American Heart Association | American Stroke Association

BCVS 2014

Recognizing 10 Years of Excellence

Basic Cardiovascular Sciences

2014 Scientific Sessions: Pathways to Cardiovascular Therapeutics

Final Program

July 14–17 | Paris Las Vegas | Las Vegas, Nev.

Sponsored and organized by the Council on Basic Cardiovascular Sciences.

my.americanheart.org/bcvssessions

Program at a Glance

	Sunday July 13	Monday July 14	Tuesday July 15	Wednesday July 16	Thursday July 17
7:00 AM		7–8:00 AM **Continental Breakfast/ Registration**	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		8:00–10:00 AM **Session 1** New Twists on Signaling Mechanisms in the Heart	8:00–9:40 AM **Session 6** Genetics and Genomics of Heart Failure	8:00–9:40 AM **Session 11** Emerging Role of Cardiac Fibroblasts	8:00–10:00 AM **Session 16** Impact of Non-myocytes on Myocardial Function
9:30 AM			9:40–10:00 AM **Refreshment Break/ Exhibits**	9:40–10:00 AM **Refreshment Break/ Exhibits**	
10:00 AM		10:00–10:20 AM **Refreshment Break**	10:00–11:40 AM **Session 7** Cardiac Epigenetics and Regulatory RNA's	10:00 AM–NOON **Session 12** Cell and Tissue Reprogramming	10:00 AM–NOON **Session 17** Mitochondrial Biology and Protein Misfolding and/ or Proteotoxicity
10:30 AM		10:20–NOON **Session 2** Cardiac Signaling, Conduction and Exercise			
11:00 AM					
NOON			NOON–1:30 PM **Early Career Workshop/Lunch** (Ticket required)	NOON–1:30 PM **Early Career Workshop/Lunch** (Ticket required)	NOON **Adjourn**
1:00 PM					
1:30 PM		1:45–2:05 PM **Welcome Remarks**	1:30–3:10 PM **Session 8** Cardiovascular Omics	1:30–2:15 PM **Session 13** Outstanding Early Career Investigator Award Finalists	
2:00 PM	2:00–10:00 PM **ACRE/AP Symposium**	2:05–4:05 PM **Session 3** Signaling Pathways and Heart Failure Progression		2:15–4:15 PM **Session 14** Joint AHA/HFSA: Myocardial Repair and Regeneration	
3:00 PM			3:10–3:30 PM **Refreshment Break/ Exhibits**		
3:30 PM			3:30–4:15 PM **Session 9** Keynote Lecture		
4:00 PM		4:05–4:50 PM **Session 4** Keynote Lecture	4:15–5:55 PM **Session 10** Epicardium vs Endocardium in Myocardial Repair and Reprogramming	4:15–4:35 PM **Refreshment Break/ Exhibits**	
4:30 PM		4:50–5:10 PM **Refreshment Break**		4:35–6:15 PM **Session 15** Engineered Heart Tissue for Modeling, Therapeutic Discovery and Repair	
5:00 PM	5:00-7:00 PM **Registration Opens**	5:10–6:50 PM **Session 5** The Emerging Importance of Diastolic Dysfunction			
6:00 PM		6:50–8:20 PM **Poster Session 1**	5:55–7:25 PM **Poster Session 2**	6:15–7:45 PM **Poster Session 3**	
6:30 PM					
7:00 PM					
7:30 PM			7:25–9:00 PM **Early Career Reception**	7:45 PM **Council Dinner** (Ticket required)	

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)
E mail *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 Council on Basic Cardiovascular Sciences, we welcome you to the Basic Cardiovascular Sciences 2014 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. This year marks the 10th anniversary of our annual BCVS meeting; thank you for joining us to celebrate this accomplishment as we further expand our reach and relevance.

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 gene and cell therapy. Invited speakers represent institutions from the United States, Europe, Asia, Latin/South America, and Australia as we further broaden our international scope and partnerships.

We continue to embrace the early career cardiovascular scientist with oral abstract presentations from early career scientists in a majority of the general sessions as well as a session dedicated specifically to the Outstanding Early Career Investigator Award presentations/competition. In addition to stimulating early career talks, over 350 Posters from submitted abstracts will be presented in three sessions. The program will once again offer two lunch workshops targeting early career development, as well as an early career reception where young investigators will have the opportunity to socialize with members of the BCVS Leadership Committee.

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. Please let us know if there is anything we can do to enrich your stay in Las Vegas and thank you for sharing your insight and expertise. We look forward to meeting you.

Sincerely,

Burns Blaxall, PhD
Program Co-Chair, BCVS 2014

Ju Chen, PhD
Program Co-Chair, BCVS 2014

Joseph Wu, MD, PhD
Program Co-Chair, BCVS 2014

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 organizations:

Celladon Corporation
Duke Cardiovascular Research Center
Fraternal Order of Eagles Diabetes Research Center
Heart Institute of Cincinnati Children's Hospital Medical Center
Lillehei Heart Institute
Pfizer, Inc.
UC San Diego Sulpizio Cardiovascular Center
Stanford Cardiovascular Institute
University of Iowa Francois M. Abboud Cardiovascular Research 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 2014 Program Committee

Invited Presenters

E. Dale Abel, MD, PhD, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA
Pilar Alcaide, PhD, Tufts Medical Center, Boston, MA
Euan Ashley, MB, DPhil, MRCP, Stanford University, Stanford, CA
Christopher Baines, PhD, University of Missouri-Columbia, Columbia, MO
Ivor Benjamin, MD, FACC, Medical College of Wisconsin, Milwaukee, WI
Burns Blaxall, PhD, Cincinnati Children's Hospital Medical Center, Cincinnati, OH
Roberto Bolli, MD, University of Louisville, Louisville, KY
Joan Heller Brown, PhD, University of California San Diego, La Jolla, CA
Ching-Pin Chang, MD, PhD, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN
Heping (Peace) Cheng, PhD, Institute of Molecular Medicine - Peking University, Beijing, China
Kenneth Chien, MD, PhD, Karolinska Institutet, Stockholm, Sweden
Wilson Colucci, MD, FACC, Boston University Medical Center, Boston, MA
Jose de la Pompa, PhD, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain
Arjun Deb, MD, University of California Los Angeles, Los Angeles, CA
Harry Dietz, MD, Johns Hopkins University School of Medicine, Baltimore, MD
Gerald Dorn, MD, Washington University School of Medicine, St. Louis, MO
Konstantinos Drosatos, MSc, PhD, Temple University School of Medicine, Philadelphia, PA
Stefan Engelhardt, MD, PhD, Technische Universitaet Muenchen (TUM), Munich, Germany
Jonathan Epstein, MD, University of Pennsylvania, Philadelphia, PA
Thomas Eschenhagen, MD, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
Loren Field, PhD, Riley Hospital for Children, Indianapolis, IN
Gemma Figtree, MBBS, PhD, Kolling Institute, Royal North Shore Hospital, Sydney, Australia
Thomas Force, MD, Vanderbilt University, Nashville, TN
Kleber Franchini, MD, PhD, Campinas State University, Campinas, Brazil
Keiichi Fukuda, MD, PhD, Keio University School of Medicine, Tokyo, Japan
Henk Granzier, PhD, University of Arizona, Tucson, AZ
Asa Gustafsson, PhD, University of California San Diego, La Jolla, CA
Roger Hajjar, MD, Mount Sinai School of Medicine, New York, NY
Saptarsi Haldar, MD, Case Western Reserve University School of Medicine, Cleveland, OH
Joshua Hare, MD, University of Miami, Miami, FL
Richard Harvey, PhD, Victor Chang Cardiac Research Institute, Darlinghurst, Australia
Steven Houser, PhD, Temple University School of Medicine, Philadelphia, PA
Patrick Hsieh, MD, PhD, Academia Sinica, Taipei, Taiwan
Masaki Ieda, MD, PhD, Keio University School of Medicine, Tokyo, Japan
Daniel Kelly, MD, Sanford-Burnham Medical Research Institute, Orlando, FL
Changwon Kho, PhD, Icahn School of Medicine at Mount Sinai, New York, NY
Il-man Kim, PhD, Georgia Regents University, Augusta, GA
Masahiro Kino-oka, PhD, Department of Biotechnology, Osaka University, Osaka, Japan
Richard Kitsis, MD, Albert Einstein College Medicine, New York, NY
Bjorn Knollmann, MD, PhD, Vanderbilt University, Nashville, TN
Peter Kohl, MD, PhD, Imperial College of London-National Heart and Lung Institute, London, United Kingdom
Richard Lee, MD, FACC, Harvard Medical School, Cambridge, MA
Hongliang Li, MD, PhD, Wuhan University, Wuhan, China
Ronglih Liao, PhD, Brigham and Women's Hospital, Boston, MA
Peter Libby, MD, Brigham and Women's Hospital, Boston, MA
Wolfgang Linke, PhD, DSc, Ruhr University Bochum, Bochum, Germany
Christoph Maack, MD, Saarland University Medical Center, Homburg, Germany
Douglas Mann, MD, Washington University School of Medicine, St. Louis, MO
Todd McDevitt, PhD, Georgia Institute of Technology, Atlanta, GA
Julie McMullen, PhD, Baker IDI Heart & Diabetes Institute, Melbourne, Australia
Elizabeth McNally, MD, PhD, University of Chicago, Chicago, IL

(continued on next page)

Invited Presenters (continued)

Jeffery Molkentin, PhD, Children's Hospital Medical Center, Cincinnati, OH
Cecilia Mundiña-Weilenmann, PhD, Centro de Investigaciones Cardiovasculares-Universidad Nacional de La Platade Medicina. UNLP, La Plata, Argentina
Sathyamangla Naga Prasad, PhD, Cleveland Clinic-Lerner Research Institute, Cleveland, OH)
Eric Olson, PhD, UT Southwestern Medical Center at Dallas, Dallas, TX
Peipei Ping, PhD, David Geffen School of Medicine at UCLA, Los Angeles, CA
Sumanth Prabhu, MD, University of Alabama-Birmingham, Birmingham, AL
William Pu, MD, Children's Hospital Boston, Boston, MA
Christoph D. Rau, PhD, University of California Los Angeles, Los Angeles, CA
Jeffrey Robbins, PhD, Cincinnati Children's Hospital, Cincinnati, OH
Howard Rockman, MD, Duke University, Durham, NC
Anthony Rosenzweig, MD, Beth Israel Deaconess Medical Center, Boston, MA
Hesham Sadek, MD, PhD, UT Southwestern Medical Center, Dallas, TX
Junichi Sadoshima, MD, PhD, Rutgers New Jersey Medical School, Newark, NJ
Michael Schneider, MD, Imperial College London, London, United Kingdom
Sarah M. Schumacher-Bass, PhD, Temple University, Philadelphia, PA
Jonathan Seidman, PhD, Harvard Medical School, Boston, MA
Karin Sipido, MD, PhD, KU Leuven, Leuven, Belgium
Henry Sucov, PhD, University of Southern California, Los Angeles, CA
Mark Sussman, PhD, San Diego State University, San Diego, CA
Michelle Tallquist, PhD, University of Hawaii, Honolulu, HI
Ling Tao, MD, PhD, The Fourth Military Medical University, Xi'an,Shaanxi, China
Jil Tardiff, MD, PhD, University of Arizona, Tucson, AZ
Leslie Tung, PhD, Johns Hopkins University, Baltimore, MD
Richard Vander Heide, MD, PhD, LSU Health New Orleans, New Orleans, LA
Inder Verma, PhD, Salk Institute for Biological Studies, La Jolla, CA
Martin Vila Petroff, PhD, Centro De Investigaciones Cv, La Plata, Argentina
Kenneth Walsh, PhD, Boston University School of Medicine, Boston, MA
Da-Zhi Wang, PhD, Boston Children's Hospital, Boston, MA
Yibin Wang, PhD, David Geffen School of Medicine at UCLA, Los Angeles, CA
Sean Wu, MD, PhD, Stanford University School of Medicine, Stanford, CA
Katherine Yutzey, PhD, Cincinnati Children's Medical Center, Cincinnati, OH
Wolfram Zimmermann, MD, University Medical Center Göttingen, Goettingen, Germany

Invited Moderators

John Calvert, Emory University School of Medicine, Atlanta, GA
Xiongwen Chen, PhD, Temple University, Philadelphia, PA
Gianluigi Condorelli, MD, PhD, Humanitas Research Hospital - University of Milan, Milan, Italy
Federica del Monte, MD, PhD, Beth Israel Deaconess Medical Center, Boston, MA
David Dostal, PhD, Texas A&M University Health Science Center, Temple, TX
Loren Field, PhD, Riley Hospital for Children, Indianapolis, IN
Nikolaos Frangogiannis, MD, Albert Einstein College of Medicine, Bronx, NY
Daniel Garry, MD, University of Minnesota, Minneapolis, MN
Roberta Gottlieb, MD, Cedars-Sinai Heart Institute, Los Angeles, CA
Joshua Hare, MD, University of Miami, Miami, FL
Joseph Hill, MD, PhD, UT Southwestern Medical Center, Dallas, TX
Michael Kapiloff, MD, PhD, University of Miami, Miami, FL
Lorrie Kirshenbaum, MD, PhD, St. Boniface Hospital Research Center, Winnipeg, Canada
Kirk Knowlton, MD, University of California San Diego, La Jolla, CA
Walter Koch, PhD, Temple University School of Medicine, Philadelphia, PA
Maria Kontaridis, PhD, Beth Israel Deaconess Medical Center, Boston, MA
Litsa Kranias, PhD, University of Cincinnati College of Medicine, Cincinnati, OH
David Lefer, PhD, Louisiana State University Health Sciences Center, New Orleans, LA
Annarosa Leri, MD, Brigham and Women's Hospital, Boston, MA
Joseph Libonati, PhD, University of Pennsylvania, Philadelphia, PA
William MacLellan, MD, University of Washington, Seattle, WA
Timothy McKinsey, PhD, University of Colorado Denver, Aurora, CO
Emiliano Medei, MD, PhD, Laboratorio de Eletrofisiologia Cardiaca, Rio de Janeiro, Brazil
Donald Menick, PhD, Gazes Cardiac Research Institute, Charleston, SC
Elizabeth Murphy, PhD, NHLBI, NIH, Bethesda, MD
Nicole Purcell, PhD, University of California San Diego, La Jolla, CA
Robert Ross, MD, University of California, San Diego, La Jolla, CA
Weinian Shou, PhD, Indiana University School of Medicine, Indianapolis, IN
Susan Steinberg, MD, Columbia University, New York, NY
Xuejun Wang, MD, PhD, University of South Dakota, Vermillion, SD
Loren Wold, Ohio State University, Columbus, OH
Joseph Woo, MD, Stanford University School of Medicine, Stanford, CA
Xiaolei Xu, PhD, Mayo Clinic, Rochester, MN
Jianyi Zhang, MD, PhD, University of Minnesota Medical School, Minneapolis, MN

Abstract Reviewers

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

Xiongwen Chen, PhD, Temple University, Philadelphia, PA
Gianluigi Condorelli, MD, PhD, University of California San Diego, La Jolla, CA
Gerald Dorn, MD, Washington University School of Medicine, St. Louis, MO
David Dostal, PhD, Texas A&M University HSC, Temple, TX
Gemma Figtree, MBBS, PhD, Royal North Shore Hospital, Sydney, Australia
Thomas Force, MD, Vanderbilt University, Nashville, TN
Nikolaos Frangogiannis, MD, Albert Einstein College of Medicine, Bronx, NY
Joshua Hare, MD, University of Miami, Miami, FL
Joseph Hill, MD, PhD, UT Southwestern Medical Center, Dallas, TX
Masahiro Kino-Oka, PhD, Osaka University, Osaka, Japan
Lorrie A. Kirshenbaum, MD, PhD, St. Boniface Hospital Research Center, Winnipeg, MB, Canada
Bjorn Knollmann, MD, PhD, Vanderbilt University, Nashville, TN
Maria Kontaridis, PhD, Beth Israel Deaconess Medical Center, Boston, MA
Richard Lee, MD, FACC, Harvard Medical School, Cambridge, MA
David Lefer, PhD, Louisiana State University Health Sciences Center, New Orleans, LA
Annarosa Leri, MD, Brigham and Women's Hospital, Boston, MA
Ronglih Liao, PhD, Brigham and Women's Hospital, Boston, MA
Joseph Libonati, PhD, University of Pennsylvania, Philadelphia, PA
William MacLellan, MD, Univeristy of Washington, Seattle, WA
Todd McDevitt, PhD, Georgia Institute of Technology, Atlanta, GA
Timothy McKinsey, PhD, University of Colorado, Aurora, CO
Emiliano Medei, MD, PhD, Laboratorio de Eletrofisiologia Cardiaca, Rio de Janeiro, Brazil
Elizabeth Murphy, PhD, NHLBI, NIH, Bethesda, MD
Sathyamangla Naga Prasad, PhD, The Cleveland Clinic Foundation, Cleveland, OH
William Pu, MD, Children's Hospital Boston, Boston, MA
Nicole Purcell, PhD, University of California San Diego, La Jolla, CA
Anthony Rosenzweig, MD, Beth Israel Deaconess Medical Center, Boston, MA
Robert Ross, MD, UCSD/VA Healthcare San Diego, San Diego, CA
Hesham Sadek, MD, PhD, UTSouthwestern Medical Center, Dallas, TX
Susan Steinberg, MD, Columbia University Medical Center, New York, NY
Henry Sucov, PhD, Univ of Southern California, Los Angeles, CA
Mark Sussman, PhD, San Diego State University, San Diego, CA
Michelle Tallquist, PhD, University of Hawaii, Honolulu, HI
Jil Tardiff, MD, PhD, University of Arizona, Tucson, AZ
Richard Vander Heide, MD, PhD, LSU Health, New Orleans, LA
Yibin Wang, PhD, David Geffen School of Medicine at UCLA, Los Angeles, CA
Da-Zhi Wang, PhD, Boston Children's Hospital, Boston, MA
Xuejun Wang, MD, PhD, University of South Dakota, Vermillion, SD
Joseph Wu, MD, Standford University School of Medicine, Stanford, CA
Sean Wu, PhD, MD, Stanford School of Medicine, Stanford, CA
Xiaolei Xu, PhD, MAYO Clinic, Rochester, MN
Katherine Yutzey, PhD, Cincinnati Children's Medical Center, Cincinnati, OH
Jianyi Zhang, MD, PhD, University of Minnesota Medical School, Minneapolis, MN

Room Locator

Time	Activity	Hotel Location, Floor
Sunday, July 13		
1:00–7:00 PM	Speaker Resource Room	Chablis, 1st
5:00–7:00 PM	Registration Opens	Champagne 1/2, 1st
2:00–10:00 PM	ACRE/APS Meeting	Versailles 1/2, 1st
Monday, July 14		
7:00 AM–5:00 PM	Speaker Resource Room	Chablis, 1st
7:00 AM–7:00 PM	Registration	Champagne 1/2, 1st
7:00–8:00 AM	Continental Breakfast	Champagne 1/2, 1st
8:00–10:00 AM	General Session	Concorde A/B, 1st
10:00–10:20 AM	Refreshment Break	Champagne 1/2, 1st
10:20 AM–NOON	General Session	Concorde A/B, 1st
1:45–4:50 PM	General Session	Concorde A/B, 1st
4:50–5:10 PM	Refreshment Break	Champagne 1/2, 1st
5:10–6:50 PM	General Session	Concorde A/B, 1st
6:50–8:20 PM	Poster Session 1	Champagne 3/4, 1st
Tuesday, July 15		
7:00 AM–5:00 PM	Speaker Resource Room	Chablis, 1st
7:00 AM–7:00 PM	Registration	Champagne 1/2, 1st
7:00–8:00 AM	Continental Breakfast/Exhibits	Champagne 1/2, 1st
8:00–9:40 AM	General Session	Concorde A/B, 1st
9:40–10:00 AM	Refreshment Break/Exhibits	Champagne 1/2, 1st
10:00–11:40 AM	General Session	Concorde A/B, 1st
NOON–1:30 PM	Career Development Workshop/Luncheon	Concorde C, 1st
1:30–3:10 PM	General Session	Concorde A/B, 1st
3:10–3:30 PM	Refreshment Break/Exhibits	Champagne 1/2, 1st
3:30–5:55 PM	General Session	Concorde A/B, 1st
5:55–7:25 PM	Poster Session 3	Champagne 3/4, 1st
7:30–8:30 PM	Early Career Reception	Versailles 4, 1st
Wednesday, July 16		
7:00 AM–5:00 PM	Speaker Resource Room	Chablis, 1st
7:00 AM–7:00 PM	Registration	Champagne 1/2, 1st
7:00–8:00 AM	Continental Breakfast/Exhibits	Champagne 1/2, 1st
8:00–9:40 AM	General Session	Concorde A/B, 1st
9:40–10:00 AM	Refreshment Break/Exhibits	Champagne 1/2, 1st
10:00–NOON	General Session	Concorde A/B, 1st
NOON–1:30 PM	Career Development Workshop/Luncheon	Concorde C, 1st
1:30–4:15 PM	General Session	Concorde A/B, 1st
4:15–4:35 PM	Refreshment Break/Exhibits	Champagne 1/2, 1st
4:35–6:15 PM	General Session	Concorde A/B, 1st
6:15–7:45 PM	Poster Session 3	Champagne 3/4, 1st
7:45 PM	Council Dinner	Rivoli A/B, 1st
Thursday, July 17		
7:00 AM–NOON	Speaker Resource Room	Chablis, 1st
7:00 AM–1:00 PM	Registration	Champagne 1/2, 1st
7:00–7:45 AM	Continental Breakfast/Exhibits	Champagne 1/2, 1st
7:45 AM–NOON	General Session/Adjourn	Concorde A/B, 1st

Program Information

Program Description

The 10th annual BCVS 2014 Conference — Pathways to Cardiovascular Therapeutics is considered by many to be the premier cardiovascular research meeting in the world. 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.

This year's program includes speakers from the United States, Europe, Japan, China, Latin/South America and Australia who were chosen on the basis of the outstanding nature and novelty of their research. New and up-and-coming junior scientists will participate in abstract talks in each session. We will hold our annual Early Career Investigator competition, and we will hear from three finalists selected from submitted competition abstracts. In addition to stimulating talks, posters from submitted abstracts will be presented in three sessions. The program will once again offer two lunch workshops targeting early career development and an early career reception where young investigators will have the opportunity to socialize with members of the BCVS Leadership Committee.

One aspect of the BCVS meeting that has remained constant over the years is the encouragement of multidisciplinary approaches to problems with the broad objective of providing a forum that promotes the relaxed exchange and discussion of cutting-edge research in molecular and translational cardiovascular biology and disease.

Learning Objectives

After completing this program, participants will be able to:

- Describe the most recent evidence on the role of fibrosis in heart failure, the molecular mechanisms that contribute to its development, and potential therapies to attenuate its progression.
- Discuss new targets for drug discovery and opportunities to participate in the translational research required to identify new compounds and bring them into clinical trials.
- Discuss current understandings of mitochondrial function in the development of heart failure and on future directions for research.
- Discuss how new models of atrial arrhythmias may alter the course of heart failure.
- Describe current understanding of the underlying mechanisms of arrhythmias in heart failure and directions for future research.

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 29.75 *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 29.75 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 CreditTM from organizations accredited by the ACCME.

Program Information (continued)

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, which is located by the registration desk in the Champagne 1/2 Ballroom.
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 2014 Sessions under the "Activity Catalog" tab and select to enroll.
- Complete the evaluation and claim your credit.
- Authorization code to claim your credit is **bcvs14**.

You are strongly encouraged to claim your CME/CE credit within 30 days of the conference, and you must claim your credit by Jan. 17, 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.

Speaker Resource Room

The Speaker Resource Room is located in Chablis on the first floor. 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 13	1:00–7:00 PM	Wednesday, July 16	7:00 AM–5:00 PM
Monday, July 14	7:00 AM–5:00 PM	Thursday, July 17	7:00 AM–NOON
Tuesday, July 15	7:00 AM–5:00 PM		

Conference Registration Hours

Sunday, July 13	5:00–7:00 PM	Wednesday, July 16	7:00 AM–7:00 PM
Monday, July 14	7:00 AM–7:00 PM	Thursday, July 17	7:00 AM–1:00 PM
Tuesday, July 15	7:00 AM–7:00 PM		

Abstract Presentations

Abstract presentations for the Basic Cardiovascular Sciences 2014 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 August online edition of the AHA journal *Circulation Research*.

Abstracts will be presented as follows:

Poster Abstracts

Poster Session 1	Monday, July 14	6:50–8:20 PM	1–119
Poster Session 2	Tuesday, July 15	5:55–7:25 PM	120–238
Poster Session 3	Wednesday, July 16	6:15–7:45 PM	239-362

Poster Presenters, please note the schedule below:

Poster Session 1

Set-up time:	Monday, July 14	NOON–5:00 PM
Attended time:	Monday, July 14	6:50–8:20 PM
Tear-down time:	Tuesday, July 15	before 9:00 AM

Poster Session 2

Set-up time:	Tuesday, July 15	NOON–5:00 PM
Attended time:	Tuesday, July 15	5:55–7:25 PM
Tear-down time:	Wednesday, July 16	before 9:00 AM

Poster Session 3

Set-up time:	Wednesday, July 16	NOON–5:00 PM
Attended time:	Wednesday, July 16	6:15–7:45 PM
Tear-down time:	Thursday, July 17	before 9:00 AM

Program Information (continued)

Web Resources

HealthJobsPlus
The American Heart Association and Lippincott Williams & Wilkins (a Wolters Kluwer business) are proud to offer HealthJobsPlus. HealthJobsPlus provides 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 powerful 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.

Conference Highlights

Keynote Lectures

Hal C. Dietz, III, MD, will present **Found in Translation: New Insights into the Pathogenesis and Treatment of Marfan Syndrome and Related Disorders** on Tuesday, July 15, at 3:30 PM. Dr. Dietz is Victor A. McKusick Professor of Pediatrics, Medicine, and Molecular Biology & Genetics in the Institute of Genetic Medicine at the Johns Hopkins University School of Medicine. He is also an Investigator in the Howard Hughes Medical Institute. His undergraduate training in biomedical engineering was performed at Duke University and his MD degree was received from the Health Sciences University of Syracuse. Clinical and research training in pediatrics, pediatric cardiology, and genetics occurred at Johns Hopkins University School of Medicine. Dr. Dietz heads a multidisciplinary clinic for the diagnosis and management of individuals with heritable forms of cardiovascular disease, with a special emphasis on Marfan syndrome and related connective tissue disorders. He is Director of the William S. Smilow Center for Marfan Research, a group of dedicated molecular biologists focused on improvement of the lives of individuals with Marfan syndrome and related disorders through the development of novel diagnostic and treatment strategies. Dr. Dietz has received multiple prestigious awards including the Curt Stern Award from the American Society of Human Genetics, the Taubman Prize for excellence in translational medical science, and the Harrington Prize for Innovation in Medicine. He is an inductee of the American Society for Clinical Investigation, American Association for the Advancement of Science, Academy of American Physicians, Institute of Medicine, Association of American Physicians, and the National Academy of Sciences.

Inder M. Verma, PhD, will present **Cancer Stem Cells: Lessons Learned From Glioblastomas** on Monday, July 14 at 4:05 PM. Dr. Verma, Laboratory of Genetics professor, holds the Irwin and Joan Jacobs Chair in Exemplary Life Science and is an American Cancer Society Professor of Molecular Biology. He received his PhD degree from the Weizmann Institute of Science in 1971 and completed his postdoctoral training with David Baltimore at MIT in 1974. Dr. Verma received an Outstanding Investigator Award from the NIH (1988), was elected a member in the Third World Academy of Sciences (1995), the National Academy of Sciences, India (1997), the National Academy of Sciences, USA (1997), the Institute of Medicine (1999), the American Academy of Arts and Sciences (2000), the European Molecular Biology Organization, EMBO (1998) and the American Philosophical Society (2006). He was awarded the Vilcek Foundation Prize (2008), ASGT Outstanding Achievement Award (2009) Spector Prize (2010), and the Pasarow Award in Cancer Research (2010). Dr. Verma now serves as the Editor-in-Chief of PNAS.

