15.6%) total genes. Importantly, there were 79 alternatively spliced genes among 213 genes (37.1%) known to be critical for heart development. This is a significant enrichment in the cardiac network genes ($p<0.001$). Most of the alternative isoforms are known protein coding variants. In addition, we saw changes in expression of several scaRNAs. scaRNA1 is reduced in the right ventricle of children with TOF and we targeted it for knockdown in the quail embryo model system. Preliminary results revealed dramatic alterations in cardiac morphogenesis and embryonic lethality. At higher levels of the antisense oligo, cells appeared to undergo apoptosis, aggregated inappropriately and failed to gastrulate. Lower concentrations resulted in initiation of gastrulation with some cells from the cardiac lineage traversing the embryonic milieu to contribute to the heart and other organs. Interestingly, these cells appeared to lack protrusive phenotypes, and may be passively moved by neighboring groups of non-electroporated motile cells. Taken together, our results suggest scaRNAs are necessary to maintain the fidelity of the spliceosome and thus play an important role in vertebrate heart development. These observations provide additional new insights into regulatory mechanisms underlying cardiac development.

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DNA Methylation Promotes Metabolic Remodeling of Hypertrophic Cardiomyocytes

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Cardiac hypertrophy and failure is characterized by major alterations in the bioenergetics of cardiomyocytes. However, the mechanisms underlying myocardial energy metabolism alterations in the hypertrophic heart are not completely understood. DNA methylation is a key element of transcriptional repression; genome-wide studies have indicated that this epigenetic modification could be involved in the pathogenesis of heart failure. By performing massive DNA sequencing on cardiomyocytes isolated from adult mice subjected to transverse aortic constriction (TAC) for one week, we found genome-wide alterations in the DNA methylation profile, and, more specifically, increased DNA methylation at promoters of genes involved in the metabolic remodeling of the hypertrophic heart. Expression screening of genes involved in DNA modifications revealed that the Uhrf1 gene - which encodes for an epigenetic cofactor absent under normal conditions and in post-mitotic cells - was highly re-expressed in hypertrophic cardiomyocytes. Mechanistically, we found that UHRF1 epigenetically silenced genes involved in the TCA cycle, OXPHOS/ETC, and ATP synthesis pathways. In addition, hyper-methylated promoters were specifically enriched in binding sites for nuclear respiratory factor-1 (NRF1), a transcription factor co-activated by PGC1α and responsible for transcriptional activation of many genes encoding mitochondrial proteins. Importantly, methylation prevented the binding of NRF1 to the promoters of several metabolic genes. Finally, in vivo knock down of Uhrf1 with serotype 9 adeno-associated vectors (AAV9) expressing anti-Uhrf1 shRNAs, prevented the development of cardiac and mitochondrial dysfunction in TAC mice. This is the first study showing a causal role of DNA methylation in the metabolic remodelling of the hypertrophic heart and identifying a key player and its underlying mechanism of action during this process. Since UHRF1 is not expressed in the adult heart under physiological conditions, it could represent a potentially interesting target for therapy.

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