Outstanding Early Career Investigator Award Finalists' Presentations

The three finalists will present their abstracts on Wednesday, July 16, 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

Special programs are planned for early career attendees on Tuesday, July 14, and Wednesday, July 15. Featured topics include:

- How to Successfully Transition From Your SDG/BGIA Early Investigator Award to the Coveted R01
- Publish or Perish: How to Write a Manuscript for Publication in Circulation Research
- Balancing Professional and Personal Responsibilities: How to Achieve Career Success Without Missing Out on Life
- The Key to Successfully Revising Your Fellowship or Grant Application: The Importance of Responding to the Reviewers' Critiques

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

Council on Basic Cardiovascular Sciences Dinner

Please plan to join us for dinner in the Rivoli A/B on Wednesday, July 16, at 7:45 PM. The cost of the dinner is $60 for each conference registrant and their guest. Tickets may be purchased at the AHA registration desk.

Exhibits

Beginning Tuesday, July 15, at 7:00 AM, visit the Exhibitors, located in Champagne 1/2. This year we welcome:

- American Heart Association
- American Physiological Society
- Eckard Global, Inc.
- emka TECHNOLOGIES Inc.
- Feel Good, Inc.
- IonOptix
- Miltenyi Biotec

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

Conference 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

Name/Institution	Abstract Number
Konstantinos Drosatos, MSc, PhD Temple University School of Medicine	O-1
Changwon Kho, PhD Icahn School of Medicine at Mount Sinai, New York, NY	O-2
Il-man Kim, PhD Georgia Regents University, Augusta, GA	O-3

The finalists for the Outstanding Early Career Investigator Award will present their abstracts during a special oral session scheduled for 1:30–2:15 on Wednesday, July 16. The winner will be announced at the Council Dinner that evening.

Cardiovascular Outreach Award Recipients

Name	Abstract/Poster Number
Alejandro Chavez	n/a
Michael Coronado	83
Robert Davis	40
Sarah Jimenez	340
Pius Nde	313
Amabel Orogo	206
Jose Renato Pinto	n/a
Xiaojing Yue	270

New Investigator Travel Award Recipients

Name	Abstract/Poster Number
Luiza Bagno	214
Kedryn Baskin	201
Bianca Bernardo	50
Jingjing Cai	97
Alessandro Cannavo	51
Young Wook Chun	121/122
Venkata Garikipati	3
David Grubb	33
Shuichiro Higo	76
Luke Hoeppner	117
Zhan-Peng Huang	65
Genesio Karere	278
Dieter Kubli	149
Ji Li	341
Xiaojun Liu	238
Qinqiang Long	328
Eliana Martinez	106/107
Catherine Passariello	190
Ashraf Rangrez	181
Christoph Rau	163
Sushma Reddy	61
Priscila Sato	208
Vivek Singh	135
Amanda Stram	210
Jun Wang	260
Andrew Wojtovich	110
Fu-li Xiang	23
Xiangsheng Yang	288
Tomohiro Yokota	287
Xiaoying Zhang	72

Policy Information

Disclaimer
The Basic Cardiovascular Sciences Scientific Sessions is a scientific and educational conference for the purpose of 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.

Embargo Guidelines
Abstracts, lectures and presentations in BCVS 2014 are embargoed for release at the time of presentation. Information may not be released before the scheduled presentation time.

Photography/Recording Policy
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Exceptions to this policy of prohibition are as follows: (1) no flash photography of the speaker and his/her presentation materials is permitted by attendees or AHA/ASA accredited reporters upon the prior written consent of the AHA/ASA and of the speaker; (2) other photography and videotaping by AHA/ASA-accredited reporters is permitted if not disruptive; (3) audiotape recording for strictly personal and noncommercial use is permitted if not disruptive; and (4) exhibitors may photograph their own booth for promotional purposes upon prior written consent of the AHA/ASA.

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Please be aware that during BCVS 2014, attendees, vendors and guests may be photographed and videotaped by AHA vendors in capturing the course of the event. Some of these photographs or videos may be displayed by the AHA in future publications or materials connected with the event. If you do not wish your image to be displayed by the AHA, please contact the AHA in writing at 7272 Greenville Avenue, Dallas, TX 75231, Attention: Manager, Scientific Conferences, BCVS 2014 Scientific Sessions, no later than Aug. 31, 2014.

No Smoking Policy
AHA policy prohibits smoking in conference meeting rooms and exhibits/registration areas. Thank you for your cooperation.

Seating/Badge Requirement
Seating is on a first-come, first-served basis. According to fire code, a session must be closed if the room fills to capacity. You must wear your name badge at all times during the symposium. Nonregistered guests may not be permitted into the sessions or food and beverage events. Be sure to remove your badge when you leave the conference or your hotel room.

The American Heart Association reserves the right to revoke or deny attendance to any registered participant, speaker, exhibitor, news media reporter or photographer of presentations or activities at AHA/ASA scientific conferences and meetings.

Please note: The American Heart Association shall not be liable for cancellation of the Basic Cardiovascular Sciences 2014 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

The BCVS Council strives to highlight the work of early career investigators. More than 20 percent of the talks in this program are presented by early career speakers

MONDAY

MONDAY, JULY 14

7:00–8:00 AM
Continental Breakfast/Registration
Champagne 1/2

8:00–10:00 AM
Concorde A/B
Session 1
New Twists on Signaling Mechanisms in the Heart

Moderators:
Xiongwen Chen, PhD, Temple University, Philadelphia, PA
Robert Ross, MD, UC San Diego/VA San Diego Healthcare System, San Diego, CA

8:00 **Drug Discovery Targeting Novel Necrotic Signaling Pathways**
Richard Kitsis, MD, Albert Einstein College Medicine, New York, NY

8:20 **New Mechanisms in Myocardial Autophagy**
Junichi Sadoshima, MD, PhD, UMDNJ New Jersey Medical School, Newark, NJ

8:40 **Focal Adhesion Kinases in Cardiac Hypertrophy**
Kleber Franchini, MD, PhD, Campinas State University, Campinas, Brazil

9:00 **Exploring CaMKII Signaling in Myocardial Digitalis Toxicity**
Martin Vila Petroff, PhD, Centro de Investigaciones Cardiovasculares, La Plata, Argentina

9:20 **The Role of Diabetes in Myocardial Ischemia-reperfusion Injury**
LingTao, MD, PhD, The Fourth Military Medical University, Xi'an, Shaanxi, China

9:40 **Non-canonical GRK2 Function Modulates Myocardial TNFalpha Signaling**
Sathyamangla Naga Prasad, PhD, Cleveland Clinic-Lerner Research Institute, Cleveland, OH

10:00–10:20 AM
Refreshment Break
Champagne 1/2

10:20 AM–NOON
Concorde A/B
Session 2
Cardiac Signaling, Conduction and Exercise

Moderators:
Joshua Hare, MD, University of Miami, Miami, FL
Joseph Libonati, PhD, University of Pennsylvania, Philadelphia, PA

10:20 **Exercise-based Discovery of Novel Targets in Heart Disease**
Anthony Rosenzweig, MD, Beth Israel Deaconess Medical Center, Boston, MA

10:40 **IGF1 Signaling and Exercise Induced Hypertrophy**
Julie McMullen, PhD, Baker IDI Heart and Diabetes Institute, Melbourne, Australia

11:00 **Calcium, CaMKII and ROS Microdomains in Ischemic Cardiomyopathy**
Karin Sipido, MD, PhD, Katholieke University Leuven, Leuven, Belgium

11:20 **Disease Modeling of Brugada Syndrome Using iPS Cells**
Keiichi Fukuda, MD, PhD, Keio University School of Medicine, Tokyo, Japan

11:40 **Catecholaminergic Polymorphic Ventricular Tachycardia – Paradigm for Calcium Triggered Arrhythmia**
Bjorn Knollmann, MD, PhD, Vanderbilt University, Nashville, TN

1:45–2:05 PM
Welcome Remarks
Concorde A/B

1:45 **Elliott Antman, MD, President American Heart Association, Brigham and Women's Hospital, Associate Dean for Clinical/Translational Research, Harvard Medical School, Boston, MA**

2:05–4:05 PM
Concorde A/B
Session 3
Signaling Pathways and Heart Failure Progression

Moderators:
John Calvert, Emory University School of Medicine, Atlanta, GA
Susan Steinberg, MD, Columbia University, New York, NY

2:05 **Biased Signaling in Cardiovascular Therapeutics**
Howard Rockman, MD, Duke University, Durham, NC

Program Agenda (continued)

2:25 **Redox Modifications of Proteins Involved in Cardiac Arrhythmogenesis During Reperfusion**
Cecilia Mundiña-Weilenmann, PhD, Centro de Investigaciones Cardiovasculares-Universidad Nacional de La Platade Medicina, La Plata, Argentina

2:45 **Targets of CaMKII – Mediated Cell Death and Heart Failure Progression**
Joan Heller Brown, PhD, University of California San Diego, La Jolla, CA

3:05 **Something Old, Something New: L- and T-type Calcium Channels in Heart Failure Progression**
Steven Houser, PhD, Temple University School of Medicine, Philadelphia, PA

3:25 **HDAC4 Controls Histone Response to Elevated Cardiac Load**
Christopher Maack, MD, Saarland University Medical Center, Homburg, Germany

3:45 **Molecular Mechanisms of Anthracycline Cardiotoxicity**
Richard Vander Heide, MD, PhD, LSU Health New Orleans, New Orleans, LA

4:05–4:50 PM
Concorde A/B
Session 4
Keynote Lecture

Moderator:
Kirk Knowlton, MD, University of California San Diego, La Jolla, CA

4:05 **Cancer Stem Cells: Lessons Learned From Glioblastomas**
Inder M. Verma, PhD, Salk Institute for Biological Studies, La Jolla, CA

4:50–5:10 PM
Refreshment Break
Champagne 1/2

5:10–6:50 PM
Concorde B/C
Session 5
The Emerging Importance of Diastolic Dysfunction

Moderators:
Joseph Woo, MD, Stanford University School of Medicine, Stanford, CA
Michael Kapiloff, MD, PhD, University of Miami, Miami, FL

5:10 **Titin-the Giant Protein of Heart Failure**
Henk Granzier, PhD, University of Arizona, Tucson, AZ

5:30 **Role of Titin in Myocardial Diastolic Stiffness**
Wolfgang Linke, PhD, DSc, Ruhr University Bochum, Bochum, Germany

5:50 **Mechanisms of Diastolic Dysfunction in Metabolic Heart Disease**
Wilson Colucci, MD, FACC, Boston University Medical Center, Boston, MA

6:10 **Modeling Diastolic Dysfunction in Engineered Heart Muscle**
Wolfram Zimmermann, MD, University Medical Center Göttingen, Göttingen, Germany

6:30 **Mechanisms and Implications of Redox Regulation of the Na+-K+ Pump in the Cardiovascular System**
Gemma Figtree, MBBS, PhD, Royal North Shore Hospital, Sydney, Australia

6:50–8:20 PM
Champagne 3/4
Poster Session 1

TUESDAY, JULY 15

7:00–8:00 AM
Continental Breakfast/Registration/Exhibits
Champagne 1/2

8:00–9:40 AM
Concorde B/C
Session 6
Genetics and Genomics of Heart Failure

Moderators:
Emiliano Medei, MD, PhD, Instituto de Biofisica Carlos Chagas Filho, Rio de Janeiro, Brazil
Elizabeth Murphy, PhD, NHLBI, NIH, Bethesda, MD

8:00 **Inherited Cardiomyopathies and Heart Failure**
Jonathan Seidman, PhD, Harvard Medical School, Boston, MA

8:20 **Genotype-phenotype Interactions in Cardiomyopathy**
Elizabeth McNally, MD, PhD, University of Chicago, Chicago, IL

8:40 **Integrative Genomics of Heart Failure**
Euan Ashley, MB, DPhil, MRCP, Stanford University, Stanford, CA

9:00 **Knockout Rats to Investigate Cardiovascular Function and Disease**
Hongliang Li, MD, PhD, Wuhan University, Wuhan, China

9:20 **An Integrative Approach to Sarcomeric Cardiomyopathies: From Computation to Animal Models**
Jil Tardiff, MD, PhD, University of Arizona, Tucson, AZ,

9:40–10:00 AM
Refreshment Break/Exhibits
Champagne 1/2

Program Agenda (continued)

10:00–11:40 AM
Concorde A/B
Session 7
Cardiac Epigenetics and Regulatory RNA's

Moderators:
Gianluigi Condorelli, MD, PhD, Humanitas Clinical and Research Center, Milan, Italy
Lorrie Kirshenbaum, PhD, St. Boniface Hospital Research Center, Winnipeg, Canada

10:00 **HDACs in Cardiovascular Development, Physiology and Disease**
Jonathan Epstein, MD, University of Pennsylvania, Philadelphia, PA

10:20 **Targeting Epigenetic Reader Proteins in Heart Failure**
Saptarsi Haldar, MD, Case Western Reserve University School of Medicine, Cleveland, OH

10:40 **Alternative Splicing in Heart Failure Pathogenesis**
Yibin Wang, PhD, David Geffen School of Medicine at UCLA, Los Angeles, CA

11:00 **miRNAs in Cardiomyocyte Proliferation and Cardiac Regeneration**
Da-Zhi Wang, PhD, Boston Children's Hospital, Boston, MA

11:20 **LncRNA Mechanism of Heart Failure**
Ching-PinChang, MD, PhD, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN

NOON–1:30 PM
Concorde C
Early Career Workshop/Lunch
(Ticket required for lunch)

Moderators:
Maria Kontaridis, PhD, Beth Israel Deaconess Medical Center, Boston, MA
Loren Wold, Ohio State University, Columbus, OH

12:00 **Balancing Professional and Personal Responsibilities: How to Achieve Career Success Without Missing Out on Life**
Asa Gustafsson, PhD, University of California, La Jolla, CA

12:45 **The Key to Successfully Revising Your Fellowship or Grant Application: The Importance of Responding to the Reviewers' Critiques**
Burns Blaxall, PhD, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

1:30–3:10 PM
Concorde A/B
Session 8
Cardiovascular Omics

Moderators:
Joseph Hill, MD, PhD, UT Southwestern Medical Center, Dallas, TX
Xiaolei Xu, PhD, Mayo Clinic, Rochester, MN

1:30 **The Cardiosplenic Axis in Heart Failure**
Sumanth Prabhu, MD, University of Alabama-Birmingham, Birmingham, AL

1:50 **A Systems Approach to Decipher the Metabolic Origins of Heart Failure**
Daniel Kelly, MD, Sanford-Burnham Medical Research Institute, Orlando, FL

2:10 **Metabolic Signaling and Myocardial Dysfunction in Insulin Resistance and Obesity**
Dale Abel, MD, PhD, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA

2:30 **Cardiac Amyloidosis: Model Organisms Inform Human Disease**
Ronglih Liao, PhD, Brigham and Women's Hospital, Boston, MA

2:50 **Myocardial Reductive Stress**
Ivor Benjamin, MD, FACC, Medical College of Wisconsin, Milwaukee, WI

3:10–3:30 PM
Refreshment Break/Exhibits
Champagne 1/2

3:30–4:15 PM
Concorde A/B
Session 9
Keynote Lecture

Moderator:
Walter Koch, PhD, Temple University School of Medicine, Philadelphia, PA

3:30 **Found in Translation: New Insights into the Pathogenesis and Treatment of Marfan Syndrome and Related Disorders**
Hal Dietz, MD, Johns Hopkins University School of Medicine, Baltimore, MD

4:15–5:55 PM
Concorde A/B
Session 10
Epicardium vs Endocardium in Myocardial Repair and Reprogramming

Moderators:
David Lefer, PhD, Louisiana State University Health Sciences Center, New Orleans, LA
Weinian Shou, PhD, Indiana University School of Medicine, Indianapolis, IN

4:15 **Role of the Epicardium in Mammalian Cardiac Development and Repair**
William Pu, MD, Children's Hospital Boston, Boston, MA

4:35 **Epicardium-derived Mesenchymal Stem-like Cells in Cardiac Homeostasis and Disease**
Richard Harvey, PhD, University of New South Wales, Darlinghurst, Australia

4:55 **Endocardial Notch Signaling**
Jose de la Pompa, PhD, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain

5:15 **Aortic Valve Development and Disease**
Katherine Yutzey, PhD, Cincinnati Children's Medical Center, Cincinnati, OH

5:35 **Wnt1/βcatenin Signaling in Epicardial Activation**
Arjun Deb, MD, University of California Los Angeles, Los Angeles, CA

5:55–7:25 PM
Champagne 3/4
Poster Session 2

7:25–9:00 PM
Early Career Reception
Versailles 4

WEDNESDAY, JULY 16

7:00–8:00 AM
Continental Breakfast/Registration/Exhibits
Champagne 1/2

8:00–9:40 AM
Concorde A/B
Session 11
Emerging Role of Cardiac Fibroblasts

Moderators:
Nikolaos Frangogiannis, MD, Albert Einstein College of Medicine, Bronx, NY
Timothy McKinsey, PhD, University of Colorado Denver, Aurora, CO

8:00 **Epicardial Derived Cells in Fibrotic Remodeling**
Michelle Tallquist, PhD, University of Hawaii, Honolulu, HI

8:20 **miRNAs Modulate Cardiac Fibrosis**
Stefan Engelhardt, MD, PhD, Technische Universitaet Muenchen (TUM), Munich, Germany

8:40 **The Odd Couple: Myocyte-fibroblast Coupling in the Heart**
Peter Kohl, MD, PhD, Imperial College of London-National Heart and Lung Institute, London, United Kingdom

9:00 **The Interactive Roles of Inflammation and Fibrosis**
Douglas Mann, MD, Washington University School of Medicine, St Louis, MO

9:20 **Discovery, Progress, and Challenges of Direct Cardiac Reprogramming for Heart Repair**
Masaki Ieda, Keio University School of Medicine, Tokyo, Japan

9:40–10:00 AM
Refreshment Break/Exhibits
Champagne 1/2

10:00 AM–NOON
Concorde A/B
Session 12
Cell and Tissue Reprogramming

Moderators:
W. Robb MacLellan, MD, University of Washington, Seattle, WA
Litsa Kranias, PhD, University of Cincinnati College of Medicine, Cincinnati, OH

10:00 **Reprogramming of Human Fibroblasts Toward a Cardiac Fate**
Eric Olson, PhD, UT Southwestern Medical Center at Dallas, Dallas, TX

10:20 **Modified mRNA Directs the Fate of Heart Progenitor Cells**
Kenneth Chien, MD, PhD, Karolinska Institutet, Stockholm, Sweden

10:40 **Heart Regeneration and the Potential Role of Nerves**
Richard Lee, MD, Harvard Medical School, Cambridge, MA

11:00 **Incomplete Transdifferentiation with Cardiac Reprogramming Factors**
Sean Wu, MD, PhD, Stanford University School of Medicine, Stanford, CA

11:20 **Genetic Lineage Tracing for Ckit Progenitor Cell Contribution to the Heart**
Jeffery Molkentin, PhD, Children's Hospital Medical Center, Cincinnati, OH

11:40 **Where Are We With Cell-based Therapy Translational Research and Clinical Trials**
Joshua Hare, MD, University of Miami, Miami, FL

NOON–1:30 PM
Concorde C
Early Career Workshop/Lunch
(Ticket required for lunch)

Moderators:
Nicole Purcell, PhD, University of California San Diego, La Jolla, CA
Donald Menick, PhD, Gazes Cardiac Research Institute, Charleston, SC

12:00 **How to Successfully Transition From Your SDG/BGIA Early Investigator Award to the Coveted R01**
Christopher Baines, PhD, Dalton Cardiovascular Research Center, University of Missouri Columbia, MO

12:45 **Publish or Perish: How to Write a Manuscript for Publication in *Circulation Research***
Roberto Bolli, MD, University of Louisville, Louisville, KY

1:30–2:15 PM
Concorde A/B
Session 13
Outstanding Early Career Investigator Award Finalists

Moderators:
Kirk Knowlton, MD, University of California San Diego, La Jolla, CA
W. Robb MacLellan, MD, University of Washington, Seattle, WA

1:30 **Klf5 Regulates Cardiac Pparα and Med13 and Affects Cardiac Fatty Acid Metabolism and Obesity**
Konstantinos Drosatos, MSc, PhD, Temple University School of Medicine, Philadelphia, PA

1:45 **The Role of Post-translational Modifications in SERCA2a-related Cardiac Dysfunction**
Changwon Kho, PhD, Icahn School of Medicine at Mount Sinai, New York, NY

2:00 **A β1-Adrenergic Receptor/β-Arrestin1-Regulatable MicroRNA, MiR-150 Protects the Mouse Heart from Ischemic Injury by Repressing Pro-apoptotic Egr2 and P2x7r**
Il-man Kim, PhD, Georgia Regents University, Augusta, GA

2:15–4:15 PM
Concorde A/B
Session 14
Joint AHA/HFSA: Myocardial Repair and Regeneration

Moderators:
Daniel Garry, MD, University of Minnesota, Minneapolis, MN
Annarosa Leri, MD, Brigham and Women's Hospital, Boston, MA

2:15 **PDGFRα Demarcates the Cardiogenic Clonogenic Sca-1+ Stem Cell in Adult Myocardium**
Michael Schneider, MD, National Heart and Lung Institute, London, United Kingdom

2:35 **Continuity of Heart Development and Regeneration: Molecules and Cells**
Henry Sucov, PhD, University of Southern California, Los Angeles, CA

2:55 **Myocardial Regeneration in Mouse Hearts**
Loren Field, PhD, Riley Hospital for Children, Indianapolis, IN

3:15 **Gene Therapy for Heart Failure**
Roger Hajjar, MD, Mount Sinai School of Medicine, New York, NY

3:35 **The Role of Prostaglandin E2 in Regulating Endogenous Cardiac Regeneration**
Patrick Hsieh, MD, PhD, National Cheng Kung University, Tainan City, Taiwan

3:55 **Teaching an Old Heart New Tricks: Lessons from the Neonate**
Hesham Sadek, MD, PhD, UT Southwestern Medical Center, Dallas, TX

4:15–4:35 PM
Refreshment Break/Exhibits
Champagne 1/2

Program Agenda (continued)

4:35–6:15 PM
Concorde A/B
Session 15
Engineered Heart Tissue for Modeling, Therapeutic Discovery and Repair

Moderators:
TBD
Loren Field, PhD, Riley Hospital for Children, Indianapolis, IN

4:35 **Human Engineered Heart Tissue**
Thomas Eschenhagen, MD, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

4:55 **Facility Design and Bioreactor System for Cell Production**
Masahiro Kino-Oka, PhD, Osaka University, Osaka, Japan

5:15 **Scalability for Stem Cell Bioprocessing and Engineered Tissue Modules**
Todd McDevitt, PhD, Georgia Institute of Technology, Atlanta, GA

5:35 **Optical Imaging of Cardiac Microtissues for Drug Discovery**
Leslie Tung, PhD, John Hopkins University, Baltimore, MD

5:55 **TBD**

6:15–7:45 PM
Champagne 3/4
Poster Session 3

7:45 PM
Rivoli A/B
Council Dinner
(TIcket required)

THURSDAY, JULY 17

7:00–8:00 AM
Continental Breakfast/Registration
Champagne 3/4

8:00–10:00 AM
Concorde A/B
Session 16
Impact of Non-myocytes on Myocardial Function

Moderators:
David Dostal, PhD, Texas A&M University Health Science Center, Temple, TX
Jianyi Zhang, MD, PhD, University of Minnesota Medical School, Minneapolis, MN

8:00 **Cancer Drug Toxicity in the Heart**
Thomas Force, MD, Vanderbilt University, Nashville, TN

8:20 **T cells in the Heart: Their Role in the Pathogenesis of Heart Failure**
Pilar Alcaide, PhD, Tufts Medical Center, Boston, MA

8:40 **Inflammatory Cell Functions in the Injured Myocardium**
Peter Libby, MD, Brigham and Women's Hospital, Boston, MA

9:00 **Advances and Lessons Learned From Clinical Trials with Cardiac Stem Cells**
Roberto Bolli, MD, University of Louisville, Louisville, KY

9:20 **Myocardial Regeneration: Uncommon Sense for Common Problems**
Mark Sussman, PhD, San Diego State University, San Diego, CA

9:40 **Genetic Basis of Isoproterenol-Induced Cardiac Fibrosis**
Christoph D. Rau, PhD, University of California Los Angeles, Los Angeles, CA

10:00 AM–NOON
Concorde A/B
Session 17
Mitochondrial Biology and Protein Misfolding and/or Proteotoxicity

Moderators:
Roberta Gottlieb, MD, San Diego State University, San Diego, CA
Xuejun Wang, MD, PhD, University of South Dakota, Vermillion, SD

10:00 **Mitochondrial Triage in the Heart**
Gerald Dorn, MD, Washington University School of Medicine, St. Louis, MO

10:20 **Mitofusins in Heart**
Kenneth Walsh, PhD, Boston University School of Medicine, Boston, MA

10:40 **Proteotoxicity and Cardiac Dysfunction**
Jeffrey Robbins, PhD, Cincinnati Children's Hospital, Cincinnati, OH

11:00 **Calcium Regulation of Mitochondrial Energetics**
Heping (Peace) Cheng, PhD, Institute of Molecular Medicine – Peking University, Beijing, China

11:20 **Understanding Molecular Signatures of Cardioprotection: A Data to Knowledge Strategy**
Peipei Ping, PhD, David Geffen School of Medicine at UCLA, Los Angeles, CA

11:40 **Domain-Specific Roles for GRK2 in Cardiac Hypertrophy and Heart Failure**
Sarah M. Schumacher-Bass, PhD, Temple University, Philadelphia, PA

NOON
Adjourn

O-1

Klf5 Regulates Cardiac Pparα and Med13 and affects Cardiac Fatty Acid Metabolism And Obesity

Konstantinos Drosatos, Temple Univ, Philadelphia, PA; Nina Pollak, Inst of Molecular Biosciences, Univ of Graz, Graz, Austria; Panagiotis Ntziachristos, Dept of Pathology, New York Univ Sch of Med, New York, NY; Chad M. Trent, Yunying Hu, Shunichi Homma, Columbia Univ, New York, NY; Iannis Aifantis, Howard Hughes Medical Inst & Dept of Pathology, New York Univ Sch of Med, New York, NY; Ira J. Goldberg, Columbia Univ, New York, NY

Krüppel-like factors (KLF) have been associated with metabolic phenotypes. Our study focused on the metabolic role of cardiac KLF5, as it showed the highest increase among all KLFs that were detected by whole genome microarrays of energy-starved hearts obtained from lipopolysaccharide (LPS)-treated mice. Analysis of ppara promoter indicated two potential binding sites for c-Jun (AP-1 sites), the transcriptional factor that is activated by LPS and reduces cardiac PPARα expression: -792/-772 bp and -719/-698 bp prior to the transcription initiation site. This analysis showed that both AP-1 sites overlap with potential KLF-binding sites. Adenovirus-mediated expression of constitutively active c-Jun in a mouse cardiomyocyte cell line (HL-1) reduced PPARα gene expression, while treatment with Ad-KLF5 had the opposite effect. Chromatin immunoprecipitation analysis (ChIP) showed that c-Jun binds both -792/-772 bp and -719/-698 sites of ppara promoter while KLF5 binds on -792/-772 bp. ChIP analysis also showed that LPS promotes c-Jun binding on -792/-772 bp, which prohibits occupation of this region by KLF5. A cardiomyocyte-specific KLF5 knockout mouse (αMHC-KLF5-/-) had normal cardiac function but reduced cardiac expression of PPARα (50%) and other fatty acid metabolism-associated genes such as CD36 (40%), LpL (20%), PGC1α (45%), AOX (28%) and Cpt1 (45%). High fat diet (HFD)-fed αMHC-KLF5-/- mice had a more profound body weight increase (35%) compared to HFD-fed WT mice (15%), as well as larger white adipocytes and brown adipocytes (H&E) and increased hepatic neutral lipid accumulation (Oil-Red-O). The obesogenic effect of cardiomyocyte-specific deletion of KLF5 resembles the phenotype of the αMHC-MED13-/- mice. We showed that KLF5 ablation reduced cardiac MED13 levels despite lack of changes in the expression levels of miR-208, a known regulator of MED13. Infection of HL-1 cells with Ad-KLF5 increased MED13 gene expression. ChIP identified a KLF5 binding site on med13 gene promoter region (-730/-714 bp). Thus, KLF5 regulates cardiac PPARα and MED13 and affects cardiac and systemic fatty acid metabolism and obesity, thus indicating KLF5 as a potential target for cardiac dysfunction associated with energetic complications, as well as for obesity

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O-2

The Role of Post-translational Modifications in SERCA2a-related Cardiac Dysfunction

Changwon Kho, Dongtak Jeong, Ahyoung Lee, Shinichi Mitsuyama, Jae Gyun Oh, Roger Hajjar, Icahn Sch of Med at Mount Sinai, New York, NY

The cardiac sarcoplasmic reticulum calcium ATPase (SERCA2a) has become a validated target for the treatment of heart failure (HF). The relationship between reduced SERCA2a activity and decreases in protein expression in the setting of HF has been found to be non-linear and the toxic intracellular milieu in HF contributes to SERCA2a's dysfunction. Post-translational modification (PTM) of SERCA2a has been recently described to as an important mechanism that can explain a reduction in SERCA2a activity in HF. Based on a comprehensive proteomic analysis, we found that the levels and activity of SERCA2a in cardiomyocytes are modulated in parallel with the levels of small ubiquitin-like modifier type 1 (SUMO-1). Moreover, our work has shown that SUMO-1 plays a critical role in protecting SERCA2a from pathological conditions (Kho et al, Nature, 2011). More recently, we demonstrated that SUMO-1 gene transfer and its combination with SERCA2a led to a reversal of HF in a porcine model of ischemic induced HF (Tilemann et al, Sci Transl Med, 2013). In our analysis of SERCA2a PTM in animal models of HF, we observed that SERCA2a is acetylated, and that this acetylation is more prominent in failing hearts. The acetylation of SERCA2a was

validated by acetylation assays with acetyltransferase and HDAC inhibitors. We identified several lysine residues on SERCA2a for susceptible to acetylation. In addition, we found that Sirt1 enzyme deacetylates SERCA2a. Sirt1 down-regulation in HL-1 cells using small interfering RNA increased SERCA2a acetylation and thereby decreased its activity. Moreover, SERCA2a acetylation was increased when Sirt1 was depleted by recombinant adeno-associated virus carrying short hairpin RNA for Sirt1 in mice model, which reflected a decrease in intensity of interaction between Sirt1 and SERCA2a. Reduced acetylation was accompanied by an increase in SERCA2a SUMOylation in the heart. Decreased acetylation, combined with increased SUMOylation, of SERCA2a may contribute to the cardioprotective effects of Sirt1. Our results show that SERCA2a acetylation increases during HF and negatively impacts SERCA2a's function, suggesting that the down-regulation of SERCA2a acetylation may afford a novel intervention in the setting of heart failure.

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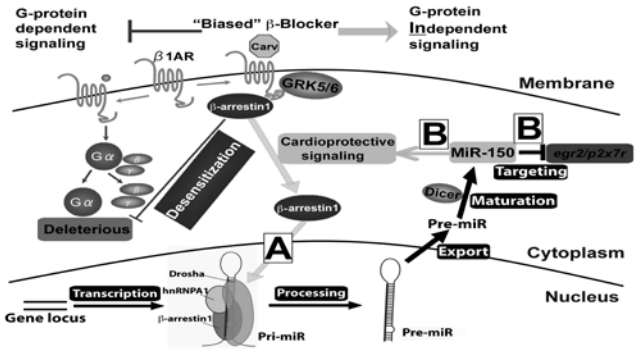
O-3

A β1-Adrenergic Receptor/β-Arrestin1-Regulatable MicroRNA, MiR-150 Protects the Mouse Heart from Ischemic Injury by Repressing Pro-apoptotic Egr2 and P2x7r

Il-man Kim, Yaoping Tang, Yongchao Wang, Kyoung-mi Park, Qiuping Hu, Georgia Regents Univ, Augusta, GA

MicroRNA (miR)-150 is down-regulated in patients with acute myocardial infarction (AMI), atrial fibrillation, dilated and ischemic cardiomyopathy as well as in various mouse heart failure (HF) models. Circulating miR-150 has been recently proposed as a better biomarker of HF than clinically used markers such as brain natriuretic peptide. We recently showed that β-arrestin1-biased β1-adrenergic receptor (β1AR) cardioprotective signaling activated by the β-arrestin-biased β-blocker, carvedilol (Carv) stimulates the processing of miR-150 in the heart (see figure A). However, the potential role of miR-150 in ischemic injury and HF is unknown. Here, we show that genetic deletion of miR-150 in mice causes abnormalities in cardiac structural and functional remodeling after MI. The cardioprotective roles of miR-150 during ischemic injury were attributed to repression of the pro-apoptotic genes egr2 (zinc binding transcription factor induced by ischemia) and p2x7r (pro-inflammatory ATP receptor) [see figure B]. These findings reveal a pivotal role for miR-150 as a regulator of cardiomyocyte survival during cardiac injury. In conclusion, our study will help to stratify HF patients that may respond better to β-arrestin-biased β-blockers, which is guided by circulating levels of miR-150.



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ABSTRACTS

1

Hypoxia-Induced Decrease In Cardiac Stem Cell Proliferation Is Associated With Downregulation Of Sirtuin 1

Michael Bellio, Claudia O Rodrigues, Victoria Florea, Wayne Balkan, Joshua M Hare, Ivonne H Schulman, Univ of Miami, Miami, FL

Background: Myocardial infarction (MI) produces severe hypoxia within regions of the myocardium, resulting in the formation of scar tissue and significant cardiac cell death. Adult hearts contain endogenous cardiac stem cells (CSCs) that have regenerative capacity and participate in myocardial tissue homeostasis and repair post-MI, but are insufficient to promote complete regeneration. The histone deacetylase Sirtuin 1 (SIRT1) likely mediates this hypoxia-induced decrease in regeneration via roles in cell cycle progression and conferring protection from senescence and oxidative damage. Hypothesis: Hypoxia decreases CSC proliferation through reduced SIRT1-mediated deacetylation.
Methods and Results: Murine CSCs were grown at room air (21% O2), physiologic (5% O2), and ischemic hypoxic (0.5% O2) conditions for 72 hours. Ischemic hypoxia, but not physiologic, reduced CSC proliferation and DNA synthesis to 25±2.0% (N=3; $p<0.05$) and 54±7.0% (N=7; $p<0.05$), respectively, relative to cells grown in room air. SIRT1 protein expression was decreased by 58±10.0% (N=4; $p<0.05$) and acetylation of Histone H3 Lys9 (2.4-fold; N=5; $p<0.05$) and p53 Lys379 (1.64-fold; N=4; $p<0.05$) were increased after 72 hours of growth in 0.5% compared to 21% O2. However, SIRT1 mRNA transcripts remained unchanged. Furthermore, SIRT1 protein was 59±6.0% less stable (N=4; $p<0.05$) following 8 hours of cyclohexamide treatment in CSCs exposed to 0.5% O2 for 72 hours compared to CSCs exposed to 21% O2. SIRT1 knockdown by RNA interference significantly reduced proliferation of CSCs grown in room air (N=3 and, $p<0.05$), similar to that observed in un-transfected cells grown under ischemic hypoxia conditions. Conclusion: The decrease in CSC proliferation with hypoxia is in part due to a reduction in SIRT1 protein stability. These results suggest that SIRT1 expression is regulated post-translationally and support a role for SIRT1 in preserving CSC self-renewal under hypoxic conditions.

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2

Automated Dissociation Of Neonatal Hearts And Immunomagnetic Purification Of Cardiomyocytes, Cardiac Fibroblasts And Endothelial Cells

Manuel Kernbach, Josephine Riesen, Anne Maria Wiencierz, Andreas Bosio, Peter Christalla, **Dominik Eckardt**, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Pure cardiovascular cell lineages are a requisite for an unbiased and cell-type specific investigation of (patho-) physiological mechanisms involved in heart failure. Moreover, pure heart cells are needed as building blocks for cardiac tissue engineering. Therefore, we developed a rapid and automated heart dissociation protocol followed by an immunomagnetic enrichment of the three major heart cell types, namely cardiomyocytes (CMs), cardiac fibroblasts (Fibs) and endothelial cells (Endos) from both, mouse and rat neonatal hearts.
First, we developed an enzyme mix optimal for the dissociation of neonatal mouse and rat hearts by screening our enzyme library. Next, we optimized the dissociation by utilizing our gentleMACS technology resulting in a fast (1 h), robust, and fully automated heart dissociation protocol. Analysis of the dissociated heart cells prior to cell purification showed: (i) high cell vitalities (>90%), (ii) high frequencies of α-actinin-positive CMs (ca. 60%) and (iii) vimentin-positive non-CMs with a frequency of ca. 40%. In order to selectively enrich various cell-types from these heterogenous cell populations, we performed a cell-surface marker screen. We identified several candidates for the composition of antibody cocktails enabling selective enrichment of mouse and rat CMs, Fibs or Endos with purities of up to 97%. CMs grown in 2D cultures showed spontaneous beating activity and the expression of sarcomeric proteins. Additionally, heart cells were cultured on decellularized mouse heart slices. Analysis of

the slice cultures after recellularization showed (i) partial restoration of synchronous contractions and (ii) orientated and elongated heart cells. Purified Fibs were highly proliferative and could be expanded 34-fold over an analyzed culture period of 15 days. Finally, functionality of the purified endothelial cells was proven by Dil-LDL endocytosis.
In summary, we established an automated protocol for the dissociation of neonatal hearts, enabling the subsequent immunomagnetic enrichment of CMs, Fibs and Endos which can readily be utilized for 2D cell culture assays or to generate in vitro heart muscle models and surrogate tissue for myocardial repair.

M. Kernbach: 1. Employment; Significant; Miltenyi Biotec full-time employee. **J. Riesen:** 1. Employment; Significant; Miltenyi Biotec full-time employee. **A. Wiencierz:** 1. Employment; Significant; Miltenyi Biotec full-time employee. **A. Bosio:** 1. Employment; Significant; Miltenyi Biotec full-time employee. **P. Christalla:** 1. Employment; Significant; Miltenyi Biotec full-time employee. **D. Eckardt:** 1. Employment; Significant; Miltenyi Biotec full-time employee.

3

Il-10 Regulated Mir-375 Enhances Endothelial Progenitor Cell Mediated Myocardial Repair And Survival After Myocardial Infarction.

Venkata N Garikipati, Prasanna Krishnamurthy, Suresh K Verma, Alexandra R Mackie, Erin E Vaughan, Mohsin Khan, Veronica Ramirez, Tatiana Abramowa, Kenneth L Ellingson, Sol Misener, Aiko I klingler, Gangjian Qin, Walter J Koch, Raj Kishore, Temple Sch of Med, Philadelphia, PA

We hypothesized that IL-10 regulates miR-375 signaling in EPCs to enhance their survival and function in ischemic myocardium after MI. miR-375 knock down EPC were transplanted intramyocardially after induction of MI. Mice receiving EPC treated with miR-375 inhibitor showed increased number of GFP+EPCs retention that was associated with reduced EPC apoptosis in the myocardium. The engraftment of EPC into the vascular structures and the associated capillary density was significantly higher in miR-375-treated mice. The above findings further correlated with reduced infarct size, fibrosis and enhanced LV function (echocardiography) in miR-375 knock down EPC group as compared to scrambled EPC. Our in vitro studies revealed that the knockdown of miR-375 enhanced EPC proliferation, migration; tube formation ability and inhibited cell apoptosis, while the up-regulation of miR-375 with the mimic had the opposite effects. In addition, we found that miR-375 negatively regulates the expression of 3-phosphoinositide-dependent protein kinase 1 (PDK1) by directly targeting the 3'UTR of the PDK1 transcript. Interestingly, EPC isolated from IL-10-deficient mice has elevated basal levels of miR-375 and exhibited poor proliferation and tube formation ability where as miR-375 knock down in EPC isolated from IL-10 deficient mice attenuated these effects. Furthermore, transplantation of miR-375 knock down IL-10 deficient EPC after MI resulted in attenuated cardiac functions compared to scramble IL-10 deficient EPCs. Taken together, our studies suggest that IL-10 regulated miR-375 enhances EPC survival and function, associated with efficient myocardial repair via activation of PDK-1/AKT signaling cascades.

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4

Lymphatic Vessels Mediate the Mobilization of Cardiac Progenitor Cells after Myocardial Infarction

Polina Goichberg, Maria Cimini, Antonio Cannata, Sergio Signore, Kanako Waight, Marcello Rota, Piero Anversa, Annarosa Leri, Brigham and Women's Hosp/Harvard Medical Sch, Boston, MA

The delivery of adult cardiac progenitor cells (CPCs) or their activation in situ constitute an evolving approach for the treatment of heart failure. CPCs are endowed with regenerative capacity, producing differentiating myocytes and vascular structures in the course of homeostasis and upon injury. The regenerative function of CPCs is contingent to their ability to migrate to and engraft within the wounded area. Yet, the mechanisms governing CPC trafficking in the diseased myocardium are largely unknown. The lymphatic system is vital for tissue repair, and

the role of the lymphatic vasculature in the trafficking of hematopoietic and cancer cells is well documented. We examined whether cardiac lymphatic vessels mediate the translocation of CPCs in the infarcted myocardium. By imaging of the heart from transgenic c-kit-GFP reporter mice, we found that as early as 4 hours after myocardial infarction (MI), uncommitted lineage-negative progenitors accumulated in the vicinity of the lymphatic vessels located in the region bordering the necrotic area. Histologically, extensive lymphangiogenesis was documented in the mouse heart in the acute (8-48 hours) and chronic (15-35 days) phases of infarct healing and scar formation. CPCs were detected traversing the wall of lymphatic vessels at different stages after MI, indicative of the functional role of the lymphatic circulation in the recruitment of primitive cells to the site of injury. Furthermore, isolated human CPCs exhibited chemotaxis and specific binding to the human lymphatic endothelial cells (LECs) in steady-state conditions and, increasingly, after exposure to an inflammatory cytokine, TNFα. CPCs performed trans-endothelial migration in vitro, and actively intravasated into the lumen of microvessels formed by LECs in three-dimensional matrices. Finally, our data suggest that sphingosine-1-phosphate (S1P)-stimulated signaling governs the interactions of CPCs with LECs. These findings on the direct role of lymphatic vasculature in CPC trafficking may contribute to the development of novel therapeutic modalities to increase mobilization of endogenous or transplanted CPCs, promoting myocardial repair in patients with ischemic heart diseases.

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5

Neovascularization By Substance-p-Mobilized Epc And Msc In Vitro And In Vivo

Hyun Sook Hong, Kyung Hee Univ, Seoul, Korea, Republic of; Suna Kim, Youngsook Son, Kyung Hee Univ, Yong In, Korea, Republic of

Bone marrow stem cells, especially, endothelial precursor cells (EPC), mesenchymal stem cells (MSC) or hematopoietic stem cell (HSC) are expected as reparative cells for the repair of a variety of tissue damages such as stroke and myocardial infarction, even though their role in the repair is not demonstrated. This report was investigated to find a role of Substance-p (SP) as a reparative agent in the tissue repair requiring EPC and MSC.
In order to examine EPC (EPC^{SP}) and MSC (MSC^{SP}) mobilized by SP, we injected SP intravenously for consecutive 2 days and saline was injected as a vehicle. At 3 post injection, peripheral blood (PB) was collected.To get mesenchymal stem cells or endothelial progenitor cells, MNCs were incubated in MSCGM or EGM-2 respectively for 10 days. Functional characteristics of the EPC^{SP} were proven by the capacity to form endothelial tubule network in the matrigel in vitro and in the matrigel plug assay in vivo. In contrast, MSC^{SP} did not form a tube-like structure but formed a pellet-structure on matrigel. However, when both cells were premixed before the matrigel assay, much longer and branched tubular network was formed, in which a-SMA expressing MSC^{SP} were decorating outside of the endothelial tube, especially enriched at the bifurcating point. MSC^{SP} may contribute and reinforce elaborate vascular network formation in vivo by working as pericyte-like cells. Thus, the EPC^{SP} and MSC^{SP} were labeled with PKH green and PKH red respectively and their tubular network was examined. Well organized tubular network was formed, which was covered by PKH green labeled cells and was decorated in a punctate pattern by PKH red labeled cells. In order to investigate the role of EPC^{SP} and MSC^{SP} specifically in vivo, rabbit EPC^{SP} and MSC^{SP} were transplanted to full thickness skin wound. The vessel of EPC^{SP}-transplanted groups was UEA-lectin+, which was not covered with a-SMA+ pericytes but EPC^{SP} + MSC^{SP}-transplanted groups showed, in part, a-SMA+ pericyte-encircled UEA-lectin+ vessels. This proved the specific role of MSC^{SP} as pericytes. From these data, we have postulated that the collaboration of MSC and EPC is essential for normal vessel structure and furthermore, accelerated wound healing as ischemia diseases, which can be stimulated through by SP injection.

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6

Formation of Human Heart Muscle Directly from Embryonic and Induced Pluripotent Stem Cells

James E Hudson, The Univ of Queensland, Brisbane, Australia; Malte Tiburcy, Wolfram-Hubertus Zimmermann, Heart Res Ctr, Goettingen, Germany

Tissue engineering enables the simulation of human heart physiology and pathology. It typically requires a mixture of cardiomyocytes, stromal cells, and extracellular matrix for fabrication. Here, we hypothesised that bioengineered heart muscle (BHM) can be formed directly from undifferentiated pluripotent stem cells by triggering processes of embryonic cardiogenesis.
Methods and Results:
We tested our hypothesis by applying an optimized serum-free cardiac differentiation protocol to undifferentiated pluripotent stem cells in a collagen type 1 hydrogel. During this process BHMs traversed through distinct developmental stages: early mesoderm (3 days), cardiac specification (10 days), and cardiac maturation (up to 50 days). Flow cytometry demonstrated that BHMs are comprised of primarily cardiomyocytes (α-actinin positive cells, 51 ± 5%, n = 6) and stromal cells (CD90 positive cells, 41 ± 5%, n = 6), with low yields of contaminating cells. By 22 days the BHMs exhibited measurable contractile force (207 ± 19 μN) and contained elongated cross-striated cardiomyocytes.
We next sought to optimize the force of contraction and also the maturity of the BHM, by investigating the effect of mechanical stimuli and growth factors. Mechanical stimulation was essential for BHM formation. Additionally, we found that 2 developmentally important growth factors, FGF2 and TGFβ1, induced pathological and physiological hypertrophy, respectively. This was characterized by an increase in cell size in both conditions coupled with reduced force and higher ANP expression in FGF2 treated BHM, and a higher force with reduced ANP expression and elevated β-MHC/α-MHC ratio TGFβ1 treated BHM.
Using our optimized protocol, 28 day old BHM responded to electrical pacing, preloading, and inotropic stimuli similarly as bona fide myocardium.
Conclusion:
BHM can be formed directly with undifferentiated pluripotent stem cells by recapitulating normal cardiac development. The serum-free protocol with developmentally defined stimuli provides us with a useful in vitro model to study cardiac biology and potentially provides a method of producing cardiac tissue for regenerative applications.

J.E. Hudson: None. **M. Tiburcy:** None. **W. Zimmermann:** None.

7

Intracoronary Autologous Cardiac Progenitor Cell Transfer In Children With Hypoplastic Left Heart Syndrome: 2-year Results Of The Ticap Trial

Shuta Ishigami, Suguru Tarui, Michihiro Okuyama, Daiki Ousaka, Junko Kobayashi, Shingo Kasahara, Shunji Sano, Hidemasa Oh, Okayama university hospital, Okayama, Japan

Backgrounds-Hypoplastic left heart syndrome (HLHS) is a severe congenital heart malformation. **Objective-** The aim of this study is to determine whether intracoronary delivery of autologous cardiosphere-derived cells (CDCs) was feasible and safe to treat the children with HLHS.
Methods- Four-teen patients with HLHS undergoing staged palliations were prospectively enrolled in this trial between January, 2011, and January, 2012. Seven patients constitutively assigned to receive intracoronary CDCs injection followed by 7 patients allocated to a control group with standard care alone. The primary endpoint was to assess the safety and the secondary endpoint was the preliminary efficacy by assessing the improvements of the right ventricular function during the follow-up. **Results-** No major complications were reported within 24 months of CDC infusion. Echocardiography showed that improvement of right ventricular ejection fraction (RVEF) was greater in the CDC-treated group (+5.3±3.2%) than in controls (+0.1±3.4%, P=0.01) at 3-month follow up. This cardiac function enhancement was manifested even in long-term observation (+7.8±4.9% vs. +2.2±3.1% at 1 year, P=0.03; +8.8±3.7% vs. +3.4±6.4% at 2 years, P=0.04). The absolute improvements in RVEF between 2 groups was confirmed by using right ventriculogram (RVG: +8.9±7.6% vs. +2.0±2.8%

at 1 year, P=0.02; +8.1±6.0% vs. +2.9±3.9% at 2 years, P=0.04). In addition, RVEF on cMRI was also markedly improved in CDC-treated patients from 36.1±7.5% at baseline to 42.7±8.7% at 1 year (P=0.04) and to 42.4±7.6% at 2 years (P=0.047). Heart failure status was reduced in CDC-treated group as shown by significant decrease in Ross Heart Failure Class (2.6±0.8 at baseline vs. 1.4±0.5 at 2 years, P=0.01). Moreover, Z scores for weight-for-age was significantly increased from -4.0±2.7 at baseline to -2.2±1.4 at 2 year (P=0.02), whereas all of these parameters did not change in control subjects. **Conclusion-** These results of 2-year follow-up of TICAP trial suggest that intracoronary infusion of autologous CDCs is feasible and safe to treat the children with HLHS. This novel therapeutic strategy may impact on cardiac function as well as clinical symptom of heart failure status and somatic growth in long-term outcome.

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8

Transplantation of Mouse Adipose Derived Stem Cell Sheet into Infarcted Rat Myocardium Increase Cell Engraftment and Cardiac Function

Jong-Ho Kim, Hyung Joon Joo, Ha-Rim Seo, Long-Hui Cui, Mi-Na Kim, Seung-Cheol Choi, Chi-Youn Park, Ji-Hyun Choi, Soon Jun Hong, Korea Univ Anam Hosp, Seoul, Korea, Republic of; Jeong Ik Lee, Konkuk Univ, Seoul, Korea, Republic of; Do-Sun Lim, Korea Univ Anam Hosp, Seoul, Korea, Republic of

Background : Cell sheet technology has magnified as an important transplantation skill. Mouse adipose derived stem cells (mADSCs) can secrete various growth factors, which promote the repair of damaged cardiomyocyte and protecting cells from death. In addition, autologous cell source to easily obtain from patients are promising candidates for cell therapy in cardiovascular field. **Methods** : mADSCs were confirmed stem cell properties and secreted cytokines were evaluated in vitro. Eighteen acute myocardial infarction (AMI) rats were divide into 3 group; sham (n=6), suspended mADSCs (n=6), and mADSCs sheet (n=6) groups. In the mADSCs sheet group, 60×106 cells were cultured for 2 days onto temperature-responsive polymers and the sheets were then transplanted over the infarct region. In additional, the sheet was made of carboxyfluorescein diacetate succinimidyl ester (CFDA) -labelled mADSCs to confirmed cell survival. Engraftment and differentiation were blindly assessed after 28 days. **Results** : The mADSCs expressed Sca-1+ and represented multi-differentiation potential. Interestingly, EGF and IGF levels significantly increased in the mADSCs sheet. Significant improvements in ejection fraction and fraction shortening value were observed in the mADSCs sheet and suspended mADSCs groups compared with the sham group at 14 and 28 days. But, it was not higher significance level in the mADSCs sheet group than in the suspended mADSCs group. Engraftment range and fibrosis area of infarct region were significantly higher in the mADSCs sheet group compared to the other two groups at 4, 14 and 28 days. In significant expressed cytokines (bFGF, IL-1a, IL-1ra, CT-1, EGF, TGFb1, IGF-1, IGF-2 and MCP-1) were observed in the mADSCs sheet group compared with the other 2 groups at 28 days after transplantation. In addition, in the mADSCs sheet was confirmed endothelial differentiation by Von Willebrand factor (vWF) at 4, 14 and 28 days.
Conclusions : Transplantation of mADSCs sheet into rat infarcted myocardium increased engraftment and survival of transplanted cells. The mADSCs sheet is very useful for the study of stem cell proliferation and differentiation as well as for cell therapy in cardiovascular field.

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9

Development And Testing Of A Human Induced Pluripotetent Stem Cell Derived Cardiac Scaffold

Jordan J Lancaster, Pablo Sanchez, Elizabeth Juneman, Kyle Weigand, Josehp J Bahl, Steven Goldman, Univ of Arizona, Tucson, AZ

Introduction: Cell-based regenerative therapies hold promise in providing treatment strategies for heart failure. Previous work from our laboratory has shown that tissue engineered cardiac constructs enhance improvements in

cardiac function by providing structural and nutrient support, potentially aiding in transplanted cell survival, integration and re-population of injured tissues. Our current studies focus on the development and testing of second-generation cardiac constructs utilizing high purity human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs).
Methods: High purity hiPSC-CMs were seeded and co-cultured onto a vicryl matrix embedded with biologically active human dermal fibroblasts. The hiPSC-CM constructs were maintained at 37°C and 5% CO2. Patches were constructed maintained as described for both in vitro and in vivo evaluation. Patches for in vivo evaluation were seeded, cultured and implanted onto the rat heart 3 weeks after left coronary artery ligation to assess improvements in LV function. Patches prepared for in vitro evaluation were seeded and cultured 1-10 days.
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. In addition hiPSC-CM constructs respond to electrical stimulation with increased rate of contraction while maintaining their synchrony. Post pacing, the hiPSC-CM constructs return to their intrinsic rhythm.
Conclusion: These findings show that high purity hiPSC-CMs can be seeded and co-cultured onto a vicryl and fibroblast construct in manner permitting adhesion and electromechanical coupling of the hiPSC-CMs to form a fully contractile construct. This is supported by the observation that the hiPSC-CMs contract spontaneously and in a synchronized manner in a directional fashion.

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10

Cardiac-specific Yap Activation Improve Cardiac Function And Survival In An Experimental Murine Mi Model

Zhiqiang Lin, Alexander von Gise, Pingzhu Zhou, Qing Ma, Jinghai Chen, Boston Children Hosp, Boston, MA; Jianming Jiang, Jonathan G Seidman, Harvard Medical Sch, Boston, MA; Da-zhi Wang, William T Pu, Boston Children Hosp, Boston, MA

Background- Yes-Associated Protein (YAP), the terminal effector of the Hippo signaling pathway, is crucial for regulating embryonic cardiomyocyte proliferation. We hypothesized that YAP activation after myocardial infarction would preserve cardiac function and improve survival.
Methods and results
In this study, we used a cardiac-specific, inducible expression system to activate YAP in adult mouse heart. Activation of YAP in adult heart promoted cardiomyocyte proliferation and did not deleteriously affect heart function. Furthermore, YAP activation after myocardial infarction (MI) preserved heart function and reduced infarct size. Using adeno-associated virus subtype 9 (AAV9) as a delivery vector, we expressed human YAP in the adult murine myocardium immediately after MI. We found that AAV9:hYAP significantly improved cardiac function and mouse survival. AAV9:hYAP did not exert its salutary effects by reducing cardiomyocyte apoptosis. Rather, AAV9:hYAP stimulated adult cardiomyocyte proliferation. Gene expression profiling indicated that AAV9:hYAP stimulated cell cycle gene expression, activated of components of the inflammatory response, and promoted a less mature cardiac gene expression signature.
Conclusions
Cardiac specific YAP activation after MI mitigated myocardial injury, improved cardiac function, and enhanced survival. These findings suggest that therapeutic activation of YAP or its downstream targets, potentially through AAV-mediated gene therapy, may be a strategy to improve outcome after MI.

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Xenogen-free In Vitro Differentiation and Expansion of Human Pluripotent Stem Cell-Derived Cardiovascular Progenitor Cells

Xianmei Meng, Univ of California, Los Angeles, Los Angeles, CA; Anne Knopf, Fraunhofer Inst for Interfacial Engineering and Biotechnology, Stuttgart, Germany; Agustin Vega-Crespo, James Byrne, Univ of California, Los Angeles, Los Angeles, CA; Ben Van Handel, Novogenix Labs, LLC, Los Angeles, CA; Ali Nsair, Univ of California, Los Angeles, Los Angeles, CA

Background_ Human pluripotent stem cell-derived cardiovascular progenitor cells (hPSC-CPCs) represent a tractable option for cell-based therapy for heart disease. However, to be clinically relevant, these cells must be derived under good manufacturing practices (GMP)-compatible conditions and produced in great enough quantities to treat adult patients. Here we sought to demonstrate for the first time the generation and expansion of clinically relevant numbers of hPSC-CPCs in xenogen-free protocol.
Methods and Results_ GMP-grade human induced pluripotent stem cells (GMP-hiPSCs) and human embryonic stem cells (H1 and H9) were dissociated into single cells and cultured in low attachment dishes to differentiate into CPCs in StemPro medium including small molecules and human cytokines with high efficiency of 86%, 80% and 66% for GMP-hiPSCs, H1 and H9, respectively (Figure 1). All hPSC-CPCs possessed trilineage differentiation potentials, as shown by differentiation into endothelial and smooth muscle cells and functional cardiomyocytes (Figure 2). Moreover, sorted hPSC-CPCs expanded >5 fold in 10 days in xenogen-free conditions while still maintaining trilineage differentiation potential and an efficiency of ~70% (Figure 3).
Conclusions_ Here we demonstrate a xenogeny-free CPC derivation and expansion protocol that can generate clinically relevant numbers of GMP-grade cardiovascular progenitors that could be used in a clinical setting.
Key words: cardiovascular progenitor cells (CPCs), human pluripotent stem cells (hPSCs), differentiation, expansion, xenogeny free, cell-based therapy

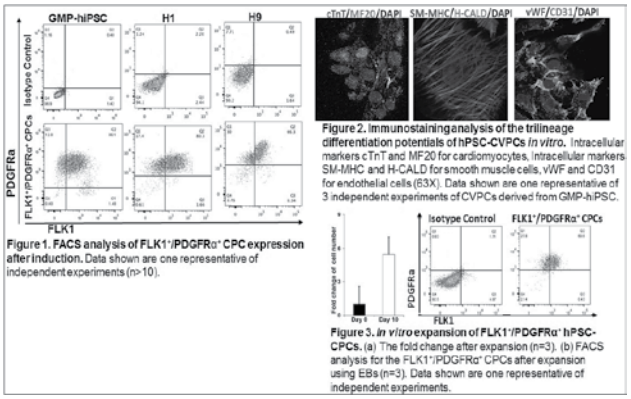

Figure 1. FACS analysis of FLK1+/PDGFRα+ CPC expression after induction. Data shown are one representative of independent experiments (n>10).

Figure 2. Immunostaining analysis of the trilineage differentiation potentials of hPSC-CVPCs *in vitro*. Intracellular markers cTnT and MF20 for cardiomyocytes, intracellular markers SM-MHC and H-CALD for smooth muscle cells, vWF and CD31 for endothelial cells (63X). Data shown are one representative of 3 independent experiments of CVPCs derived from GMP-hiPSC.

Figure 3. *in vitro* expansion of FLK1+/PDGFRα+ hPSC-CPCs. (a) The fold change after expansion (n=3). (b) FACS analysis for the FLK1+/PDGFRα+ CPCs after expansion using EBs (n=3). Data shown are one representative of independent experiments.

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12

The Effect of Extracellular Matrix Stiffness on Human Bone Marrow-Derived Mesenchymal Stem Cell Differentiation

Alison L Müller, Yun Li, Univ of Manitoba, Winnipeg, MB, Canada; Boris Hinz, Univ of Toronto, Toronto, ON, Canada; Darren H Freed, Univ of Alberta, Edmonton, AB, Canada

Differentiation of human mesenchymal stem cells (hMSCs) has been shown to be influenced by the surrounding microenvironment. It is important to understand the physiological implications of the hMSC microenvironment regarding differentiation within the body, especially in patients with cardiovascular disease. We are interested in understanding the influence of differing extracellular matrix (ECM) stiffness found in the body that the MSC encounters during its journey from the bone marrow to the infarct scar in patients recovering from a myocardial infarction. As hMSCs must respond rapidly to their environment, we also investigated the influence of microRNA at different surface tensions.
HMSCs were isolated from the bone marrow of patients undergoing open heart surgery and cultured in standard DMEM/F12 with 20% FBS. We plated these cells on fibronectin-coated plates with surface tensions of 2kPa, simulating bone marrow; 15kPa, simulating left ventricle; and 100kPa, simulating a fibrotic environment. Protein and mRNA were collected for further analysis.
Our data have revealed that softer surface tensions, representing a bone marrow-

undifferentiating environment, cause a decrease in the protein expression of EDA-fibronectin and alpha-smooth muscle actin. There were also increases in mRNA of myosin heavy chain-9 and 10, and collagen-1. Softer surface tensions also show a slight increase in miR-301a, although at 15kPa, miR-301a expression is increased even further. Our lab has previously shown that miR-301a is involved in maintaining a proliferative phenotype of hMSCs. Interestingly, Dicer1, responsible for processing microRNAs, is upregulated at softer surface tensions and attenuated at 15kPa. Dicer1 mRNA expression is attenuated at 15kPa. These results indicate that ECM stiffness influences hMSC differentiation and the increase in Dicer1 found with softer matrices could represent an umbrella miRNA inhibition effect to effectively suppress hMSC differentiation.

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13

Nkx2-5-notch Signaling Axis Regulates The Proliferation Of The Atrial Myocytes And Conduction System

Austin Nakano, Yasuhiro Nakashima, Diana A Yanez, Marlin Touma, Haruko Nakano, Artur Jarodzewicz, Maria C Jordan, Matteo Pellegrini, Kenneth P Roos, Kenneth P Roos, UCLA, Los Angeles, CA

Rationale: Tight control of cardiomyocyte proliferation is essential for the formation of four-chambered heart. Although human mutation of NKX2-5 is linked to septal defects and atrioventricular conduction abnormalities, early lethality and hemodynamic alteration in the mutant models have caused controversy as to whether Nkx2-5 regulates cardiomyocyte proliferation.
Objective: In this study, we circumvented these limitations by atrial-restricted deletion of Nkx2-5.
Method and Results: Atrial-specific Nkx2-5 mutants died shortly after birth with hyperplastic working myocytes and conduction system including two nodes and internodal tracts. Multicolor reporter analysis revealed that Nkx2-5-null cardiomyocytes displayed clonal proliferative activity throughout the atria, indicating the suppressive role of Nkx2-5 in the cardiomyocyte proliferation after chamber ballooning stages. Transcriptome analysis revealed that aberrant activation of Notch signaling underlies hyperproliferation of mutant cardiomyocytes, and forced activation of Notch signaling recapitulates hyperproliferation of working myocytes but not conduction system.
Conclusion: Collectively, these data suggest that Nkx2-5 regulates proliferation of atrial working and conduction myocardium in coordination with Notch pathway.

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14

The Cytoskeletal Architecture of Myocytes Derived from Human Cardiac Progenitor Cells

Francesco Pasqualini, Moises Di Sante, João D Pereira, Piero Anversa, Marcello Rota, Annarosa Leri, Brigham and Women's Hosp, Boston, MA

The stem cell antigen c-kit characterizes a heterogeneous pool of human cardiac progenitor cells (hCPCs) that exhibit a remarkable degree of regenerative potential and are currently employed in clinical trials. While this hCPC pool contains distinct subpopulations of c-kit+ cells that preferentially differentiate into muscular or vascular cardiac cells, we hypothesize that hCPCs may be coerced to specify only along the cardiomyogenic lineage by manipulating the Wnt/β-catenin pathway.
We report that pharmacological inhibition of the non-canonical Wnt pathway facilitated the commitment of more than >95% c-kit+ hCPCs to the cardiomyocyte lineage after 4 days in-vitro: this constitutes a substantially more homogeneous population than previously reported with dexamethasone treatment.
The hCPC-derived myocytes stained positive for Nkx2.5, a transcription factor that orchestrates cardiomyogenic differentiation, and for the contractile protein sarcomeric α-actin. To test if

we could push the cells towards a more mature phenotype, we mimicked the cyclic modulation of the Wnt pathway observed during development. While activation of Wnt signaling resulted in widespread cell death and reduction in cell size, subsequent Wnt inhibition prompted the spared cells to proliferate. With this protocol, hCPC-derived myocytes increased in size and displayed more mature cytoskeletal architectures. In contrast with dexamethasone treated cells, where the localization of α-sarcomeric actinin is mostly diffuse in the cytoplasm, here we observed both Z-bodies and Z-disks like structures. The latter exhibited a periodicity of ~1.6 um and were clustered in larger, more aligned actin bundles. This finding suggests that the tension developed along these cytoskeletal components may play a role in the recruitment of sarcomeric proteins. In conclusion, Wnt signaling inhibition in hCPCs may be sufficient to obtain a homogeneous population of cells with features of myocytes, characterized by improved cytoskeletal organization than dexamethasone treated cells and similar to that observed in myocytes derived from human induced pluripotent stem cells.

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15

Limiting GRK2 in the Ischemic Heart can Promote Cardiac Regeneration

Maria Cecilia Scimia, Lin Zuo, Kate E. Sydnes, Daniel A. Zuppo, Erhe Gao, Walter J. Koch, Temple Univ, Philadelphia, PA

The detrimental role of G protein-coupled receptor (GPCR) kinase (GRK2) following cardiac injury/stress has been documented over the last two decades. Importantly, our lab has shown that inhibition or deletion of GRK2 in cardiomyocytes can prevent and also rescue heart failure (HF) phenotypes. Its role in GPCR desensitization including regulation of β-adrenergic receptors (βARs) during HF development has been well characterized. However, recently our lab and others have found that GRK2 can have novel GPCR-independent effects in the heart that appear to contribute to its pathological effects and thus, inhibition of these actions of GRK2 may contribute to therapeutic effects seen. In this study we explored whether the cardiac repair observed with lower myocardial GRK2 might involve regenerative processes. In cardiac-specific GRK2 knockout (KO) mice and also transgenic mice with cardiac-targeted expression of the βARKct, a peptide inhibitor of GRK2 activation via Gβγ sequestration, we induced HF via coronary artery ligation and subsequent myocardial infarction (MI) and measured aspects of cardiac repair including potential regeneration indices. Post-MI mice (GRK2 KO, βARKct mice and wild-type and non-transgenic control mice) were treated with 5-ethynyl-2'-deoxyuridine (EdU) or Bromodeoxyuridine (BrDU) to examine indices of DNA proliferation in myocytes as well as Ki67 staining. We also quantitated c-kit+ cells and myocytes in the post-MI hearts to compare how either loss of GRK2 expression or inhibition via its C-terminus altered potential regeneration mechanisms compared to control mice with endogenous GRK2 levels and activity. We found significantly more BrDU positive myocytes in post-MI hearts with lower GRK2 and this correlated with increased myocytes that were also cKit+. Thus, it appears that the myocardial functional improvement seen in the post-MI heart with targeted lowering of GRK2 involves, to at least a certain extent, regenerative mechanisms. This adds novel insight into the therapeutic potential of GRK2 inhibition for HF.

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16

Cardiosphere Derived Cells from Pediatric End-Stage Heart Failure Patients Have Enhanced Functional Activity Due to the Heat Shock Response

Sudhish Sharma, Rachana MIshra, David Simpson, Ling Chen, Univ of Maryland, Baltimore, MD; Evan Colletti, Univ of Nevada, Reno, NV; Savitha Deshmukh, Sunjay Kaushal, Univ of Maryland, Baltimore, MD

Background: The potential of novel cell based therapies using cardiosphere derived cells (CDCs) to replace or repair damaged cardiac tissue is an attractive therapy and Phase I clinical trials in adults report encouraging results. We have previously demonstrated that age plays an important role in controlling the

functional activity of CDCs and now we will determine how the heart's physiology may change the functional activity of pediatric end-stage heart failure (ESHF) derived CDCs when compared to age-match control derived CDCs. Methods and Results: Our results shows that ESFH derived CDCs have significantly increased numbers of CSCs, including c-kit+, ISL-1+ and Sca-1+ cells. They are functionally more potent in an infarcted rodent model as compared to hCDCs derived from congenital heart disease (CHD) age-matched controls which have normal myocardium. The functional recovery was mediated in part by increased secretion of cytokines, SDF-1α and VEGF-A, which stimulated more angiogenesis, recruitment of endogenous stem cells, and proliferation of cardiomyocytes. The possible mechanism for this increased cytokine secretion was due to the activation of heat shock response (HSR), supported by three lines of evidence. First, gain of function studies demonstrated that the HSR induced the low functioning CHD-derived CDCs to significantly recover the injured myocardium, even more than the ESHF-derived CDCs by increasing cytokine secretion. Secondly, loss-of function studies targeting the HSR down regulating the ability of the ESHF-derived CDCs to secrete cytokines and thus functionally recover the injured myocardium. Finally, the HSR alone increased the number of the endogenous cardiac stem cell population and recovered the injured myocardium. We will present the results showing that activation of HSR expands cardiac stem cells (CSCs) without losing their phenotypic characteristics. Conclusion: Collectively, the present work demonstrates a novel biological activity of the HSR in its ability to increase the number of functional CSCs while maintaining the 'stemness' of these cells that have direct implications for future clinical trials.

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The Comparison of Adipose Derived Regenerative Cells and Bone Marrow Mononuclear Cells as Transplanted Cells for Therapeutic Angiogenesis

Satoshi Shintani, Changning Hao, Yuuki Shimizu, Kazuhisa Kondo, Toyoaki Murohara, Nagoya Univ, Nagoya, Japan

Background—Transplantation of adipose-derived regenerative cells (ADRCs) enhances ischemia-induced angiogenesis, but the underlying mechanism remains unknown. Here, we compared the efficacy of ADRC transplantation with bone marrow mononuclear cell (BM-MNC) treatment using a hindlimb ischemia (HLI) rabbit model, and examined if the anti-inflammatory phenotypic polarization of macrophages regulates postnatal neovascularization.
Methods and Results_ADRCs and BM-MNCs were isolated from New Zealand White (NZW) rabbits and C57BL/6J mice. In the rabbit studies, ADRCs were incorporated into the existing vascular formation in vitro and in vivo. ADRC-conditioned media (CM) and BM-MNC-CM similarly enhanced the migratory ability and prevented apoptosis induction in human umbilical vein endothelial cells. Four weeks after treatment, NZW rabbits administered with either ADRCs or BM-MNCs revealed enhanced collateral vessel formation and functional blood flow recovery. In mice, lipopolysaccharide and/or hypoxic stress increased the level of prostaglandin E2 (PGE2), and led to the polarization of M2 macrophages in cultured ADRCs. Gene expressions of several angiogenic cytokines were amplified in macrophages cultured in ADRC-CM rather than BM-MNC-CM. The expression of interleukin (IL)-10 was increased in ischemic muscles of mice treated with ADRCs compared with those from BM-MNC- treated and control groups. The blockade of IL-10 using a neutralizing antibody attenuated ischemia-induced angiogenesis in vivo.
Conclusions—The therapeutic angiogenesis potential of ADRCs was comparable with those of BM-MNCs in healthy animals. Anti-inflammatory phenotypic polarization of macrophages plays an important role in the regenerative action of ADRCs through the PGE2-EP2/4 axis.

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Coxsackievirus B3 Escapes Infected Cardiac Stem Cells via Ejected Autophagosomes

Jon Sin, Heart Inst, Cedars Sinai Medical Ctr, West Hollywood, CA; Vrushali Mangale, Laura McIntyre, Ralph Feuer, San Diego State Univ, San Diego, CA; Roberta A Gottlieb, Heart Inst, Cedars Sinai Medical Ctr, West Hollywood, CA

Many viruses have been shown to upregulate host autophagic processes during the course of infection. Originally this process was thought to be a response mechanism of the host cell in order to engulf viral particles and destroy them to prevent viral spread and host cell death. However, certain viruses have been shown to strategically hijack the autophagic pathway to evade host immunity and increase viral replication. Coxsackievirus B (CVB) is a non-enveloped picornavirus that most commonly causes a self-limited febrile illness in young children, but in rare severe cases can progress to myocarditis, pancreatitis, or meningo-encephalitis. It has also been associated with late-onset idiopathic dilated cardiomyopathy. CVB type 3 (CVB3) has previously been documented to upregulate host autophagic machinery upon infection, and we report here that the virus can infect cardiac stem cells and other muscle progenitors and allow itself to be engulfed in autophagosomes which later get shed from the surface of the host cell. This is a novel method of viral dissemination especially for a “naked” virus which would typically escape the infected host by triggering cytolysis. However, cytolytic viruses subsequently become exposed to neutralizing antibodies. The fact that CVB3 can hide itself in host membranes presents several fascinating possibilities. Not only could the ejected autophagosome mask the virus from adaptive immunity, but it may also bypass receptor-mediated endocytosis and infect cell-types that might lack CVB3 receptors. This mode of viral escape represents a unique evolutionary adaptation which obscures the line dividing enveloped and non-enveloped viruses.

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Reversal of Adverse Remodeling in Chronic Rat Myocardial Infarction by Cardiospheres: Role of the Tgf-beta Pathway

ELENI TSELIOU, Geoffrey de Couto, John Terrovitis, Baiming Sun, Weixin Liu, Ryan Middleton, Linda Marbán, Eduardo Marbán, Cedars - Sinai Medical Ctr, Los Angeles, CA

Background: Self-assembling three-dimensional cardiospheres (CSps) are known to be effective in improving function and attenuating remodeling as adjunctive therapy in acute myocardial infarction (MI), and they exert functional benefits in large animals with convalescent MI. The effect of cardiospheres in chronically-remodeled myocardium post-MI, and the mechanisms of benefit are unknown. **Methods**: We performed permanent LAD ligation in Wistar Kyoto female rats. One month later the animals underwent repeat thoracotomy and were randomly assigned to one of two groups: vehicle-treated controls, or CSp-treated, where CSps (at a seeding dose of $2X10^6$ cells) were injected in the peri-infarct area. Mechanisms were also probed *in vitro*. **Results**: CSps downregulated the canonical Tgfβ1-smad2/3 pathway in co-cultures with fibroblasts and reduced fibroblast proliferation *in vitro*. Soluble endoglin, abundantly expressed in CSps, was found to limit the Tgfβ1-induced fibrosis in a dose-dependent manner. *In vivo*, left ventricular function gradually increased one month post-CSp injection with further improvement after up to six months of follow up. Immunoblots verified Tgfβ1/smad cascade downregulation one month post-CSp injection and upregulation of soluble endoglin. In addition, scar mass was reduced one month post-CSp injection compared to controls. Collagen density was attenuated within the infarcted region of the treated group, while immunostaining showed enhanced vessel density in the same region. Six months post-transplantation, cardiomyocyte hypertrophy was reduced in the CSp-treated group compared to control. **Conclusions**: CSps injected into the peri-infarct zone enhance angiogenesis and reduce established fibrosis in chronically-infarcted myocardium, thereby reversing, at least partially, adverse remodeling of the heart. The underlying mechanism involves modification of Tgfβ1/smad signaling, a key pathway in the cardiac response to injury.

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Manipulating PHD2 to Promote Efficacy of Stem Cell Therapy for Myocardial Infarction

Wei Eric Wang, Dezhong Yang, Liangpeng Li, Yulan Peng, Wei Wang, Dept of Cardiology, Daping Hosp, Third Military Medical Univ, Chongqing, China; Linda Ye, Dayue Duan, Dept of Pharmacology, Univ of Nevada Sch of Med, Reno, NV; Xiongwen Chen, Steven R. Houser, Cardiovascular Res Ctr, Temple Univ Sch of Med, Philadephia, PA; Chunyu Zeng, Dept of Cardiology, Daping Hosp, Third Military Medical Univ, Chongqing, China

OBJECTIVE:
Stem cell transplantation has had modest success as a treatment for myocardial infarction (MI). One of the limitations is the poor stem cell survival and function in the diseased microenvironment. We studied whether and how prolyl hydroxylase domain protein 2 (PHD2), a cellular oxygen sensor, enhances stem cell cardioprotective effects after transplantation into infarcted hearts.
METHODS and RESULTS:
Both adipose-derived stem cells (ADSCs) and bone marrow mesenchymal stem cells (BM-MSCs) were used. Stem cells were transduced with lentiviral short hairpin RNA to silence PHD2 (shPHD2) and intramyocardially injected into infarcted heart in mice. ADSCs reduced cardiomyocyte apoptosis, fibrosis, and infarct size (MI+ADSCs: 39.4±3.3%;MI+PBS: 48.4±4.5%) and improved cardiac function (MI+ADSCs: 43.8±5.6%; MI+PBS: 37.2±3.0%). shPHD2-ADSCs exerted significantly more protection (infarct size: 22.6±3.0%; LVEF: 67.3±6.8%; $p<0.05$ vs. MI+ADSCs). PHD2 silencing induced greater ADSC survival (survival rate at 7 days post-transplantation: shPHD2-ADSCs: 14.7±4.2% vs. ADSCs: 3.4±0.8%, $p<0.05$), which was abolished by HIF-1alpha silencing. No ADSC gave rise to cardiomyocyte and ADSCs induced cardioprotection was mainly induced by paracrine function. Conditioned medium from shPHD2-ADSCs decreased cardiomyocyte apoptosis. Insulin-like growth factor-1 (IGF-1) levels were 3.8 times higher in the conditioned medium of shPHD2-ADSCs than ADSCs, and depletion of IGF-1 attenuated the cardioprotective effects of shPHD2-ADSC-conditioned medium. NF-kappaB activation was induced by shPHD2 to stimulate IGF-1 secretion via binding to the IGF-1 gene promoter. A combination of HIF-1alpha silencing and IGF-1 neurtralization blocked the beneficial effects of shPHD2-ADSCs for MI. Similar findings were observed with BM-MSCs.
CONCLUSIONS:
PHD2 silencing promotes stem cell survival in infarcted hearts and enhances their paracrine function to protect cardiomyocytes. Its prosurvival effect on stem cells is HIF-1alpha dependent, while it enhances stem cell paracrine function through NF-kappaB-mediated IGF-1 upregulation. PHD2 silencing in stem cells may be a novel strategy for enhancing the effectiveness of stem cell therapy after MI.

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Quantification of Total Myocyte Regeneration Following Intracoronary Cardiosphere-Derived Cell (icCDC) Treatment in Swine with Chronic Myocardial Ischemia

Brian R Weil, Amanda Ribbeck, Gen Suzuki, Merced M Leiker, James A Fallavollita, John M Canty Jr., Univ at Buffalo, Buffalo, NY

Objective: icCDCs improve function of ischemic myocardium and increase cardiomyocyte nuclear density but the extent to which this is explained by an increase in nuclei per cell vs. the formation of new myocytes is unclear. We aimed to address this issue and quantify new myocyte formation throughout the left ventricle (LV) after global icCDC treatment in swine with hibernating myocardium.
Methods: Swine with a chronic LAD stenosis (N=29) received either ~35 x 10^6 icCDCs or no treatment. Regional function (echocardiography) and myocyte morphometric indices were assessed 1 month later. Transverse and longitudinal tissue sections were used to measure regional myocyte nuclear density, diameter, length, and nuclei/cell, from which myocyte volume and myocytes per gram of myocardium were calculated.
Results: icCDC-mediated improvements in

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regional function were accompanied by an increase in myocyte nuclear density without a change in the number of nuclei/cell (Table). Compared with untreated animals, icCDC-treated animals exhibited a reduction in myocyte volume and a reciprocal increase in the number of myocytes per gram of tissue. Anatomic LV hypertrophy did not occur, however, as LV mass/body mass ratio was not different between groups (untreated: 2.5 ± 0.1 vs. icCDCs: 2.3 ± 0.1).

Conclusion: These data demonstrate that icCDCs produce significant myocyte regeneration throughout the regionally ischemic heart that is dissociated from alterations in LV mass. Changes in myocyte nuclear density do not reflect an altered number of nuclei/cell and quantitative estimates suggest that intracoronary delivery of CDCs to the entire LV increases the number of myocytes by ~25% in just 4 weeks.

	Ischemic LAD Region		Non-Ischemic Remote Region	
	Untreated (n=14)	icCDCs (n=15)	Untreated (n=14)	icCDCs (n=15)
Regional Function				
Wall Thickening (%)	32.5 ± 2.8	50.9 ± 3.6*	66.5 ± 5.6	99.5 ± 8.5*
Myocytes in Cross Section				
Myocyte Nuclei/mm^2	912 ± 44	1530 ± 65*	1014 ± 28	1557 ± 77*
Nuclei/Myocyte	4.4 ± 0.1	4.6 ± 0.2	4.3 ± 0.2	4.4 ± 0.2
Myocytes per Gram of Myocardium				
Myocyte Diameter (μm)	20.2 ± 0.6	17.9 ± 0.5*	18.7 ± 0.7	17.4 ± 0.4
Myocyte Cell Volume (x10^3 μm^3)	50.9 ± 4.8	37.0 ± 2.5*	43.8 ± 4.9	33.1 ± 2.0*
Myocytes per Gram (x10^6 cells/gram)	16.7 ± 1.5	22.0 ± 1.6*	19.5 ± 1.6	24.2 ± 1.5*

Values are mean ± SEM; *p<0.05 vs. Untreated

B.R. Weil: None. **A. Ribbeck:** None. **G. Suzuki:** None. **M.M. Leiker:** None. **J.A. Fallavollita:** None. **J.M. Canty:** None.

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CXCR4 Expression on Endogenous Bone Marrow Stem/Progenitor Cells is Crucial for Preservation of Myocardial Function After MI

Marcin Wysoczynski, Mitesh Solanki, Rakesh Ponnapureddy, Rakesh Gadde, Roberto Bolli, Gregg Rokosh, Univ of Louisville, Louisville, KY

Background. Studies examining the role of bone marrow CXCR4 in the response to myocardial infarction (MI) using the CXCR4 antagonist AMD3100 in animal myocardial infarction (MI) models are inconclusive. Chronic AMD3100 administration exacerbated injury and myocardial dysfunction while bolus injection immediately post-MI reduced size of the injury with improvement of systolic function. ***Aims:*** As MI mobilizes bone marrow stem/progenitor cells and immune cells into peripheral blood which then home to injured myocardium to facilitate and impair, respectively, regeneration and healing we were interested in the role of bone marrow CXCR4 on functional recovery, angiogenesis, and cardiomyogenesis. ***Experimental approach:*** To define the role of CXCR4 in the bone marrow we generated chimeras with bone marrow from CXCR4$^{flox/flox}$ mice. Wild type mice were transplanted with CXCR4$^{flox/flox}$ or CXCR4$^{flox/flox}$ UBQ Cre bone marrow cells after a lethal dose of irradiation. CXCR4 deletion was induced 5 weeks after transplant with Tamoxifen. The coronary artery was ligated after another two weeks. Function was evaluated five weeks post MI function by echo and pathology analysis was performed to measure scar size, collagen content, hypertrophy, capillary counts, CSCs counts, angiogenesis and cardiomyogenesis. ***Results***: compared to wild type, mice with CXCR4 KO bone marrow had impaired LV systolic function evaluated 5 weeks post-MI by echocardiography. Morphometric analysis of Masson's Trichrome stained sections confirmed LV exacerbated chamber enlargement (expansion index), larger scar and

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decreased infarcted wall thickness in mice with bone marrow cells deficient for CXCR4. More detailed analysis revealed c-kit positive CSC numbers were decreased as were proliferating c-kit CSC indicated by Ki67 staining. Decreased proliferation also correlated with reduced numbers of newly formed myocytes (αsarcomeric actin^{pos}/BrdU^{pos}). Capillary and arteriole counts were also reduced in infarcted hearts of mice with CXCR4 KO bone marrow compared to wild type. ***Conclusion:*** based on these findings we can conclude that CXCR4 is necessary for bone marrow cell homing to infarcted myocardium to preserve LV function, regenerate lost myocardium, and promote angiogenesis.

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Overexpression Of Tbx20 In Adult Cardiomyocytes Promotes Cardiomyocyte Proliferation And Improves Cardiac Function Post Myocardial Infarction

Fu-li Xiang, Katherine Yutzey, CCHMC, Cincinnati, OH

Background: Adult mammalian cardiomyocytes (CM) have the potential to proliferate, but this is not sufficient to compensate for the massive loss of functional CMs after myocardial infarction (MI), which remains a leading cause of death in the US. During embryonic heart development, the transcription factor Tbx20 is required for CM proliferation, and Tbx20 overexpression promotes fetal characteristics in adult CMs when initiated before birth in mice. We hypothesize that Tbx20 overexpression (Tbx20OE), when induced in adult CMs after injury, improves cardiac function and repair via dedifferentiation of CMs, thus promoting cell cycle re-entry and repair in mice post-MI. Methods and Results: αMHCMerCreMer (STG) and the inducible cardiomyocyte-specific Tbx20 transgenic (αMHCMerCreMer/CAG-CAT-Tbx20, DTG) mice were subjected to MI or sham surgeries. Tbx20OE was induced 3 days post-surgery via tamoxifen to specifically target cardiac repair post-MI. In sham-operated mice, no difference in cardiac function or morphology was observed between DTG and STG groups. However, more proliferating CMs as labeled by Ki67 were found in DTG sham myocardium compared to STG. Expression of cyclin D1, E1 (cell cycle markers) and IGF1 mRNA was increased, while p21 (cell cycle inhibitor) and Meis1 (negative regulator of proliferation) were decreased, in DTG sham hearts compared to STG controls. In mice subjected to MI, cardiac function, as measured by echocardiography, was significantly improved, and the infarct scar size was smaller (58.1% vs 38.3%) in the DTG group compared to STG controls 2 and 4 weeks post-MI. Myocardial hypertrophy determined by heart to body weight ratio and myocyte diameter was also significantly reduced in DTG heart compared to STG 4 weeks post-MI. Thus, induction of Tbx20OE post-MI injury leads to improved cardiac performance, decreased scar size, and decreased maladaptive cardiac remodeling. Ongoing studies will determine if proliferation indices (Ki67, pHH3, aurora kinase B) and cytokinesis of CM post-MI are increased in myocardium and isolated adult cardiomyocytes with Tbx20OE. Conclusions: Tbx20OE in adult CM activates cell proliferation markers and also improves cardiac function and repair in mice when induced post-MI.

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Bone Marrow Cells Transdifferentiate into Cardiomyocytes and Repair the Infarcted Heart

Anna Czarna, Junghyun Kim, Fumihiro Sanada, Sergio Signore, Antonio Cannata, Marcello Rota, Annarosa Leri, Piero Anversa, Brigham and Women's Hosp, Boston, MA

A major controversy concerns whether c-kit-positive bone marrow cells (c-kit-BMCs) can acquire cell lineages different from their organ of origin. Technical challenges and variability in the protocols employed for cell isolation and detection of donor-derived structures may account only in part for the conflicting results obtained in different laboratories. We raised the hypothesis that c-kit-BMCs are functionally heterogeneous and possess distinct transdifferentiation potential. To address this issue, two methodologies were employed to label individual c-kit-BMCs and define their

clonal fate in vivo: viral gene-tagging and fluorescent protein-based multicolor cell marking. c-kit-BMCs were infected with EGFP lentiviruses and injected in infarcted hearts. At 2 weeks, the regenerated myocardium was enzymatically digested and EGFP-labeled myocytes, endothelial cells, fibroblasts, and c-kit-cells were sorted. By employing a PCR-based method of detection of viral integrants, we searched for sites of viral insertion in the isolated cells. Common insertion sites were found in the DNA of c-kit-BMCs and specialized cells, documenting that single c-kit-BMCs transdifferentiated into multiple cell lineages. However, only specific subsets of c-kit-BMCs generated cardiomyocytes in vivo, strongly indicating that the c-kit-BMC pool is composed of myogenic and non-myogenic cells. To strengthen these observations, FACS-sorted c-kit-BMCs were simultaneously transduced with 3 lentiviruses, each encoding red, green or blue (RGB) fluorescent proteins. Different combinations of inserted vectors in individual c-kit-BMCs resulted in a variety of mixed colors, each indicative of the development of clonal populations. RGB-infected c-kit-BMCs were delivered to infarcted rats and gave rise to homogeneously colored colonies containing fluorescently labeled myocytes and vascular cells. The homogenous pattern of expression of the fluorescent proteins documented that the regenerated structures derived from the expansion and transdifferentiation of single c-kit-BMCs. Our findings demonstrate that c-kit-BMCs are functionally heterogeneous and have a different ability to survive, engraft, and transdifferentiate in the ischemic heart.

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The Role of cAMP-Phosphodiesterase 1C Signaling in Pathological Cardiac Remodeling and Dysfunction

Walter E Knight, Univ of Rochester, Rochester, NY; Masayoshi Oikawa, Fukushima Medical Univ, Fukushima, Japan; Clint Miller, Stanford Univ, Stanford, CA; Chen Yan, Univ of Rochester, Rochester, NY

The cyclic nucleotides cAMP and cGMP play important roles in mediating both protective and detrimental signaling in heart failure. By acting as regulators of cAMP and cGMP, the phosphodiesterases play important roles in modulating this signaling. A comparison of PDE expression between control mice and mice given transverse aortic constriction revealed that expression of phosphodiesterase 1C (PDE1C) was increased significantly by TAC, both on the mRNA and protein levels. To determine whether this was protective or maladaptive, we performed TAC on mice with a genetic deletion of PDE1C. While TAC-operated WT mice experienced significant overall cardiac hypertrophy and cardiomyocyte hypertrophy, these were reduced in PDE1C KO mice. Cardiac function, as assessed by echocardiography, was also reduced significantly in WT TAC mice, but was preserved in PDE1C KO TAC mice. Histological analysis indicated that TAC-operated PDE1C KO mice also experienced reduced cardiomyocyte apoptosis compared to WT mice, indicating a potential cardioprotective mechanism for PDE1C deletion. Cardiomyocytes isolated from PDE1C KO mice experienced reduced Ang II or Iso-induced cell death compared to WT myocytes, indicating that this was a cardiomyocytes-specific effect of PDE1C deletion. Ang II-induced cardiomyocyte cell death and apoptosis were also blocked via pharmacological PDE1 inhibition. PDE1C is able to hydrolyze either cAMP or cGMP; therefore, it seemed possible that this protective mechanism was dependent on either PKA- or PKG-mediated signaling. While PKG inhibition did not alter the protective effect of PDE1 inhibition in isolated cardiomyocytes, PKA inhibition blocked it. Overexpression studies also indicated that PDE1C is localized to the cell membrane in cardiomyocytes. Therefore, we propose that by modulating a novel, membrane-localized, anti-apoptotic, cAMP/PKA-dependent pathway in cardiomyocytes, PDE1C potentially represents a novel therapeutic target in heart failure.

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A Novel Variant Cell Cycle-related Kinase Provides Cardioprotection Upon Pressure Overload By Promoting Autophagy

Hongyu Qiu, Loma Linda Unviersity, Loma Linda, CA; Lo Lai, , New Jersey Medical Sch, Rutgers, The State Univ of New Jersey, Newark, NJ

Background: One approach to discovering novel targets for heart failure (HF) therapy is to determine which genes are down-regulated in HF. We found previously that cardiac cell cycle-related kinase (cCCRK), a novel variant of CCRK in heart, is down-regulated by 50% in failed heart vs control, and over-expression of the cCCRK decreases the susceptibility of cardiac myocytes to pro-apoptotic agents, indicating that cCCRK might be a potential target of HF. We tested here our hypothesis that cCCRK provides cardiaoprotection upon stress of pressure overload.
Methods and Results: A transgenic (TG) mouse model with cardiac-specific over-expression of cCCRK was generated. Both wild type (WT) and TG mice were submitted to aortic banding for two weeks. In basal condition, no significant differences were found between TG and WT in left ventricular (LV) /body weight (3.5±0.3 in TG vs. 3.6±0.2 in WT) and contractility (LV ejection fraction (EF): 72.7±1 in TG vs 72.7±1 % in WT). After two weeks banding, EF was significantly impaired in the WT (62±2%, P<0.05 vs sham), but maintained in the TG (71.1±2 %, P<0.05 vs WT). Lung /tibial length ratio, an index of pulmonary congestion and heart failure was lower (p<0.05) in banded heart of TG (9.9±1) vs WT (13.1±1). A cyclin-dependent kinase inhibitor p21, which is also an important regulator of autophagy, was significantly upregulated in TG by 5 folds vs WT (P<0.05). Overexpression of cCCRK in rat neonatal cardiomyocyte (RNC) stimulated autophagy in a dose-dependent manner representing by the enhanced LC3-II (autophagy marker) (P<0.05 vs β-gal).
To test its effect in response to stress, glucose starvation was induced to in RNC. cCCRK induced an early response to cardiac stress via the activation of autophagy by increasing LC3-II at 0.5 hrs vs the control at 2 hrs after starvation.
Conclusions: over-expression of cCCRK in heart provides protection against pressure overload via activation of autophagy signaling pathways.

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Increased Expression Of Mitochondrial Inducible Nitric Oxide Synthase By Heat Shock Protein Hsp22/h11 Kinase Promotes Oxidative Phosphorylation In Mammalian Heart

Hongyu Qiu, Loma Linda Unviersity, Loma Linda, CA; Eman Rashed, Christophe Depre, New Jersey Medical Sch, Rutgers, The State Univ of New Jersey, Newark, NJ

Aims. Stress-inducible heat shock protein 22 (Hsp22) confers protection against ischemia through induction of the inducible isoform of nitric oxide synthase (iNOS). Hsp22 over-expression *in vivo* significantly stimulates cardiac mitochondrial respiration, whereas Hsp22 deletion *in vivo* shows a reciprocal effect. It has also been shown in *Drosophila* that Hsp22 is expressed in the mitochondria that depends on its N-terminal domain. We hypothesized that Hsp22-mediated regulation of mitochondrial function is dependent upon its mitochondrial translocation together with iNOS.
Methods and Results. Adenoviruses harboring either the full coding sequence of Hsp22 (Ad-WT-Hsp22) or a mutant lacking a 20 amino acid putative N-terminal mitochondrial localization sequence (Ad-N20-Hsp22) were generated, and infected in rat neonatal cardiomyocytes. Compared to β-Gal control, Ad-WT-Hsp22 accumulated in mitochondria by 2.5 fold (P<0.05), reduced chelerythrine-induced apoptosis by 60% (P<0.01), and increased oxygen consumption rate by 2-fold (P<0.01). This latter effect was abolished upon addition of the specific iNOS inhibitor, 1400W. Ad-WT-Hsp22 significantly increased global iNOS expression by about 2-fold (P<0.01), and also increased its mitochondrial localization by 2.5 fold vs β-gal (P<0.05). Upon comparable over-expression, the Ad-N20-Hsp22 mutant did not show significant mitochondrial translocation, protection against apoptosis or stimulation of mitochondrial respiration. Although Ad-N20-Hsp22 did increase global iNOS expression by

6-fold it did not significantly promote iNOS mitochondrial translocation. ***Conclusion***. Translocation of both Hsp22 and iNOS to the mitochondria is necessary for the stimulation of oxidative metabolism and protection against apoptosis.

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Novel Atheroma-specific Atheroprotective Factor Promotes VSMC Pro-survival Signaling

Amanda K Segedy, Vanderbilt Univ, Nashville, TN; Amy L. Pyle, Ohio State Univ, Columbus, OH; Bin Li, Youmin Zhang, Vladimir Babaev, Vanderbilt Univ, Nashville, TN; Parmjit Jat, Univ Coll London, London, United Kingdom; Sergio Fazio, James B. Atkinson, MacRae F. Linton, Pampee P Young, Vanderbilt Univ, Nashville, TN

Cardiovascular disease, the leading cause of death in the United States, is primarily driven by atherosclerosis. In recent years, studies have focused on identification of naturally expressed, atheroprotective genes for use in delaying development of or preventing complications from atherosclerosis. To this end, we previously identified SPRR3 (small proline rich protein 3) as a gene specifically upregulated in vascular smooth muscle cells (VSMCs) of atheroma versus healthy arterial tissue of humans and mice. In the present study, we generated ApoE-null mice lacking SPRR3, which displayed significantly increased atheroma burden compared with ApoE-null controls. To determine the cellular driver(s) of this phenotype, we investigated SPRR3-dependent changes in bone marrow-derived cells, endothelial cells (ECs), and VSMCs. Bone marrow transplant of SPRR3-expressing cells into SPRR3-/-ApoE-/- recipients failed to rescue atheroma burden. Similarly, no change was observed in SPRR3-deficient versus control ECs. However, apoptosis was significantly reduced in SPRR3-overexpressing VSMCs in vitro. A positive association was also observed between SPRR3 expression and PI3K/Akt activity in VSMCs. The SPRR3-dependent survival advantage observed in SPRR3-overexpressing cells was lost following treatment with PI3K/Akt pathway inhibitor. Our data indicate that SPRR3 protects the atheroma from VSMC loss by promoting survival signaling in lesion VSMCs, thereby modulating atherosclerosis development. As the first identified atheroma-specific VSMC pro-survival factor, SPRR3 represents a potential target for lesion-specific modulation of VSMC survival to inhibit plaque progression.

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Nub1l Suppresses Neddylation And Enhances Misfolded Protein Clearance In Cardiomyocytes

Huabo Su, Jie Li, Wenxia Ma, Goergia Regents Univ, Augusta, GA; Ning Hou, Faqian Li, Univ of Rochester Medical Ctr, Rochester, NY

Protein modification by ubiquitin (Ub) or Ub-like proteins such as NEDD8 (neddylation) constitutes a fundamental regulatory mechanism of protein function. In contrast to well-recognized role of Ub in protein degradation, little is known about the role of NEDD8 in protein quality control. We have previously revealed that CM-restricted inactivation of deneddylation, a process that removes NEDD8 from modified proteins, accumulates neddylated proteins and impairs proteasomal and autophagic proteolysis. Here we report that proteasome inhibitors, simulated ischemia/reperfusion and H2O2 significantly increase NEDD8 conjugates in cardiomyocytes (CMs). Immunoprecipitation analysis reveals mixed modification of these proteins by Ub and NEDD8. Expression of NEDD8 but not the conjugation-deficient mutant increases neddylated proteins and accumulates a proteasome surrogate substrate GFPu in a dose-dependent manner, suggesting that excessive neddylation disrupts proteasomal proteolysis. We further targets to NUB1L, a UBL (Ub-like domain)-UBA (Ub associating domain) family protein that was shown to negatively regulate neddylation. NUB1L expression markedly reduces free NEDD8 by promoting its degradation, and abrogates proteasome inhibition-induced neddylation in CMs.

Suppression of neddylation by NUB1L expression enhances GFPu degradation at baseline, and attenuates GFPu accumulation upon sI/R and H2O2 treatment. Furthermore, NUB1L expression promotes, while down-regulation of NUB1L impairs, the clearance of a bona fide misfolded protein in CMs. NUB1L expression also ameliorates proteotoxic stress- and sI/R-induced CM injury. Finally, increased NEDD8 conjugates are evident in the mouse hearts of a number of cardiac disease models as well as in human failing hearts. Together, our findings suggest that excessive neddylation disrupts protein quality control and that antagonizing neddylation by NUB1L promotes misfolded protein degradation. Targeting neddylation/NUB1L could be a novel therapeutic strategy for prevention and treatment of insufficient protein quality control-associated cardiac disease.

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LincRNA-p21 Regulates Neointima Formation and Atherosclerosis by Enhancing p53 Activity

Gengze Wu, Yue Cai, Yujia Yang, Zaicheng Xu, Wei Eric Wang, Ken Chen, Chunyu Zeng, Daping Hosp, Third Military Medical Univ, Chongqing, China

Background: Long non-coding RNAs (lncRNAs) recently have been implicated in many biological processes and diseases. Atherosclerosis is a major risk factor for cardiovascular disease. However, the functional role of lncRNAs in atherosclerosis is largely unknown. Methods and Results: The downstream targets of p53 also been detected by un-bias mRNA array and specific qPCR. Furthermore, bioinformatics prediction, RNA-Immunoprecipitation, biotin-labeled RNA-pulldown and deletion mapping were performed to test the potential interaction between lincRNA-p21 and MDM2. Co-IP, genome-wide ChIP-seq and ChIP-qPCR were preformed to verify the possible influence on p53 transcriptional activity. We further investigated the function of lincRNA-p21 in vivo, therefore recombinant lincRNA-p21 knockdown lentivirus were injected into the injured area of carotid arteries injury mouse model. Then the expression of lincRNA-p21 in vascular samples of clinical coronary disease patients were also detected. We identified lincRNA-p21 as a key regulator of atherosclerosis. Expression of lincRNA-p21 was dramatically down-regulated in atherosclerotic plaques of ApoE-/- mice, an animal model for AS. Through loss- and gain-of function approaches, we showed that lincRNA-p21 represses cell proliferation and induces apoptosis in VSMCs in vitro. Moreover, we found that inhibition of lincRNA-p21 results in neointimal hyperplasia in vivo in a carotid artery injury model. Mechanistically, we revealed that lincRNA-p21, which is a transcriptional target of p53, feeds back to enhance p53 transcriptional activity via binding to mouse double minute 2 (MDM2), an E3 ubiquitin-protein ligase. The association of lincRNA-p21 and MDM2 releases MDM2 repression of p53, enabling p53 to interact with p300 to regulate apoptosis, cell proliferation and suppress neointima formation. Conclusions: Our studies identified lincRNA-p21 as a novel regulator of cell proliferation and apoptosis and suggest that this lncRNA could serve as a therapeutic target to treat atherosclerosis.

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Contractile Dysfunction In The Mouse Heart Caused By Phospholipase C beta1b Mediated Activation Of Protein Kinase Calpha

David R Grubb, Yi Ma, Jieting Luo, Bryony Crook, Nicola Cooley, Helen Kiriazis, Hong Wei Qian, Paul Gregorevic, Xiao-Ming Gao, Xiao-Jun Du, Elizabeth A. Woodcock, Baker IDI Heart and Diabetes Inst, Melbourne, Australia

The activity of the early signaling enzyme, phospholipase Cβ1b (PLCβ1b), is elevated in diseased myocardium and activity increases with disease progression. PLCβ1b and the alternative splice variant, PLCβ1a, were expressed in mouse hearts using adeno-associated viral constructs (rAAV6-FLAG-PLCβ1b, rAAV6-FLAG- PLCβ1a) delivered intravenously. Functional responses were assessed in vivo and confirmatory mechanistic

studies were conducted in neonatal rat ventricular myocytes (NRVM). FLAG-PLCβ1b was expressed in all of the chambers of the mouse heart, but was highest in left ventricle, where expression was observed in >90% of the cells and was localized to the sarcolemma and T-tubules. Heightened PLCβ1b expression caused a rapid loss of contractility and down-regulation of Phospholamban expression. The loss of contractility induced by PLCβ1b was reversed by inhibition of protein kinase Cα (PKCα). PLCβ1a did not affect contractile function or phospholamban expression. Mechanistic analysis performed in neonatal rat cardiomyocytes confirmed PLCβ1b increased the membrane association of PKCα as well as downstream dephosphorylation of phospholamban and depletion of the Ca2+ stores of the sarcoplasmic reticulum, both of which were mediated by PKCα. Trans-aortic constriction (TAC) resulted in progressive hypertrophy together with reduced contractility in PLCβ1a expressing mice. In PLCβ1b-expressing mice, TAC induced a similar hypertrophic response, but did not cause further contractile depression above that due to PLCβ1b expression alone, suggesting that PLCβ1b is responsible for lowering contractility in response to pressure overload. We conclude that heightened PLCβ1b activity observed in diseased myocardium contributes to pathology by PKCα-mediated contractile dysfunction. PLCβ1b is a cardiac-specific signaling system, and thus provides an ideal therapeutic target for the development of well-tolerated inotropic agents for use in failing myocardium.

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A Human G109E Polymorphism in Inibitor-1 Compromises Cardiomyocyte Function and Induces Arrhythmias

Kobra Haghighi, Tracy J. Pritchard, Vivek P. Singh, Parthib Das, Erica L. Vanderbilt, Jack Rubinstein, Evangelia G. Kranias, Univ of Cincinnati, Cincinnati, OH

Protein phosphatase 1 (PP1) has emerged as a nodal regulator of function and survival in the heart. Indeed, the increased activity of this enzyme in failing hearts contributes to depressed SR Ca-cycling and deteriorative remodeling. PP1 is negatively regulated by endogenous inhibitor-1, which is considered a promising therapeutic target. Increases in inhibitor-1 activity and decreases in PP1 protect against ischemia/reperfusion injury, chronic isoproterenol stimulation and heart failure progression. We recently identified a polymorphism (G109E) in the inhibitor-1 gene in heart failure patients with a frequency of 6%. Expression of G109E (called mutant) in rat cardiomyocytes resulted in ~20% decreases in contractile parameters, Ca-transients and sarcoplasmic reticulum Ca-load. This depressed function was rescued by isoproterenol. Interestingly, when subjected to stress conditions (2 Hz +/- Iso), the mutant cells were more susceptible to aftercontractions. Similar findings were obtained by expression of G109E in inhibitor-1 knockout cardiomyocytes in the absence of endogenous protein. The underlying mechanisms included reduced binding of mutant to PP1, increased PP1 activity and hyper-phosphorylation of S2814 in ryanodine receptor (RyR), promoting aberrant SR Ca-release. These findings were also reflected by in vivo cardiac overexpression of G109E. Contractile and Ca-kinetic parameters were depressed by ~30% in mutant cardiomyocytes, while isoproterenol relieved these inhibitory effects. Stress conditions were associated with induction of Ca waves and aftercontractions in G109E cells. Furthermore, serial caffeine/Iso injections in vivo, elicited higher incidence of ventricular ectopy (bigeminy, trigeminy and non-sustained ventricular tachycardia) in mutant mice, whereas WTs had normal rhythm. Our findings suggest that G109E and increased PP1 may dephosphorylate the RyR and promote Ca-leak, in agreement with previous reports. Subsequently, the increased Ca-levels activate CAMK, leading to hyperphosphorylation of RyR and further increases in Ca-leak. Thus, inhibitor-1 is critical in cardiac function and represents a key control in balancing phosphatase/kinase activities to stabilize Ca-cycling in the heart.

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2-deoxy Adenosine Triphosphate Improves Contraction In Human End-stage Heart Failure

Farid Moussavi-Harami, Maria V Razumova, Yuanhua Cheng, Alice W Racca, April Stempien-Otero, Michael Regnier, Univ of Washington, Seattle, WA

We are developing a novel approach to treat heart failure that involves adeno-associated virus vectors to elevate intracellular 2 deoxy-ATP (dATP) via increased expression of the enzyme Ribonucleotide Reductase. Our studies in rodents have shown that substitution of dATP for ATP as the energy substrate increases contraction in striated muscle. Here we report for the first time the effect of dATP on human cardiac muscle contraction. We measured the contractile properties of demembranated multicellular ventricular wall preparations and isolated myofibrils from adult human heart tissue obtained from twelve patients undergoing surgery for cardiac transplantation or placement of left ventricular-assist device for end-stage heart failure. Isometric force at saturating calcium concentration was increased by about ten percent from 38.6±3.8 mN/mm2 to 42.8±4.2 mN/mm2 when dATP was substituted for ATP ($P<0.001$). The effect was even greater at physiologic calcium concentrations with an approximate increase of thirty percent. Isometric force increased from 21.2 ± 6.1 mN/mm2 to 26.4± 6.4 mN/mm2 ($p<0.001$) at pCa=5.6 and from 22.6 ± 5.9 mN/mm2 to 27.5± 6.3 mN/mm2 ($p<0.001$) at pCa=5.8 with dATP for ATP substitution. The result was an increase in the Ca2+ sensitivity of force as the [Ca2+] required to elicit half maximum force (pCa50) increased by 0.08 units from 5.68±0.03 to 5.79±0.03 ($N=24$, $P< 0.001$). The maximum rate of force redevelopment (ktr) in demembranated wall muscle increased (0.82±0.01 s-1 vs. 0.62±0.01 s-1, $P< 0.05$), as was the rate of contractile activation in isolated myofibrils (0.80±0.06 s-1 vs. 0.57±0.06 s-1, $P<0.01$) suggesting dATP may increase dP/dT in failing human myocardium. Importantly, there was no slowing of relaxation, as the time to 50% and 90% myofibril relaxation were unchanged. Purified myosin from failing human myocardium showed enhanced NTPase activity with dATP (0.89±0.17 s-1/head) vs. ATP (0.55±0.20 s-1/head, $P<0.05$). In conclusion, the data strongly suggest dATP increases cross-bridge cycling, compared with ATP in failing human myocardium and shows promise in restoring cardiac pump function. These data support a novel myofilament approach for treating heart failure that warrants further pre-clinical evaluation.

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Acute Inhibition Of O-GlcNAc Signaling Rescues Inotropic Responsiveness In Type 1 Diabetic Rat Hearts

Chengxue Qin, Rochelle S S Sleaby, Baker IDI Heart and Diabetes Inst, Melbourne, Australia; Lea M Delbridge, Univ of Melbourne, Melbourne, Australia; Amy J Davidoff, Univ of New England, Biddeford, ME; John C Chatham, The Univ of Alabama, Birmingham, AL; Rebecca H Ritchie, Baker IDI Heart and Diabetes Inst, Melbourne, Australia

Metabolism of excess glucose is an important component of the aetiology of type 1 diabetes. The cardiac phenotype includes left ventricular (LV) remodelling and LV dysfunction. Increased hexosamine biosythesis (HBP) and downstream upregulation of protein O-GlcNAcylation has been linked to diabetic complications in many organs. Its impact on LV contractile responsiveness is however not well understood. This study aimed to test the hypothesis that acute inhibition of O-GlcNAc signaling protects inotropic responsiveness in type 1 diabetic heart. Hearts isolated from adult Sprague-Dawley male rats were Langendorff-perfused (constant flow, 10ml/min). Baseline and phenylephrine-stimulated (PE, 10µmol/L) LV function was determined in diabetic (8wks post-streptozotocin diabetes, 55mg/kg i.v.) versus non-diabetic sham rats in the presence of pharmacological inhibitors of HBP/O-GlcNAc including 6-diazo-5-oxo-L-norleucine (DON, 20µM) and alloxan (5mM). Diabetic rats exhibited a marked reduction in inotropic responsiveness to PE (Table, mean±SEM, one-

way ANOVA, #P<0.05 vs non-diabetic vehicle rats, *P<0.05 vs diabetic vehicle, at 40 mins). Acute interruption of cardiac HBP/O-GlcNAc by DON and Alloxan significantly rescued LV responsiveness to PE in type 1 diabetic rat hearts. These results support further assessment of the impact of upregulated protein O-GlcNAcylation on LV function, particularly in the diabetic heart. Treatment strategies that target HBP may provide significant benefits alone or in combination with current standard treatments, to reduce progression of heart failure and death in type 1 diabetic patients.

PE-stimulated LV function (relative to baseline)	Non-diabetic rats (mean±SEM)			Diabetic rats (mean±SEM)		
	Vehicle	DON	Alloxan	Vehicle	DON	Alloxan
n	5	3	5	6	6	6
LV Systolic Pressure (%)	219±25	333±29*	326±17*	138±26#	326±28*	355±28*#
LV Developed Pressure (%)	227±38	392±68*	362±15*	121±24#	389±38*	410±37*
ΔLV End-Diastolic Pressure (fold, mmHg)	1±8	-4±5*	-15±2*	11±5	-12±2*	-17±2*#
LV+dP/dt (%)	197±32	297±27*	290±16*	124±24#	363±32*#	387±22*#
LV-dP/dt (%)	272±48	275±24	346±94	201±32	248±46	212±54
Rate Pressure Product (%)	206±39	384±48*#	358±28*	154±27	433±46*	408±53*#
Perfusion Pressure (%)	159±23	137±22	111±4	144±20	116±10	111±4
Heart Rate (%)	89±3	100±5	98±4	138±24	109±5	98±5

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The Positive Inotropic S100a1 Prevents Arrhythmogenic Sarcoplasmic Reticulum Ca2+ Leak And Ventricular Arrhythmias

Julia Ritterhoff, Mirko Völkers, Andreas Seitz, Kristin Spaich, Heidelberg Univ Hosp, Heidelberg, Germany; Karten Peppel, Thomas Jefferson Univ, Philadelphia, PA; Sven Pleger, Heidelberg Univ Hosp, Heidelberg, Germany; Wolfram Zimmermann, Georg-August-Univ Göttingen, Göttingen, Germany; Oliver Friedrich, Univ of Erlangen-Nürnberg, Erlangen, Germany; Rainer Fink, Univ of Heidelberg, Heidelberg, Germany; Walter Koch, Thomas Jefferson Univ, Philadelphia, PA; Hugo Katus, Patrick Most, Heidelberg Univ Hosp, Heidelberg, Germany

S100A1 has emerged as a key factor in the control of cardiomyocyte (CM) contractile performance. Improved sarcoplasmic reticulum (SR) function with enhanced Ca2+ resequestration appears critical for its cAMP-independent inotropic effects but raises concerns about potential diastolic SR Ca2+ leakage that might trigger fatal arrhythmias. Thus, the goal of this study was to determine the impact of S100A1 on ryanodine receptor 2 (RyR2)-mediated SR Ca2+ leakage in vitro and in vivo.
S100A1 association with the RyR2 was significantly diminished (-50%) in failing cardiomyocytes and hearts with S100A1 downregulation, as shown by co-immunofluorescence, co-immunoprecipitation and proximity ligation assay. Adenoviral-mediated S100A1 overexpression (3-4 fold vs. GFP-control) in quiescent NCs (normal CMs) and FCs (failing CMs) decreased SR Ca2+-frequency (-50 and -40% respectively) and protected from β-AR-triggered diastolic Ca2+-waves (-62 and -58% respectively) in electrically stimulated (2Hz) CMs as assessed by epifluorescent and confocal Ca2+ imaging. In multicellular rat engineered heart tissue (EHT), S100A1-overexpression (6-8 fold vs. GFP-control) protected from Ca2+-triggered after-contractions (ACs) (-50%) with preserved enhancement of isometric twitch force (TF, +40%) at 2Hz. S100A1-mediated rescue of contractile failure of endothelin-1-treated EHT (-50% decrease in TF) was associated with protection from Ca2+-triggered ACs. In mice with post-ischemic heart failure, AAV9-mediated therapeutic administration of S100A1 enhanced S100A1/RyR2 association and prevented epinephrine-induced VTs (70% in MI group vs. 30% in MI-S100A1 group). Mechanistically, S100A1-overexpression changed neither PKA/CaMKII RyR2 phosphorylation pattern nor binding of accessory proteins like FKBP12.6, calmodulin or sorcin to RyR2 but enhanced S100A1/RyR2 stoichiometry.
Our data provide evidence that S100A1 interaction with the RyR2 can beneficially modulate and reverse diastolic RyR2 function dysfunction. S100A1 appears to convey a rather unique molecular profile combining cAMP-independent inotropy with protection against Ca2+-triggered arrhythmias.

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Important Role of Adiponectin in Volume Overload Induced Heart Failure

Lili Wang, Desiree Wanders, Gayani Nanayakkara, Rajesh Amin, Robert L. Judd, Edward E. Morrison, Juming Zhong, Auburn Univ, Auburn, AL

Adiponectin (ADP) has been reported to exert cardiac protective effects during ventricular remodeling following pressure overload and myocardial ischemia. However, the potential role of ADP in the volume overload induced heart failure has not been reported. In this study we examined the effect of ADP in cardiac myocyte contractile dysfunction following sustained volume overload. Rat model of volume overload induced heart failure was created by infrarenal aorta-vena cava (A-V) fistula. Some rats were administered with adenoviral ADP (Ad-ADP) at 2-, 6-, and 9-weeks following fistula surgery. Serum total ADP levels were measured at 3 days before, 5 weeks and 10 weeks after fistula surgery. Myocyte contractility and intracellular Ca2+ transients were evaluated at 10 weeks following fistula. Results indicated a progressive reduction of serum ADP levels. In ventricular myocytes isolated from 10-week fistula rats, protein expression of ADP, AdipoR1/R2 and T-cadherin were decreased, and AMPK phosphorylation was reduced. Consistent with these, myocytes exhibited significant depression in cell shortening and intracellular Ca2+ transient. In vivo overexpression of adenovirus-mediated ADP in fistula rats significantly increased ADP serum levels, and prevented the depression of myocyte contractile performance. Moreover, in vitro treatment with ADP significantly improved myocyte contractility and intracellular Ca2+ transient from 10-week fistula rats, but had no effect on myocyte performance in control and Ad-ADP animals. These results demonstrate a positive correlation of ADP reduction and ventricular remodeling induced by volume overload. Adiponectin plays a protective role in volume overload-induced heart failure.

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Frequency of the Angiotensin Converting Enzyme (I/D) and β1 Adrenergic Receptor (Arg389Gly) Gene Polymorphisms in a Sample of Mexican Population

Juan Pablo Cervantes Minjares Sr., Clinical and Experimental Therapeutics Inst, Univ of Guadalajara, Guadalajara, Mexico; Manuel Alejandro De la Torre Zazueta Dr., Autonomous Univ of Baja California, Mexicali, Mexico; Guadalupe Hernández Pacheco Dr., Dept of Physiology, Natl Inst of Cardiology, Mexico City, Mexico

Introduction.
The angiotensin converting enzyme (ACE) is involved in blood pressure regulation. The I/D polymorphism of ACE has been associated with hypertension, left ventricular hypertrophy and metabolic syndrome.
The β1 adrenergic receptor (ARβ1) is located mainly in the heart, its activation has a chronotropic and inotropic effect. The Arg389Gly polymorphism of ARβ1 has been associated with dilated cardiomyopathy, atrial fibrillation and vasovagal syncope.
The identification of these polymorphisms in a control group, will reveal the frequencies in healthy mexican population so we can compare with the diseased population and seek association with different diseases.
Objective.
Identifying gene and allelic prevalence in a healthy population.
Material and methods.
We worked with 45 samples from healthy individuals, 36 males and 9 females, with a median age of 35.8 ± 16.1 and a BMI of 25.81 ± 4.6.
Extraction of DNA from 10 ml of peripheral blood. ACE polymorphism was determined by direct PCR. The determination of the Arg389Gly polymorphism was by PCR-RFLP.
Results.
The gene frequency of the ACE gene in 39 subjects was: DD 33.33% (13 of 39), ID 38.46% (15 of 39) and II 28.2% (11 of 39). The allelic frequency: I 47.43% and D 52.56%.

ABSTRACTS

Arg389Gly polymorphism frequency was in 11 subjects: ArgArg 9% (1 of 11), ArgGly 72.7% (8 of 11) and GlyGly 18.2% (2 of 11). The allele frequency: 45% Arg and Gly 55%. Conclusions.
DD homozygote frequency differs from that observed in the study of Vargas et al., which is lower. This is important because the D allele is associated with greater activity and concentration of ACE, which may predispose to cardiovascular disease in our population. Regarding the Arg389Gly polymorphism, it has been observed that the Gly allele is linked to cardiovascular disease and the response to beta-blockers.
Our population showed an increase of Gly allele carriers, so they may be more susceptible to these diseases.
It is important to know the frequency of these polymorphisms in our population, and identify that they can impact in medical treatment or may serve as potential predictors of cardiovascular disease.

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Genetic Deletion Of Mir-208a Induces Pathological Remodeling And Heart Failure

Robert T Davis III, Raghu S Nagalingam, Mariam Noor, Univ of Illinois at Chicago, Chicago, IL; Da-Zhi Wang, Children's Hosp Boston-Harvard Medical Sch, Boston, MA; Beata M Wolska, R.John Solaro, Madhu Gupta, Univ of Illinois at Chicago, Chicago, IL

Introduction: miR-208a, a small non-coding RNA expressed only in the heart, has a profound influence on cardiac gene expression. It has been previously demonstrated that genetic deletion of miR-208a does not affect viability or induce gross morphological heart defects, suggesting it is not required for normal cardiac growth and function (van Rooij et al., 2007; Callis et al., 2009). However, recent data from our lab indicate that miR-208a knock-out (KO) mice (developed in the laboratory of Da-Zhi Wang and subsequently maintained by inbreeding) display gross morphological cardiac abnormalities and dramatically reduced survival. Therefore, this investigation sought out to determine the mechanism(s) responsible for the maladaptive growth. Methods: Wild-type (WT) and miR-208a KO littermate mice (3-5 mon old) were used for this investigation. Cardiac function was assessed via echocardiography, cardiac fibrosis by collagen fiber staining, and gene expression by real-time PCR and Western blotting. We also measured basal Ca2+ transients and unloaded cell shortening in isolated mouse ventricular myocytes. Results: We observed KO mice have significantly lower survival rates, developed marked cardiac hypertrophy, fibrosis and have significantly reduced cardiac function. Compared to WT controls, cardiomyocytes from KO mice exhibited a significantly lower percent of cell shortening, slower rates of relaxation and contraction, lower amplitude, and slower kinetics (tau) of Ca2+ transients. Additionally, there was a reduced phosphorylation of phospholamban (Ser16) in miR-208 KO mice indicating a lower Ca2+ affinity of the SR Ca2+-ATPase . Conclusions: These data provide evidence that the observed reduction in cardiac function in miR-208a KO mice is likely due, in part, to alterations in cellular Ca2+ fluxes. To our knowledge, this is the first study to demonstrate genetic deletion of miR-208a induces a maladaptive phenotype at baseline. Our findings indicate the importance of considering variables such as genetic/environmental constraints when perturbing the expression of the miR-208a in the heart.

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Dnajb6b Is A Novel Genetic Modifier For Cardiomyopathy That Regulates ER Stress Response

Yonghe Ding, Weibin Liu, Beninio Gore, Stephen C. Ekker, Xiaolei Xu, Mayo Clinic, Rochester, MN

Background: Cardiomyopathy and heart failure affect millions of people worldwide. Because genetic modifiers contribute in large part to the highly variable phenotypic expression of cardiomyopathy in patients even with identical disease-causing mutations, the identification of modifier genes for this disease will greatly improve risk stratification, prognostic test

development, and personalized therapy. However, only a rather limited number of modifier genes for cardiomyopathy have been identified sporadically.
Objective: To identify genetic modifiers for cardiomyopathy using a novel insertional mutagenesis screening approach in adult zebrafish.
Methods and Results: We screened 476 gene break-transposon (GBT) lines and isolated 44 zebrafish insertional cardiac (ZIC) mutants. Employing doxorubicin (DOX) stress to these ZIC mutants, we identified four candidate GBT lines that modified the progression of DOX-induced cardiomyopathy. Here, we report the detailed study of the GBT0411 mutant that exacerbated DOX-induced cardiomyopathy. GBT0411 mutant was tagged to the dnajb6b gene. Mutations in the short (sarcomeric) isoform of its human homologue gene DNAJB6 was recently reported to cause limb-girdle muscular dystrophy type 1D. Interestingly, our data showed that long (nuclei) isoform (dnajb6b[L]) was the major isoform expressed in the heart, and loss-of-function of which deteriorated the progression of DOX-induced cardiomyopathy. We further found that a cardiomyocyte-specific dnajb6b(L) transgene reverted the deleterious modifying effect of GBT0411 mutant, and exerted a cardioprotective function on chronic anemia induced cardiomyopathy. Mechanistically, Dnajb6b(L) could partially localize to endoplasmic reticulum (ER) upon ER stress, and function as an ER stress suppressor. Indeed, inhibition of ER stress by using a chemical chaperon mimics the cardioprotective effect of dnajb6b(L) transgene.
Conclusions: By conducting an unbiased mutagenesis screening in adult zebrafish, we identified dnajb6b as a novel genetic modifier for cardiomyopathy. A cardioprotective function was identified by overexpressing its long isoform in cardiomyocytes, which might be conveyed by inhibition of ER stress response.

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Differential Contribution Of The N- And C-terminal Domains To The Folding And Function Of Tandem Calponin-homology Domains

Krishna Mallela, Swati Bandi, Surinder Singh, Univ of Colorado Anschutz Medical Campus, Aurora, CO; Geoffrey Armstrong, Univ of Colorado Boulder, Boulder, CO

Tandem calponin-homology (CH) domains constitute a major class of actin-binding domains that include dystrophin and utrophin, the two key proteins involved in muscular dystrophy. Despite their importance, how their structure controls their function is not understood. Here, we study the contribution of individual CH domains to the actin-binding function and thermodynamic stability of utrophin's tandem CH domain. Traditional actin co-sedimentation assays indicate that the isolated C-terminal CH2 domain binds weakly to F-actin when compared with the full-length tandem CH domain. In contrast, isolated CH1 binds to F-actin with a similar efficiency as that of the full-length tandem CH domain. Thus, the obvious question that arises is why tandem CH domains require CH2, when their actin-binding efficiency is originating primarily from CH1. To answer, we probed the thermodynamic stabilities of individual CH domains. Isolated CH1 domain is unstable and is prone to serious aggregation. Isolated CH2 is very stable, even appears to be more stable than the full-length tandem CH domain. In addition, the CH2 domain, which is more stable, is less functional. These results indicate that the main function of CH2 is to stabilize CH1. Consistently, the proposed structure of utrophin's tandem CH domain based on earlier X-ray studies indicates a close proximity between the C-terminal helix of CH2 and the N-terminal helix of CH1, and this helix in CH2 is more dynamic in the full-length protein when compared with that in the absence of CH1, suggesting the mechanism by which CH2 stabilizes CH1. These observations indicate that the two CH domains contribute differentially to the folding and function of tandem CH domains, although both domains essentially have the same native structure in the tandem CH domain. The N-terminal domain determines the function, whereas the C-terminal domain determines the stability.

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Mice With Cardiac-Specific Inactivation Of Ppap2b (Lipid Phosphate Phosphatase 3) Validates Genome-Wide Association Studies.

Manikandan Panchatcharam, Sumitra Miriyala, Natalie D Hendrix, LSU Health Sciences Ctr - Shreveport, Shreveport, LA; Diana Escalante-Alcalde, División de Neurociencias, Insto de Fisiología Celular, México, Mexico

A recent meta-analysis of Genome-Wide Association Studies (GWAS) of coronary artery disease (CAD) involving over 86,000 individuals identified *PPAP2B* (encodes lipid phosphate phosphatase-3; LPP3) as a new loci (SNP rs17114036; P= 3.81 X 10^{-19}) that independently predicts CAD. LPP3 is an integral membrane enzyme that dephosphorylates lysophosphatidic acid, sphingosine-1-phosphate and related bioactive lipids. Strikingly, we found that targeted inactivation of LPP3 in endothelial cells results in early embryonic lethality, in part to due to vascular patterning defects. In addition, we found that constitutive inactivation of LPP3 in the myocardium results in cardiac dysfunction, indicating that dysregulation of LPP3-dependent cardiomyocyte cell function. Heart rate was significantly higher in conditional *Ppap2b*$^{\Delta}$ mice (P<0.001), which may indicate a role for LPP3 in regulating heart rate and/or function. Further, we found that myocardial LPP3 levels are altered following myocardial infarction in mice and these results are in accord with our myocardial infarct data from patient that had down-regulated myocardial LPP3 levels. The major rs17114036A allele was associated with a 1.17 odds ratio for CAD. This SNP is located in the final intron of the six exon *PPAP2B* gene. At least seven SNPs are in robust linkage disequilbrium (r2>0.9) with rs17114036. The sequence surrounding the SNP rs17114036 matches a U1 spliceosome recognition sequence, and the variant base is located at the final position of a sequence with high homology to a U1 spliceosome 5' spice site recognition motif. Binding of the U1 spliceosome may alter mRNA stability or processing, perhaps by masking cryptic splice sites, thus enabling efficient splicing or promoting polyadenylation of the mature message. To address the polymorphism, we transfected MDA MB 453 cells, which are homozygous for the "T' allele of rs17114036 SNP, with a short synthetic 2'-O-methyl phosphorothioate RNA. We found a dramatic decrease in both LPP3 protein expression and LPP3 mRNA levels. These results imply that the region containing the rs17114036 SNP may be important for proper processing and/or stability of the LPP3 transcript thereby functionally validating the GWAS study.

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Interaction Of Osr1 And Tbx5 Is Involved In The Mouse Limb And Heart Development

Linglin Xie, Univ of North Dakota, Grand Forks, ND; Lun Zhou, Huazhong Univ of Science and Technology, Wuhan, China; Patrick Olson, Jacy O'Keefe, Univ of North Dakota, Grand Forks, ND; Qiang Fu, Huazhong Univ of Science and Technology, Wuhan, China

Mutations of TBX5 cause Holt-Oram syndrome (HOS) in human, a disease characterized by upper limb and heart defects. Mouse embryos of *Osr1* knockout caused similar heart defects, while the upper limb defects have never been reported. By genetically marking *Osr1*expressing cells in mice, using *Osr1:CreERT2,* we showed that *Osr1* expression cells contribute to the atrial septum progenitors between E8.0 and E11.0, and to the forelimb after E9.0. The expression of *Osr1* in the forelimb showed a gradient decreasing pattern from the digit 5 to digit 1. Conditional-*Tbx5* haploinsuffiency, using *Osr1:CreERT2*, compound with *Osr1*

haploinsuffiency induced more incidence of atrial septal defects (ASDs) and double outlet right ventricle (DORV). Forty percent of these embryos also had digit defects: the digits are either missing, fused or lack normal identity, which were not observed in mouse embryos of either *Osr1* or *Tbx5* haploinsuffiency. Detailed study of the cardiac progenitors of the compound haploinsufficinecy for Tbx5 and Osr1 showed decreased proliferation in the posterior second heart field, which was associated with lower number of cells transiting from G2 to M phase and less gene expression of *Cdk6* and *CyclinD2*. In summary, our study demonstrated that interaction of *Osr1* and *Tbx5* is involved in the mouse limb and heart development and provides a potential mechanism for HOS.

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The Identification and Hierarchy of Bone Marrow-derived Artery-resident Mesodermal Progenitor Cells and their Dynamics in Atherosclerosis

Hyun-Jai Cho, Hyun-Ju Cho, Yoo-Wook Kwon, Young-Bae Park, Hyo-Soo Kim, Seoul Natl Univ Hosp, Seoul, Korea, Republic of

Background: We recently identified bone marrow (BM)-derived artery resident calcifying progenitor cells. Sca-1+PDGFRα- cells may possess bipotent (osteoblastic/osteoclastic) characteristics. However, the nature of progenitor cells remains elusive. Therefore, we investigated developmental hierarchy of progenitor cells and *in vivo* dynamics in atherosclerosis.
Methods and Results: We harvested cells from BM and artery of C57 mice. In BM, Lin-CD29+Sca-1+PDGFRα- cells showed hematopoietic potential and differentiated into osteoclasts OC). They also possessed mesenchymal stem cell property including osteoblastic (OB) differentiation, suggesting that Sca-1+PDGFRα- cells could be mesodermal progenitor cells. Interestingly, BM-derived artery-resident, clonal Sca-1+PDGFRα- cells maintained bipotency (OB/OC) but lost hematopoietic nature. In contrast, Sca-1+PDGFRα+ cells in BM and artery only showed unipotency (OB). When we overexpressed or knocked down PDGFRα, there was no alteration in OB or OC differentiation of Sca-1+PDGFRα- cells and no effect on OB differentiation of Sca-1+PDGFRα+ cells, indicating PDGFRα as a surface marker but not a functional player. In hyperlipidemic ApoE-KO mice compared with control, Sca-1+PDGFRα- cells were less mobilized from BM to peripheral circulation and less infiltrated into atherosclerotic plaque, whereas Sca-1+PDGFRα+ cells were not significantly affected. Multiplex cytokine assay of serum and artery revealed that IL-1β was significantly increased and IL-5 was markedly decreased in atherosclerotic mice. IL-1β decreased the migration of Sca-1+PDGFRα- cells by 5 folds compared with TNFα, and IL-5 increased the migration as much as TNFα. But the migration of Sca-1+PDGFRα+ cells was not altered. These data indicate that atherosclerosis-related humoral factors mainly regulated mesodermal progenitor cells' dynamics.
Conclusion: We demonstrate that Sca-1+PDGFRα- cell is a mesodermal progenitor cell that possesses both hematopoietic and mesenchymal potentials. In atherogenesis, the mobilization and infiltration of Sca-1+PDGFRα- progenitor cells were regulated by IL-1β and IL-5. These data provide a novel mechanism regarding the role of bipotent progenitor cells in atherosclerosis.

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Temporal Ubiquitin-Proteasomal Degradation of Smad9 Mediated by its Specific E3 Ligase Asb2 is Required for Cardiac Development through the Regulation of Tbx2 Expression

Kyung-Duk Min, Masanori Asakura, Shin Ito, Miki Imazu, Kazuhiro Shindo, Masafumi Kitakaze, Natl Cerebral and Cardiovascular Ctr, Osaka, Japan

Background: The bone morphogenetic protein (BMP) pathway plays crucial roles in cardiac development. Recent studies have reported that mutations in Smad9, one of the regulatory Smad specific for the BMP pathway, might result in

cardiovascular diseases. However, both regulation and function of Smad9 in the cardiovascular system have not been elucidated. Methods and Results: We conducted DNA microarray using P19CL6 cells with forced expression of Smad9. Microarray analysis using Ingenuity Pathway Analysis elucidated that 19 genes including Tbx2 were related to BMP pathway and showed significantly altered expression levels by transient expression of Smad9. We confirmed by qRT-PCR that only Tbx2, but not other Tbx families, were induced by Smad9. Importantly, the expression of Tbx2 was more up-regulated by Smad9 than by Smad1. Moreover, we identified Asb2 as a specific E3 ligase that targets Smad9, but not Smad1/5, for proteasomal degradation. The in situ hybridization using murine embryo revealed that Asb2 is expressed predominantly in the heart during embryonic development, suggesting that Asb2 quantitatively regulates Smad9 in the developing heart. Biochemical analysis demonstrated that Tbx2 expression induced by Smad9 was attenuated by Asb2, which was restored by the treatment with proteasome inhibitor, lactacystin. Developmental studies using both P19CL6 cells and zebrafish showed that the ablation of Asb2 leads accumulation of Smad9 resulting in the up-regulation of Tbx2, which attenuates myocardial development while induces non-myocardial tissue including cardiac cushion. Indeed, alcian blue staining of morpholino-mediated knockdown of zebrafish Asb2 showed significantly dilated ventricle and thinned ventricular wall, accompanied with decreased myocardium and increased cardiac jelly.
Conclusions: Smad9 induces the expression of Tbx2 during cardiac development and is temporally and quantitatively regulated by its specific E3 ligase Asb2. This is the first study to show both the target gene and specific E3 ligase of Smad9.

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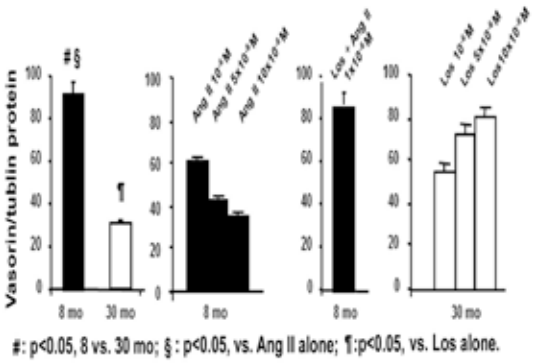
Age-associated Imbalance of Vasorin/TGF-β1 Signaling in VSMC Facilitates Collagen Production

Mingyi Wang, Gianfranco Pintus, Roberta Giordo, Jing Zhang, Liqun Jiang, Robert Monticone, Edward Lakatta, Lab Cardiovascular Science/Natl Inst on Aging, Baltimore, MD

Collagen deposition, a hallmark of arterial aging that resembles post-injury arterial restenosis, is perpetrated by angiotensin II (Ang II) signaling in arterial wall. Collagen aggregation at sites of arterial injury is regulated by the coordinated signaling of pro-fibrotic TGF-β1 and anti-fibrotic vasorin within VSMCs. The Ang II/TGF-β1/vasorin signaling relationship within VSMCs with aging, however, remains unknown. In vivo studies in old vs. young FXBN rats show that aortic transcription and translation of vasorin markedly decrease with aging. In vitro studies in VSMCs isolated from old vs. young aortae. Ang II-associated reduction of vasorin protein abundance in young VSMCs and age-associated changes in vasorin protein levels are reversed by the AT1 antagonist, Losartan (Los) (Figure). Dual immunolabeling and co-immunoprecipitation demonstrate that the co-incidence and physical interaction of vasorin and TGF-β1 within VSMCs are significantly decreased with aging. Importantly, exposure of young VSMCs to Ang II that increases p-SMAD2/3 and collagen type I production, mimicking old cells, and this effect is abolished or substantially mitigated by Los treatment, overexpression of ectopic vasorin, or exogenous recombinant human-vasorin protein. In contrast, exposure of old VSMCs to Los decreases p-SMAD2/3 and collagen type I production.Thus, an imbalance of the Ang II/TGF-β1/vasorin signaling cascade, a feature of the aged arterial wall, enhances the collagen production by VSMCs. Maintaining this signaling balance is a novel measure to retard adverse extracellular matrix remodeling, a determinant of arterial stiffening with aging. (MW and GP co-first authors)



#: p<0.05, 8 vs. 30 mo; §: p<0.05, vs. Ang II alone; ¶:p<0.05, vs. Los alone.

ABSTRACTS

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Over-activation Of Natural Killer Cells Due To Dysfunction Of Cd16-mediated Signaling In Adamts13-deficient Ttp Patients With A History Of Relapse

FANG ZHOU, Darise Farris, Oklahoma Medical Res Fndn, Oklahoma City, OK

Thrombotic thrombocytopenic purpura (TTP) is a rare life-threaten vascular autoimmune disease. There is no effective method to treat it in clinical trials since pathogenesis of TTP has not been fully elucidated. Here we investigate the role of NK cells in relapse development of TTP. Our results showed that the frequencies of $CD3^-CD56^{dim}CD16^-$ and $CD3^-CD56^{bri}CD16^-$ NK cells are increased in TTP patients with a history of relapse. Expression of CD107a, granzyme A and IFN-γ by $CD3^-CD56^{dim}$ NK cells following *in vitro* stimulation with PMA/ionomycin / monensin is improved in the relapse group, compared with those on NK cells derived from TTP patients without relapse development. NK cells isolated from TTP patients with a history of relapse indicated stronger cytotoxicity to target K562 cells than those of NK cells derived from TTP patients without relapse development, suggesting prior activation of NK cells *in vivo*. Treatment with anti-human CD16 antibody up-regulates cytotoxicity of NK cells derived from TTP patients without relapse development. However, Anti-human CD16 antibody treatment does not affect cytotoxicity of NK cells isolated from TTP patients with a history of relapse, suggesting inability of CD16-mediated signaling in NK cells derived from TTP patients with relapse development. These data provide evidence of altered NK cell activation and/or licensing in TTP patients with a history of relapse modulated by CD16-mediated signaling and a new avenue of investigation into mechanisms of TTP immunopathogenesis.

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Therapeutic Silencing of miRNA-652 Restores Cardiac Function and Attenuates Pathological Remodeling in a Mouse Model of Pressure Overload

Bianca C. Bernardo, Sally S. Nguyen, Catherine E. Winbanks, Xiao-Ming Gao, Esther J.H. Boey, Yow Keat Tham, Helen Kiriazis, Baker IDI Heart & Diabetes Inst, Melbourne, Australia; Colleen J. Thomas, La Trobe Univ, Bundoora, Australia; Ruby C.Y. Lin, Univ of New South Wales, Sydney, Australia; Xiao-Jun Du, Julie R. McMullen, Baker IDI Heart & Diabetes Inst, Melbourne, Australia

Introduction: Targeting microRNAs differentially regulated in settings of stress and protection could represent a new approach for the treatment of heart failure. miR-652 expression increased in hearts of a cardiac stress mouse model and was downregulated in a model of cardiac protection. **Aim:** To assess the therapeutic potential of silencing miR-652 in a mouse model with established pathological hypertrophy and cardiac dysfunction due to pressure overload. **Methods:** Mice were subjected to a sham operation (n=10) or transverse aortic constriction (TAC, n=14) for 4 weeks to induce hypertrophy and cardiac dysfunction. Mice were subcutaneously administered a locked nucleic acid (LNA)-antimiR-652 or LNA-control. Cardiac function was assessed by echocardiography before and 8 weeks post treatment, followed by molecular and histological analyses. **Results:** Expression of miR-652 increased in hearts subjected to pressure overload compared to sham operated mice (2.9 fold, n=3-5, $P<0.05$), but was silenced in hearts of mice administered LNA-antimiR-652 (95% decrease, n=3-7, $P<0.05$). In mice subjected to pressure overload, inhibition of miR-652 improved cardiac function (29±1% at 4 weeks post TAC compared to 35±1% post treatment, n=7, $P<0.001$) and attenuated cardiac hypertrophy. Functional and morphologic improvements in hearts of treated mice were associated with reduced cardiac fibrosis, apoptosis, cardiomyocyte size; decreased B-type natriuretic peptide gene expression; and preserved angiogenesis (all $P<0.05$, n=4-7/group). Mechanistically, we identified Jagged1, a Notch1 ligand, as a direct target of miR-652 by luciferase assay. Jagged1 and Notch1 mRNA were upregulated in hearts of

TAC treated mice (1.2-1.7 fold, n=7, P<0.05). Importantly, chronic knockdown of miR-652 was not associated with any notable toxicity in other tissues.
Conclusion: Therapeutic silencing of miR-652 protects the heart against pathological cardiac remodeling and improves heart function via mechanisms that are associated with preserved angiogenesis, decreased fibrosis and upregulation of a miR-652 target, Jagged1. These studies provide the first evidence that targeted inhibition of miR-652 could represent an attractive approach for the treatment of heart failure.

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Aldosterone Induces Cardiac Deleterious Effects Through A Grk2 And Grk5 Dependent Pathway

Alessandro Cannavo, Daniela Liccardo, Sarah M. Bass, Benjamin Woodall, Christopher J. Traynham, Jessica Ibetti, Giuseppe Rengo, Walter J. Koch, Temple Univ, Philadelphia, PA

High Aldosterone (Aldo) plasma levels are present in heart failure patients and are associated with increased cardiac fibrosis and hypertrophy. These effects have been shown to be, in part, related to the transactivation of the angiotensin II receptor (AT1R), a G-protein coupled receptor (GPCR). In this regard, the GPCR kinase 2 (GRK2) and 5 (GRK5) are both critical regulators of cardiac function through GPCR regulation. Since these GRKs have also been shown to play a role in cardiac hypertrophy and HF development we investigated whether these kinases may play a role in myocardial Aldo signaling. To test this, we first treated neonatal rat ventricular cardiomyocytes (NRVMs) with Aldo (1 μM) and looked for GRK regulation. Following 12 hrs of Aldo-stimulation we observed a significant increase in both GRK2 and GRK5 protein levels. We also found that Aldo induced the localization of GRK2 to mitochondria, which the lab has previously found to occur following ischemic injury leading to increased cell death and mitochondrial dysfuntion. Indeed, high Aldo on NRVMs led to a significant increase in ROS generation and cell death, as observed by Mitosox Red and TUNEL staining, respectively. Notably, all these effects were abolished respectively by Spironolactone (Spiro, an Aldo receptor blocker) or Losartan (Los, an AT1R antagonist) pre-treatment. Next, in nuclear fractions, purified from Aldo-treated NRVMs, we observed a consistent increase in GRK5 nuclear localization that consequently induced a significant activation of hypertrophic genes, which is consistent with previous studies showing GRK5 being a key regulator of maladaptive cardiac hypertrophy after pressure-overload. Importantly, Spiro pre-treatment efficiently abolished these effects of GRK5. Finally, we treated wild-type mice in vivo with Aldo for two and four weeks and not only found that this induced significant cardiac hypertrophy and fibrosis compared to saline-treated mice, we also found significant increases in levels of GRK2 and GRK5. Therefore, our study provide, for the first time, data demonstrating that GRK2 and GRK5 are potential regulators of Aldo-mediated cardiac pathology acting either down-stream of mineralocorticoid receptor or transactivated AT1Rs.

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Temporal Regulation Of Cardiac Cytokine Expression in Response to Chronic β-Adrenergic Receptor Stimulation

Laurel A Grisanti, Ashley A Repas, Rhonda L Carter, Temple Univ, Philadelphia, PA; Jennifer A Talarico, Jessica L Gold, Thomas Jefferson Univ, Philadelphia, PA; Walter J Koch, Douglas G Tilley, Temple Univ, Philadelphia, PA

Chronic catecholamine stimulation of β-adrenergic receptors (βAR) is ultimately deleterious during heart failure (HF). While alterations in cytokines contribute to HF pathogenesis and βAR have been demonstrated to regulate cytokines in different models of HF, a comprehensive understanding of this relationship is lacking. Thus, we sought to characterize the impact of chronic βAR signaling on cardiac cytokine expression in vivo. C57BL/6

mice underwent infusion with vehicle or isoproterenol (Iso; 3 mg/kg/day) via minipumps for 1 or 2 weeks and cardiac function was monitored via echocardiography. At study termination, hearts were excised and assessed for changes in hypertrophy, fibrosis and apoptosis, each of which were enhanced by Iso. Expression of cardiac transcripts were assessed via whole transcriptome analysis, where 780 and 689 transcripts were significantly altered at 1 and 2 weeks of Iso, respectively, with only 115 transcripts regulated similarly between the two cohorts. Significant changes in cytokine transcript expression was observed in response to chronic Iso and Ingenuity Pathway Analysis (IPA) predicted the involvement of additional upstream cytokine regulators potentially regulated by Iso. Transcriptome results and IPA predictions were confirmed via qRT-PCR. A cytokine array also confirmed temporally-distinct alterations in the expression of 42 cytokines at the protein level. Differential alterations in cytokine expression resulting from 1 versus 2 weeks of Iso infusion suggest that cytokine-directed therapies may have distinct temporally-dependent consequences on cardiac function and survival under conditions of chronic catecholamine stress.

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Pathogenic role of DNA Single Strand Break Accumulation in Heart Failure

Tomoaki Higo, Osaka Univ, Osaka, Japan; Atsuhiko Naito, Tokyo Univ, Tokyo, Japan; Yasushi Sakata, Osaka Univ, Osaka, Japan; Issei Komuro, Tokyo Univ, Tokyo, Japan

Various environmental stress including reactive oxygen species (ROS) causes nuclear DNA damage. Increased production of ROS is observed in the failing heart and is considered as one of the causes of heart failure. Accumulating evidences suggest the presence of DNA damage in the failing heart, however, mechanistic link that connects DNA damage and heart failure remains elusive. Here, we show that DNA single strand break (SSB) accumulates in the failing heart and that SSB accumulation induces cell-autonomous inflammation through activation of DNA damage response (DDR) signaling pathway. Using alkaline- and neutral comet assay, we found that SSB is increased in the failing heart of pressure overload. Using in vitro model, we found that SSB accumulation activates ataxia talengiectasia mutated (ATM) kinase, which in turn induces nuclear translocation of NF-kB and increases the expression of inflammatory cytokines. Our findings suggest that SSB accumulation in cardiomyocytes plays an important role in the pathogenesis of heart failure by activating DDR pathway and subsequent cell-autonomous inflammation. SSB accumulation is supposed to be characteristic to post-mitotic cells like cardiomyocytes because unrepaired SSB usually develops into DNA double strand break and lead to catastrophic cellular death in mitotic cells. Approaches targeting efficient SSB repair or DDR pathway may become a novel therapeutic strategy against heart failure.

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Opposing Roles Of Distinct Macrophage Lineages In Heart Failure And Cardiac Recovery

Kory J Lavine, Slava Epelman, Keita Uchida, Kassandra J Weber, Joel D Schilling, Colin G Nichols, Gwendalyn J Randolph, Douglas L Mann, Washington Univ Sch of Med, St Louis, MO

Why inflammation is simultaneously deleterious following injury and essential for tissue repair continues to be fundamentally important and debated question. Recently, a new paradigm has emerged in the macrophage field: that organs are replete with resident macrophages of embryonic origin, distinct from monocyte-derived macrophages. This added complexity raises the question of whether distinct immune cells drive inflammatory and reparative activities following injury. Previous work has demonstrated that the neonatal heart has a remarkable capacity for tissue repair compared to the adult, offering an ideal context to examine these concepts. We hypothesized that unrecognized differences in macrophage composition in the neonatal and adult heart represents a key determinant of cardiac recovery. To test this hypothesis, we generated a novel cardiomyocyte ablation model and demonstrated that following injury neonatal mice expand a population of resident cardiac macrophages derived from embryonic lineages, which generate minimal inflammation and are necessary and sufficient for cardiac recovery through promotion of cardiomyocyte proliferation and angiogenesis. During homeostasis the adult heart also contained embryonic-derived macrophages with similar properties. However, following injury these cells disappeared, and instead, the adult heart recruited pro-inflammatory monocytes and monocyte-derived macrophages that lacked reparative activities. Inhibition of monocyte recruitment into the injured adult heart preserved embryonic-derived macrophage subsets, reduced inflammatory cytokine and chemokine production, and enhanced tissue repair. Together, these findings indicate that embryonic-derived macrophages, rather than monocyte-derived macrophages, are key mediators of cardiac recovery. Therapeutics targeting distinct macrophage and monocyte lineages may serve as novel treatments for heart failure.

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Candesartan And Valsartan, Contrary To Irbesartan, Are Potent Biased Antagonists Of Adrenal (beta)arrestin-dependent, Angiotensin II Type 1 Receptor-induced Aldosterone Production And Improve Cardiac Function Post-myocardial Infarction

Anastasios Lymperopoulos, Karlee Walklett, Samalia Dabul, Ashley Siryk, Nova Southeastern Univ, Ft. Lauderdale, FL; Emmanuel Sturchler, Patricia McDonald, Scripps Florida, Jupiter, FL; Giuseppe Rengo, Fondazione “Salvatore Maugeri”, Telese Terme, Italy; Walter J Koch, Temple Univ, Philadelphia, PA

Introduction: The scaffolding protein βarrestin1 (βarr1) by the angiotensin II (AngII) type 1 receptor (AT_1R) mediates AngII-induced aldosterone production in vitro and physiologically in vivo, thereby exacerbating heart failure (HF) progression post-myocardial infarction (MI). Herein, we sought to investigate the relative potency of various AT_1R antagonist drugs (sartans) at inhibiting βarr vs. G protein activation and hence aldosterone production in vitro and in vivo. We also investigated the alterations in plasma aldosterone levels conferred by these agents and their impact on cardiac function of post-MI rats. **Methods:** For the in vitro tests, transfected CHO and adrenocortical H295R cells were used. For in vivo studies, post-MI rats overexpressing βarr1 in their adrenals received 7-day-long treatments with the drugs of interest. **Results:** Among the sartans tested, candesartan and valsartan were the most potent βarr activation and βarr-mediated aldosterone production inhibitors in vitro, as well as the most “biased” antagonists towards βarr vs. G-protein inhibition. Conversely, losartan and irbesartan were the least potent βarr inhibitors and the

least "biased" antagonists towards βarr inhibition. These in vitro findings were corroborated in vivo, since candesartan and valsartan, contrary to irbesartan, caused significant plasma aldosterone reductions in post-MI rats. Accordingly, cardiac ejection fraction (EF) and contractility were significantly augmented in candesartan- and valsartan-treated rats (EF: 41.1±1% and 40±1% respectively, vs. 35±0.3% for saline-treated), but further deteriorated in irbesartan-treated post-MI rats (EF: 32±1%, n=7 rats/group). **Conclusions:** These findings provide important insights that might aid pharmacotherapeutic decisions (i.e. individual agent selections) involving this commonly prescribed cardiovascular drug class (sartans).

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Impaired Aldehyde Dehydrogenase 2 Activity Contributes To Inhibition Of Mitochondrial Respiration In Hyperglycemic Rats

Vishal Mali, Div of Hypertension and Vascular Res, Dept of Internal Med, Henry ford health system, Detroit, MI; Mandar Deshpande, Dept of Neurology, Dept of Internal Med, Henry ford health system, Detroit, MI; Jiang Xu, Div of Hypertension and Vascular Res, Dept of Internal Med, Henry ford health system, Detroit, MI; Shailendra Giri, Dept of Neurology, Dept of Internal Med, Henry ford health system, Detroit, MI; Xiao-Ping Yang, Div of Hypertension and Vascular Res, Dept of Internal Med, Henry ford health system, Detroit, MI; Suresh Palaniyandi, Dept of Internal Med, Henry ford health system, Detroit, MI

Aldehyde dehydrogenase (ALDH) 2 is a mitochondrial isozyme of the heart involved in the metabolism of toxic aldehydes produced from oxidative stress. A decrease in ALDH2 levels and activity was reported in the hearts of experimental hyperglycemia along with an increase in 4-hydroxy-2-nonenal (4HNE). 4HNE is produced in the mitochondria upon lipid peroxidation and known to form adduct with mitochondrial proteins. We hypothesize that reduced ALDH2 activity impairs mitochondrial respiration due to 4HNE adducts formation, ultimately resulting in cardiac damage in hyperglycemia. A single dose (65 mg/kg; i.p.) of streptozotocin in rats resulted in hyperglycemia with a blood glucose level of 443 ± 9 mg/dl versus 121 ± 7 mg/dl in control animals, $p<0.0001$. N=7-11 in both groups. After 6 months of hyperglycemia, heart function was recorded and the rats were sacrificed. Increases in cardiomyocyte cross sectional area (446 ± 32 μm^2 *Vs* 221 ± 10 μm^2; $p<0.0001$) indicated cardiac hypertrophy in diabetic rats. Both diastolic and systolic dysfunctions were recorded with diabetic rats compared to controls. For instance, % fractional shortening was lower in diabetic group versus control; ($p<0.0001$). Most importantly, myocardial ALDH2 activity and levels were reduced and immunostaining for 4HNE protein adducts was increased in diabetic hearts compared to controls. The mitochondrial oxygen consumption rate (OCR), an index of mitochondrial respiration, was decreased in mitochondria isolated from diabetic hearts compared to controls ($p<0.0001$). To check the direct effect of 4HNE and ALDH2 inhibition, we treated H9C2 cardiomyocytes with 4HNE and disufiram, an ALDH inhibitor, respectively, which reduced mitochondrial respiration ($p<0.0001$) and cell viability ($p<0.0001$) along with reduced ALDH2 activity and increased 4HNE adducts formation. We conclude that chronic hyperglycemia-induced 4HNE accumulation due to low ALDH2 activity in the heart leads to defective mitochondrial respiration and cardiac damage and dysfunction.

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Identification Of miRNA-34 Networks In Pathological Cardiac Remodeling

Jenny Y.Y. Ooi, Bianca C. Bernardo, Esther J.H. Boey, Baker IDI Heart & Diabetes Inst, Melbourne, VIC, Australia; Ruby C.Y. Lin, Ramaciotti Ctr for Genomics, Sch of Biotechnology & Biomolecular Sciences, Univ of New South Wales, Sydney, New South Wales, Australia; Julie R. McMullen, Baker IDI Heart & Diabetes Inst, Melbourne, VIC, Australia

Introduction: MicroRNAs (miRNAs) are altered in heart disease and have emerged as promising therapeutic targets. We recently reported that therapeutic inhibition of the miR-34 family (with an 8 mer antimiR) was effective in attenuation of pathological cardiac remodeling. We hypothesized the efficacy of antimiR-34 may be due to its regulation of other miRNAs, producing direct and indirect target effects. Objectives: To identify miRNAs regulated by miR-34 and understand miRNA networks involved in miR-34 inhibition therapy for pathological cardiac hypertrophy. Methods: MiRNA sequencing (Illumina HiSeq 2000) was performed on hearts of mice subjected to transverse aortic constriction (TAC) for 5 weeks, and subcutaneously administered a locked nucleic acid (LNA)-antimiR-34 for 6 weeks (n=3-4). Results: Expression of miR-34 family members (miR-34a, miR-34b, miR-34c) is increased in the hearts of TAC mice compared to Sham controls (1.7, 2.5, 4-fold, $P<0.05$) and attenuated with antimiR-34 treatment (85% decrease [0.85 of 1], $p<0.05$). TAC mice developed pathological cardiac hypertrophy (60% increase [4.8 of 8] in heart weight/tibial length ratio compared to Sham controls, $P<0.05$) and treatment with antimiR-34 significantly attenuated heart size ($P<0.05$). To uncover the underlying miRNA interactions, we carried out miRNA-Seq to identify other miRNAs that might be dysregulated in response to antimiR-34-based therapy. We identified several miRNAs (e.g., miR-3083 and miR-20b) that were downregulated in hearts from TAC vs Sham but not significantly downregulated in the TAC antimiR-34 group. Conversely, two miRNAs (miR-8103, miR-1933) were increased in response to TAC vs Sham but not significantly upregulated in TAC antimiR-34 treated mice. Our data suggests that miR-34 can regulate other miRNA and that these miRNAs can work together to regulate pathways leading to pathological cardiac hypertrophy. Conclusion: The identification of other miRNAs regulated by miR-34 may improve our understanding of miRNA networks for optimization of better therapeutic targets for treatment of complex diseases such as heart failure.

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A Protective Role Of Adenine Nucleotide Translocator 1 In The Inflammatory Response Following Myocardial Infarction

Shi Pan, Nadan Wang, Shey-Shing Sheu, Thomas Jefferson Univ, Philadelphia, PA

Background: Inflammation following myocardial infarction (MI) contributes significantly to the pathogenesis of heart failure. Although the initial stage of inflammatory response is necessary for the repair and healing of myocardium, excessive inflammation post MI causes adverse remodeling leading to heart failure. Recent studies suggest that mitochondrial dysfunction plays an important role in inflammation. However, the molecular target in cardiac mitochondria and the mechanisms of action remain unclear. We hypothesized that mitochondrial ADP/ATP carrier adenine nucleotide translocator isoform 1 (ANT1) is protective in post MI inflammation. Methods and Results: MI was induced by permanent occlusion of left anterior descending artery in C57BL/6 mice. In the inflamed heart following MI, ANT1 protein levels were significantly decreased in the left ventricle (0.48±8.94%), indicating ANT1 down-regulation occurs in myocardial inflammation following MI. To delineate the mechanisms involved, we performed siRNA knockdown of ANT1 in myocardium-derived H9c2 cells and cardiomyocytes, and examined tumor necrosis factor alpha (TNFα)-induced pro-inflammatory response. Knocking down ANT1 significantly increased the expression of TNFα (~10-fold) and interleukin-6 (IL-6) (~3.6-fold) concomitant with increased reporter gene activity of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) (~61±1.22%) in response to TNFα. These effects were not due to mitochondria loss because no changes were detected for voltage-dependent anion channel, or cyclophilin D when ANT1 is knocked down. Confocal microscopy analysis indicated that ANT1 knockdown increased TNFα-induced donut/blob-shaped mitochondria (~4.9-fold). Interestingly, exogenous TNFα and lipopolysaccharide (LPS) in turn significantly decreased ANT1 protein levels (~37±13%; ~37±6%, respectively) suggesting a feed forward regulation of pro-inflammatory cytokine expression activated by ANT1 down-regulation. Conclusion: These data suggest that cardiac

inflammation is dependent on, in part, the function of ANT1. Preventing ANT1 down-regulation can be a novel molecular target to attenuate post MI inflammation and heart failure.

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The Role of Mononuclear Phagocytes in Pressure Overload Heart Failure

Bindiya Patel, Mohamed Ismahil, Mehak Goel, Tariq Hamid, Shyam Bansal, Sumanth D. Prabhu, Univ of Alabama at Birmingham, Birmingham, AL

We previously reported that chronic ischemic HF induced by coronary ligation results in an exacerbated pro-inflammatory cell profile in the systemic circulation, spleen, and failing heart. In addition, this pro-inflammatory cell profile was strongly influenced by the cardiosplenic axis; however, the role of this axis in other etiologies of HF is unknown. Immune cells infiltrate the heart early (1-4 weeks) after pressure-overload injury, but it is unknown whether these cells persist and are of pathological import during chronic pressure-overload HF. We hypothesized that phenotypic changes in mononuclear phagocytes are necessary for the transition from compensatory hypertrophy to chronic HF during sustained pressure-overload, and that inhibition of myocardial immune cell infiltration would ameliorate this progression. Pressure-overload was induced by transverse aortic constriction (TAC) in wild-type (WT) C57BL/6 mice, and in macrophage Fas-induced apoptosis (MAFIA) Tg mice, which allows ablation of c-fms expressing cells upon administration of the AP20187 dimerizer. Compared to sham mice, TAC mice developed progressive cardiac dysfunction beginning at 2 w with significantly ($p<0.05$) reduced LVEF (59 ± 10 vs 67 ± 3 %), increased left atrial size (2.29 ± 0.1 vs 2.04 ± 0.1 mm), and increased E/E' Doppler ratio (31.8 ± 6 vs 24.0 ± 3). Circulating CD11b+Lineage- monocytes were elevated in TAC mice at 2 w (1.7 ± 0.2 vs 1.0 ± 0.1 %, $p<0.05$); however, these levels normalized by 8 w. Moreover, at 8 w, no significant differences in the levels of splenic and cardiac mononuclear phagocytes were seen between TAC and sham mice. Systemic ablation of mononuclear phagocytes beginning 2 w after TAC in MAFIA AP20187-treated mice did not rescue cardiac function or delay HF progression compared to TAC MAFIA vehicle-treated mice over the course of 16 w (LVEF 43 ± 18 vs 45 ± 18 %). We conclude that in contrast to chronic ischemic HF, inflammatory cell-mediated cardiac injury does not have primacy in chronic non-ischemic HF induced by pressure-overload. These findings highlight the importance of the index injury in regard to immune cell activation in HF, and suggest that therapeutic immunomodulatory approaches may not be equivalent across the various etiologies of this disease.

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miR-28 Induces Oxidative Stress via Nrf2 in Right Ventricular Failure but not in Left Ventricular Failure

Sushma Reddy, Lucile Packard Children's Hosp, Los Altos, CA; Dong-Qing Hu, Mingming Zhao, Lucile Packard Children's Hosp, Palo Alto, CA; Eddie Blay, Duke Univ, Durham, NC; Giovanni Fajardo, Daniel Bernstein, Lucile Packard Children's Hosp, Palo Alto, CA

MicroRNAs (miRs) are crucial regulators of cardiac remodeling in left ventricular hypertrophy and failure (LVH/LVF). However, there is minimal data on their role in right ventricular hypertrophy and failure (RVH/RVF), a risk for patients with congenital heart disease or pulmonary hypertension. Utilizing a murine model of RVH/RVF, we have described RV-specific overexpression of miR-28, not found in LVH/LVF. We used this model to evaluate miR-28 regulation of its downstream target Nrf2, a master regulator of antioxidant defenses, and a potential mechanism of the enhanced susceptibility of the RV to fail under pressure overload.
Methods: miR-28 and Nrf2 gene and protein expression and ROS production and antioxidant defenses were assessed at 10d in RVH/RVF (pulmonary artery banding) and LVH/LVF (aortic banding). miR-28 was overexpressed in HEK293 cells and Nrf2 and ROS production assessed. Plasma miRs were also profiled. Results: Mice developed RVH by d4, at which time miR-28 was not increased vs. sham, and

RVF by d10, when miR-28 was increased 2-fold. This was accompanied by decreases in Nrf2 gene (2-fold) and protein (0.4±0.2 vs. 0.8±0.1, $p<0.05$) expression, Nrf2-regulated SOD expression (2-fold), and SOD activity (80±15% vs. 90±18%, $p<0.05$). ROS production (4HNE) was increased (1.5±0.1 vs. 1.0±0.1, $p<0.05$). In contrast, at the same stage of LVH/LVF, miR-28 is not increased, Nrf2 expression is increased (0.45±0.2 vs. 0.1±0.02, $p<0.05$) and SOD is unchanged. Lentiviral miR-28 overexpression in HEK293 cells showed downregulation of Nrf2, SOD and heme oxygenase expression (1.6-2.1 fold) with a 35% increase in ROS production ($p<0.05$). Finally, plasma miR-28 decreased with the progression from RVH to RVF in mice, and this was confirmed in children undergoing pulmonary valve replacement for RV failure (patients vs controls, n=4/group). Conclusions: Our data show that RV-specific miR-28 enhances RVH/RVF through suppression of Nrf2 signaling and increased oxidative stress. Although we did not find Nrf2 downregulation at the same stage of LVH/LVF, others have shown this at 4-6 wks, suggesting this process may occur earlier in RVF vs. LVF. Finally, miR-28 plasma expression may be a biomarker for early RVF in patients.

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The Coxsackie Virus B3 modulates Cardiac Ion Channels

Guiscard Seebohm, Univ Clinic Münster, Münster, Germany; Katja Steinke, Ulrike Henrion, Ruhr Univ Bochum, Bochum, Germany; Nicole Ettischer, Univ Hosp of Tuebingen, Tübingen, Germany; Frank Sachse, Univ of Utah, Salt Lake City, UT; Matthias Rohbeck, Univ Clinic Münster, Münster, Germany; Karin Klingel, Univ Hosp of Tuebingen, Tübingen, Germany; Nathalie Strutz-Seebohm, Univ Clinic Münster, Münster, Germany

Infections with coxsackieviruses of type B (CVB) induce severe forms of acute and chronic myocarditis that are often accompanied by ventricular arrhythmias. The mechanisms underlying the development of virus-induced, life-threatening arrhythmia, remain largely elusive. Here, we show time-dependent CVB3-induced modulation of the cardiac ion channels Kv7.1, hERG1 and CaV1.2 in vitro. Channel protein localizations within cells and plasma membrane abundance are altered in infected mouse cardiac cells. In silico analyses of infected human myocytes suggest increased risk of arrhythmogenesis. These modifications are attenuated by the common Asian polymorphism KCNQ1-P448R, a genetic determinant preventing coxsackievirus-induced effects in vitro. This study provides a previously unknown explanation for the development of arrhythmias in enteroviral myocarditis, which will help to develop therapeutic strategies for arrhythmia treatment.

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Potential Genetic and Pharmacological Protection from Virus-induced Ventricular Arrhythmias and Sudden Cardiac Death

Nathalie Strutz-eebohm, Univ Clinic Münster, Münster, Germany; Katja Steinke, Ruhr Univ Bochum, Bochum, Germany; Ulrike Henrion, Ruhr Univ Bochum, Münster, Germany; Matthias Rohbeck, Univ Clinic Münster, Münster, Germany; Karin Klingel, Univ Hosp of Tuebingen, Tübingen, Germany; Guiscard Seebohm, Univ Clinic Münster, Münster, Germany

In patients as well as in mouse models, enteroviral infections, especially Coxsackie group B viruses (CVB1-6), frequently induce ventricular arrhythmias and sudden cardiac death. The cardiac action potential requires proper function of cardiac ion channels. CVB3 alters Kv7.1 channel trafficking potentially leading to changes in action potentials and increasing likelihood of arrhythmias. Genetic variants of cardiac ion channels can cause changes in channel trafficking that may preserve from CVB3 modulations and present an evolutionary advantage. Here, we show that a common polymorphic Kv7.1 channel variant uses alternative trafficking pathways and may

thus exert a benefit during CVB3 infections. Genetic and pharmacological disruption of a CVB3-stimulated Serum- and Glucocorticoid inducible Kinase 1 (SGK1) pathways blunts Kv7.1 channel dysfunctions. Our results suggest that escape from CVB3-induced SGK1-stimulation by genetic variation in Kv7.1 may be protective and inhibition of SGK1 may present a pharmacological approach to reduce the pro-arrhythmic risk associated with acute coxsackievirus infections.

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Regulation of Cardiac Hypertrophy and Dilated Cardiomyopathy by CIP

Zhan-Peng Huang, Masaharu Kataoka, Jinghai Chen, Da-Zhi Wang, Boston Children's Hosp, Boston, MA

Cardiac hypertrophy is one of the primary responses of the heart to pathophysiological stress. However, the mechanism of the transition from compensative hypertrophic growth to cardiac dilation is poor understood. Recently, we identified a cardiac-specific expressed gene CIP. The expression of CIP is unchanged in hypertrophic heart but significantly down-regulated in dilated hearts, suggesting CIP may play an important role in the transition from cardiac hypertrophy to dilated cardiomyopathy. We generated CIP knockout mice and found that CIP is dispensable for cardiac development. Interestingly, CIP-null mutant mice developed severe cardiac dilation 4 weeks after TAC (transverse aortic constriction) surgery, while control mice were still at the stage of compensative hypertrophic growth. Echocardiography and histological examinations showed that mutant hearts had enlarged chamber with thinner ventricle wall and decreased cardiac performance compared to controls. The expression of marker genes of cardiac disease, BNP and Myh7, was elevated. Consistently, deletion of CIP in Myh6-CnA transgenic mice result in premature death, displaying severe left ventricle dilation. Conversely, cardiac-specific CIP overexpression inhibited pressure overload-induced cardiac hypertrophy. CIP transgenic mice exhibit decreased ventricle weight/body weight ratio, decreased cardiomyocyte cross-section area and repressed expression of hypertrophic related marker genes. CIP overexpression also protected the heart from developing cardiac dilation and preserved the cardiac function after prolonged pressure overload. We performed unbiased microarray assay to document the transcriptome in CIP knockout and control mice which were subjected to pressure overload (TAC). The analysis of Gene Ontology term indicated the Negative Regulation of Apoptosis was down-regulated while the Collagen/Extracellular Structure Organization was up-regulated in CIP-null hearts under TAC condition.
In summary, our studies established CIP as a key regulator of the transition from cardiac hypertrophy to dilated cardiomyopathy. The protective effect of CIP in cardiac remodeling indicates that CIP could become a therapeutic target for cardiac diseases.

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Unique Cardiac Fibroblast Expression Profiles in Pathological and Physiological Hypertrophy

Janet K Lighthouse, Lissette S Velasquez, Eric M Small, Univ of Rochester, Rochester, NY

The heart undergoes hypertrophic growth in response to both physiological and pathological stimuli. Pathological hypertrophy results from various humoral, mechanical, or ischemic insults, and often leads to cardiac fibrosis, diminished contractility, and heart failure. In contrast, physiological hypertrophy is an adaptive response to the excessive demands of exercise or pregnancy and does not lead to fibrosis. We hypothesized that cardiac fibroblasts, the main cellular source of extracellular matrix in the heart, exhibit distinct expression profiles in physiological or pathological remodeling that influence the

divergent fibrotic response. To investigate these differences, we obtained the expression profile of cardiac fibroblasts isolated from mice subjected to swim training, pressure-overload induced cardiac remodeling, or myocardial infarction by RNA-sequencing. Although we observed cardiac growth in all conditions, pressure-overload induced hypertrophy and myocardial infarction induced the predicted fibrotic gene expression signature, which was absent in physiological hypertrophy. Utilizing these validated datasets, we identified novel genes and molecular pathways that are differentially expressed in physiological and pathological hypertrophic remodeling and will correlate transcriptional programs with altered gene profiles. Fibroblast gene expression profiles in pathological and physiological are expected to lead to diagnostic or prognostic markers of fibrotic remodeling as well as genes that may serve as novel therapeutic strategies to prevent or reverse cardiac fibrosis.

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67

GSK-3 Dephosphorylation by PP2A Modulates Age-dependent Cardiac Growth and Hypertrophic Response

Maradumane L Mohan, Elizabeth E Martelli, Sathyamangla V Naga Prasad, Cleveland Clinic, Cleveland, OH

Glycogen synthase kinase-3 (GSK-3) is a known negative regulator of cardiac hypertrophic response and much remains unknown about its dephosphorylation process that activates GSK-3. Since GSK-3 function is regulated by PI3K, age based assessment of cardiac hypertrophic response was measured in PI3Kγ knockout (PI3Kγ KO) mice. Interestingly, we observed marked reduction in heart size in PI3Kγ KO mice compared to littermate controls. Consistent with the reduced size, we observed elevated PP2A activity and dephosphorylation mediated activation of GSK-3 in PI3Kγ-KO hearts. Mechanistically, we found that higher PP2A activity in PI3Kγ-KO was due to PP2A methylation mediated by elevated PP2A methyl transferase (PPMT-1) activity. To test in vivo whether PI3K activity regulates GSK-3 dephosphorylation mediated cardiac growth, we generated mice with cardiac specific overexpression of inactive PI3Kγ (PI3Kγinact) and constitutively active PI3Kγ (myrPI3Kγ) in the PI3Kγ-KO background. Age based hypertrophic response and cardiac function was assessed by heart weight/body weight ratios and echocardiography. The heart size was significantly increased in PI3Kγinact (LVEDD- 3.41+/- 0.12) and myrPI3Kγ (LVEDD- 3.73 +/- 0.14) overexpressing mice, when compared to WT (LVEDD- 3.00 +/- 0.15) mice at 18 months age, indicating that kinase activity of PI3Kγ is inconsequential for age-dependent regulation of cardiac growth. Similar cardiac growth due to overexpression of active and inactive form of PI3Kγ suggests that the kinase independent function potentially overrides the kinase activity of PI3Kγ. Correspondingly, PP2A activity was normalized and was associated with decreased GSK-3 dephosphorylation with overexpression of PI3Kγinact. This led to normalization of heart size in these mice compared to PI3Kγ-KO littermates correlating with the decreased GSK-3 dephosphorylation and consequent inhibition of GSK-3 activity. Regulation of PP2A mediated dephosphorylation of GSK-3 by PI3Kγ combined with similar level of increased cardiac growth in PI3Kγ-KO mice with cardiac overexpression of PI3Kγinact or myrPI3Kγ, suggests a non-canonical role of PI3Kγ in hypertrophy of the heart due to age related increase in mechanical demand.

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Caveolae-specific Phosphorylation of L-type Calcium Channel β2a Subunit exaggerates Cardiac Hypertrophic Responses after α1 Adrenergic Stimulation in Mice

Hiroyuki Nakayama, Shohei Kumagai, Sachi Matsunami, Nao Hayamizu, Kota Tonegawa, Wataru Otsuka, Yasushi Fujio, Osaka Univ, Osaka, Japan

Rationale: Ca2+ influx via L-type Ca2+ channels (LTCCs) plays a pivotal role in excitation-contraction coupling and cardiac hypertrophy. Phosphorylation of LTCC β2a subunit (β2a) by CaMKII enhances channel activity. LTCCs are

localized in both T-tubules and caveolae, but the functional role of phosphorylation of β2a in caveolae remains unelucidated. Objective: To develop a novel tool to analyze caveolae specific activation of CaMKII and to determine the functional roles of caveolae-specific CaMKII signaling in LTCC-related cardiac hypertrophy. Methods and Results: To evaluate caveolae-specific activation of CaMKII, we generated a fusion protein composed of the cytosolic domain of phospholamban (PLN) as a phosphopeptide tag and caveolin3 (cPLN-Cav3). Activation of CaMKII was assessed by phospho-specific antibody for PLN (Thr17). To inhibit caveolae-specific activation, we generated a GFP fusion protein with caveolae-targeting sequences fused to CaMKII inhibitory peptide (CTS-GFP-AIP). In neonatal rat cardiomyocytes (NRCM), adenoviral expression revealed that CTS-GFP-AIP co-localizes with caveolin3 and mediates caveolae specific inhibition of CaMKII, thus validating this novel method. CTS-GFP-AIP inhibited CaMKII phosphorylation of β2a in NRCM, thus suggesting that phosphorylation of β2a occurs exclusively in caveolae. Phenylephrine stimulation mediates CaMKII activation in caveolae, which leads to CaMKII-specific phosphorylation of β2a in vitro and in vivo. Finally, we generated non-phospho mutant β2a -overexpressing mice and assessed hypertrophic responses in both wild-type and mutant β2a transgenic animals (TG). Protein expression by transgenes in mutant TG was similar to those previously reported in wild-type β2a overexpressing mice. Wild-type β2a TG showed exaggerated cardiac hypertrophic responses at two weeks after phenylephrine stimulation when compared to controls (4.8 ± 0.2 vs. 5.5 ± 0.3 for heart weight to body weight ratio; $p<0.05$), which was completely abolished in mutant TG. Conclusions: We developed a novel method to analyze caveolae-specific activation of CaMKII and confirmed that caveolae-specific phosphorylation of β2a exaggerates cardiac hypertrophy caused by phenylephrine stimulation.

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69

Angiotensin II- Canonical TGF-β Signaling Downregulates Apelinergic Pathway in Hypertension

Rahul Rai, Varun Nagpal, Amanda E Boe, Northwestern Univ, Chicago, IL; Douglas E Vaughan, Dept of Internal Med, Northwestern Univ, Chicago, IL

Background: Apelin is a novel peptide which along with its receptor, APJ, mediates apelinergic signaling. Apelinergic signaling plays a critical role in cardiovascular homeostasis, including regulation of blood pressure. While exogenous apelin blocks Angiotensin II (AngII) mediated nuclear signaling, the role and regulation of endogenous apelin in hypertension (HTN) remains obscure. We hypothesize that apelinergic pathway is downregulated in HTN, which is primarily mediated by aberrant AngII signaling.
Approach: To test our hypothesis we utilized two mouse models of HTN, including AngII infusion and oral administration of N (ω)-nitro-l-arginine methyl ester (L-NAME). Blood pressure was monitored via noninvasive tail-cuff device. To determine the signaling involved we investigated the effect of AngII on apelinergic pathway in vitro.
Results: Cardiac apelin was decreased significantly in both murine models of HTN. Downregulated apelin also corresponded with increased deposition of collagen, and up-regulation of senescence markers including PAI-1. Meanwhile, APJ levels were unaffected in both these hypertensive models. In our in vitro studies AngII downregulated apelin expression in human aortic endothelial cells (HAECs) and human cardiac fibroblasts (HCFs). Furthermore, our studies in AngII infused mice and in HCFs highlight the role of TGF-β-pSMAD signaling, independent of MEK involvement, in AngII induced apelin downregulation. Conclusion and Significance: Our studies demonstrate that aberrant AngII signaling downregulates apelin in HTN. This downregulation involves canonical Tgf-β1 signaling and affects apelin transcription. Importantly, we propose that AngII mediates its hypertensive pathology by decreasing apelinergic regulation. Since exogenous apelin blocks AngII signaling, further knowledge and negation of AngII induced apelin downregulation

ABSTRACTS

could result in the development of novel anti-hypertensive therapies.

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

Sarah M Schumacher, Erhe Gao, Jess I Gold, Maya Cohen, Kurt Chuprun, Walter J Koch, Temple Univ, Philadelphia, PA

During heart failure (HF), cardiac levels and activity of the G protein-coupled receptor (GPCR) kinase (GRK) GRK2 are elevated, increasing phosphorylation, desensitization and down-regulation of β-adrenergic receptors (βARs) and other cardiac GPCRs. Increased GRK2 participates in adverse remodeling and contractile dysfunction during HF, while inhibition via a carboxy-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 novel GRK2 interacting partners are important for adaptive and maladaptive myocyte growth including Gq, the signaling trigger for maladaptive cardiac hypertrophy, leading to HF. Importantly, GRK2 contains a putative amino-terminal Regulator of G protein Signaling (RGS) domain (βARK-RGS). This domain directly interacts with Gq and appears to inhibit signaling without altering Gq enzymatic activity. Therefore, this domain may alter hypertrophic responses in the heart and represent a novel role for GRK2 and a potential therapeutic target to limit maladaptive cardiac hypertrophy. We have begun to address this by generation of novel transgenic (Tg) mice with cardiac-specific expression of the RGS domain of GRK2. Using a trans-aortic constriction (TAC) model of pressure overload hypertrophy, we found that expression of βARK-RGS demonstrates anti-hypertrophic effects. Echocardiographic analysis post-TAC revealed reduced left ventricular posterior wall thickness (LVPW) in βARK-RGS compared to non-transgenic littermate controls (NLC) (0.85 vs 1.0 mm LVPWd at 4 weeks). RT-PCR analysis found decreased hypertrophic factor transcripts, such as ANF for which the nearly 18-fold increase post TAC was completely inhibited in βARK-RGS mice. Further, the progression to HF was inhibited in βARK-RGS mice, but not NLCs, 14 weeks post-TAC. While mechanistic characterization is underway, these data support our hypothesis that the RGS domain of GRK2 may serve as a non-canonical inhibitor of Gq-mediated hypertrophic signaling in the heart and highlight how this research may pave the way for novel GRK2-based therapeutic approaches to prevent hypertrophy and HF.

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A Dual PI3K-mTOR Inhibitor Induced Hyperglycemia and Increased Insulin Signals in the Heart

Xinhua Yan, Jillian Onufrak, Yongyao Yang, Genesys Res Inst, Boston, MA; Juyong Lee, Calhoun Cardiology Ctr, Univ of Connecticut Health Ctr, Farmington, CT; John Fuseler, Univ of South Carolina Sch of Med, Columbia, SC; Maoyun Sun, Sharath Sasi, David Goukassian, Lynn Hlatky, Genesys Res Inst, Boston, MA; Robert L. Price, Univ of South Carolina Sch of Med, Columbia, SC; Joseph Carrozza, St. Elizabeth's Medical Ctr, Boston, MA; Thomas K. Borg, Univ of South Carolina Sch of Med, Columbia, SC; James P. Morgan, St. Elizabeth's Medical Ctr, Boston, MA

We previously reported that a dual PI3K-mTOR inhibitor BEZ235 (BEZ) induced cardiac hypertrophy. Here, we investigated potential mechanisms. Methods: three month old FVB/n female mice were treated with BEZ for five weeks. Cardiac function was monitored by serial echocardiography during the treatment and hemodynamic measurements at the end of the study. Cell signaling was analyzed by RT-PCR, Western blotting and ELISA. Results: BEZ induced a dose-dependent increase of left ventricular (LV) wall thickness and systolic function. These were associated with increased hypertrophic markers ANP, BNP, β-MHC and α-skeletal actin in the heart. In addition, in chronic BEZ-treated mouse hearts, the activations of PI3Ks, mTOR and ERK were increased. We conducted further studies to understand these contradictory results. We found that BEZ induced an increase of hepatic gluconeogenesis gene expression which was associated with

increased fasting glucose, increased serum insulin level, a worsened glucose and pyruvate tolerance and increased IGFR/Insulin receptor activation in the heart. Injections of insulin lowered blood glucose, improved glucose and pyruvate tolerance, but further aggravated BEZ-induced cardiac dysfunction. On the other hand, OSI-906 (an IGFR/IR inhibitor) normalized cardiac function in BEZ-treated mice. Conclusions: Chronic BEZ treatment induced cardiac hypertrophy may be caused by increased insulin receptor activation in the heart.

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Pka Is A Master Regulator Of Pathological Cardiac Hypertrophy

Xiaoying Zhang, Temple Univ, Philadelphia, PA; Ying LI, The Third Military Medical Univ & Temple Univ, Chongqing, China & Philadelphia, USA, PA; Mingxin Tang, Xiaojie Ai, Christopher Szeto, Xiang Hua, Xiongwen Chen, Temple Univ, Philadelphia, PA

Aims: The role of PKA in pathological cardiac hypertrophy (PCH) is not clear. The literature suggests both prohypertrophic and antihypertrophic effects of PKA. Furthermore, there are endogenous PKA inhibitors, PKI, highly expressed in the heart to regulate PKA activity but their roles in PCH have not been studied. We aim to explore the role of PKI/PKA in PCH induced by isoproterenol, phenylephrine, angiotensin II and pressure overload. Methods and results: 1. PKIα and PKIγ were highly expressed in the heart but only PKIα was reduced by transaortic banding (TAB); TAB induced a significant increase in cardiac PKA activity at 1 week post TAB. 2. Four transgenic mouse lines with high (HE), medium (ME), low (LE) and very low (VLE) expression of PKI-GFP were obtained with the inhibition of maximum PKA activity induced by 1μM cAMP by 95%, 57%, 20% and 10% in the cardiac homogenates; 3. In the VLE hearts, some myocytes were PKI-GFP+ and some were PKI-GFP-, GFP- LVMs had significantly larger surface area than GFP+ LVMs; 4. PKA inhibition by PKI-GFP abolished PCH induced by isoproterenol, phenylephrine, angiotensin II in HE mice; 5. TAB for 8 weeks did not change HW/BW, myocyte cross-sectional area and myocardial fibrosis in HE mice but induced significant increases in HW/BW, myocyte cross-sectional area, myocardial fibrosis and depressed cardiac fractional shortening in control mice. 6. In cultured neonatal rat ventricular myocytes, PKI-GFP prevented myocyte hypertrophy induced by isoproterenol (ISO), phenylephrine (PE) and angiotensin II, as evidenced by no significant increases in protein synthesis (protein/DNA ratio), myocyte surface area, sarcomere organization. 7. PKI-GFP in NRVMs prevented the translocation of NFAT3 and HDAC5 induced by ISO and PE and increased the secretion of antihypertrophic ANF at baseline; 8. TAB induced PKA-dependent phosphorylation of GSK-3α and GSK-3β, inactivating them to relieve their antihypertrophic effect and promote protein synthesis (increased phosphorylation of mTORC1, eIF-4EBP1, p70 S6K); PKA inhibition abolished these effects. Conclusions: PKA is regulated by PKI and is a master regulator of PCH induced by pressure overload.

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A Novel Role of E2F1 in Stress-induced Cardiac Fibrosis

Dauren Biyashev, Chan Boriboun, Gangjian Qin, Northwestern Univ, Chicago, IL

Rationale: E2F1 transcription factor is best known as a cell cycle regulator. Recent reports indicate the importance of E2F1 in cardiovascular system, though its exact role is not clear. TGF-beta/Smad2,3 signaling pathway, on the other hand, has been long implicated in the regulation of cardiovascular health and numerous disease, including cardiac fibrosis.

Interaction between these two major pathways has been reported in the cancer settings. Objective: To identify the possible interactions between E2F1 and TGF-beta/Smad2,3 signaling pathways in cardiovascular system and determine the functional outcome of these interactions in cardiac health. Methods and Results: E2F1-/- mice developed significantly higher degree of cardiac fibrosis than wild type mice in the Angiotensin II - induced cardiac fibrosis model. The levels of phosphorylated Smad2 and Smad3 were significantly higher in the hearts of E2F1-/- mice, as well as in mouse embryonic fibroblasts derived from E2F1-/- animals. Associated expression of collagen I was significantly increased in mouse embryonic fibroblasts derived from E2F1-/- animals, and treatment with TGF-beta resulted in higher collagen deposition compared to wild type fibroblasts. Treating animals with SB 431542, chemical inhibitor of Smad2,3 signaling, obliterated the difference in the degree of cardiac fibrosis between wild type and E2F1 knockout animals in the Ang II model. We discovered that levels of syndecan-4, heparan sulfate proteoglycan transmembrane protein implicated in fibrosis and known to interact with TGF-beta are significantly increased in both E2F1-/- fibroblasts and hearts. siRNA-mediated knockdown of syndecan-4 using siRNA resulted in decreased Smad2,3 phosphorylation in E2F1-/- MEFs. Similarly, down regulation of syndecan-4 in-vivo using morpholino lead to decreased cardiac fibrosis in E2F1-/- mice in Ang II model. Conclusions: E2F1 suppresses activation of TGF-beta/Smad 2,3 pathway. The E2F1-dependent suppression of cardiac fibrosis through TGF-beta/Smad 2,3 pathway is at least partially regulated by syndecan-4.

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The Inflammatory Phenotype Of Mesenchymal Fibroblasts And Its Role In Aging Dependent Cardiac Fibrosis- A Target For Statins?

Katarzyna A Cieslik, JoAnn Trial, Mark L Entman, Baylor Coll of Med, Houston, TX

In the aging mouse (C57BL/6) myocardium fibrosis steadily increases after 14 months of age and is accompanied by elevated numbers of myeloid derived fibroblasts. Recently, we proposed a mechanism by which inflammatory mesenchymal fibroblasts (IMF) derived from mesenchymal stem cells secrete monocyte chemoattractant protein-1 (MCP-1) necessary for myeloid fibroblast induction in the aging heart. The current study extends the characterization of this inflammatory phenotype by describing elevated interleukin-6 (IL-6) secretion and increased expression of IL-6 receptor (IL-6R) in IMF. Since IL-6R lacks an intracellular domain it requires a co-receptor gp130 (generally expressed) to induce an intracellular signal. Thus, generation of an IL-6R soluble receptor allows IL-6 signaling on cells that do not express IL-6R (or expression is low), such as endothelial cells. We investigate the function of IL-6 and IL-6R in the promotion of transendothelial migration of monocytes through cardiac endothelium and their maturation into myeloid fibroblasts in in vitro assay. Treatments with IL-6 and more extensively IL-6+IL-6R resulted in a 3-5 fold increase (above the control level) in myeloid cell migration and maturation into myeloid fibroblasts. Thus IMF can contribute both IL-6 and IL-6R to endothelial cells and facilitate myeloid cell transendothelial migration. In agreement with these data, analysis of the aged mouse heart revealed the presence of fibroblasts expressing IL-6 (procollagen type $I^+IL\text{-}6^+$ cells), M1 macrophages ($CD86^+$ cells) and M2 macrophages ($CD301^+$procollagen type I^+ cells) that were absent in hearts from young mice. The mechanisms by which expression of these factors is upregulated in IMF are being investigated; our data suggest that MCP-1 and IL-6 expression are controlled by the farnesyltransferase (FTase)-Ras-Erk1/2 pathway. Interestingly, since atorvastatin interferes with farnesyl synthesis it also reduced MCP-1 and IL-6 expression in IMF. These data may introduce a new use of this class of drugs in the prevention of the age-related fibrosis.

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ABSTRACTS

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TNF Receptor 1 Signaling: a Mechanistic Link between Cardiac Inflammation and Fibrosis

Clemens Duerrschmid, Yanlin Wang, Mark L Entman, Sandra B Haudek, Baylor Coll of Med, Houston, TX

Background: We previously showed in several models of cardiac hypertrophy and failure that, in the absence of cell death, cardiac interstitial fibrosis was mediated by the uptake of monocyte-derived fibroblast precursor cells; fibrosis and concurrent cardiac remodeling were blocked by deletion of monocyte chemoattractant protein-1 (MCP-1), as well as of tumor necrosis factor receptor-1 (TNFR1). We now investigated the cellular origin, kinetics of uptake, and subpopulation of fibroblast precursors in the angiotensin-II (Ang-II)-challenged heart.

Methods: Mice with genetic deletion of TNFR1 (TNFR1-KO mice) were irradiated and rescued with bone marrow from wild-type (WT) mice, and were subjected to continuous infusion of Ang-II for 7 days. Flow cytometry was performed on isolated cells, immunostaining on perfusion-fixed tissue, quantitative PCR on whole heart mRNA isolations.
Results: In WT mice, Ang-II induced the early uptake of monocytes which became M1 macrophages (CD86+CD45+) in an M1/Th1 environment. Monocytes entering the heart after 2-3 days polarized to M2 macrophages (CD301+CD45+) in an M2/Th2 milieu with concurrent appearance of collagen-producing CD301+ fibroblasts. M1 cells produced TNF, whereas M2 cells did not; both expressed TNFR1. Ang-II-exposed TNFR1-KO hearts showed similar cardiac infiltration of M1 cells, but the amount of M2 cells was significantly lower. They also had reduced expression of M1 and M2 cytokines, but not of Th1 and Th2 interleukins. Transplantation of WT bone marrow to TNFR1-KO mice before Ang-II exposure restored cardiac fibrosis, uptake of M2 cells, and expression of cytokines to levels observed in Ang-II-exposed WT animals. Many TNFR1+ cells in chimeric TNFR1-KO/WT were committed to the fibroblast lineage.
Conclusion: Our data show that monocytic fibroblast precursor cells originated in the bone marrow, and that signaling through TNFR1 was required for their uptake and maturation into collagen-producing M2/fibroblasts that mediated the development of cardiac fibrosis in response to Ang-II. They also suggest a mechanistic link between inflammation and fibrosis, i.e. pro-inflammatory, M1-produced TNF initiates pro-fibrotic M2/fibroblast maturation via TNFR1 signaling.

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Meox1 is a Cell-cycle Oscillator Required for Mitotic Transition and Proliferation in Cardiac Fibroblasts

Shuichiro Higo, Yoshihiro Asano, Yuki Masumura, Yasushi Sakata, Osaka Univ Graduate Sch of Med, Suita, Japan; Masafumi Kitakaze, Natl Cerebral and Cardiovascular Ctr Res Inst, Suita, Japan; Seiji Takashima, Osaka Univ Graduate Sch of Med, Suita, Japan

Background
Tissue fibrosis plays important roles in the pathogenesis of chronic diseases, including heart failure. The mechanism underlying interstitial fibroblast proliferation is a promising analytical target for therapeutic applications. Here we developed quantitative epigenome profiling to identify a critical regulator in interstitial cell populations that emerges during the progression of heart failure.
Methods and Results
We subjected pressure-overloaded hearts of mice to trimethylated histone H3 lysine 4 (H3K4me3) ChIP-sequence and RNA-sequence. Expression analysis followed by quantitative H3K4me3 profiling identified 45 fibrosis-related genes with significant H3K4me3 enrichment in failing hearts, including Meox1 transcription factor. Meox1 emerged in the interstitial fibrotic region in failing heart, and intriguingly Meox1 was expressed in the limited population of cardiac fibroblasts both in vivo and in vitro. Meox1-positive fibroblasts were increased in response to a paracrine signal from cardiomyocytes, and knockdown of Meox1 completely inhibited the reactive proliferation of cardiac fibroblasts stimulated by conditioned medium from cardiomyocytes. Gene expression profiling combined with siRNAs clarified that

Meox1 depletion resulted in down regulation in the mitosis-related genes including Aurora B kinase. Indeed, Meox1 depletion decreased the cells under mitosis, but conversely increased the proportion of DNA synthesizing cells, thereby inhibited mitotic transition. The cell-cycle synchronization analysis and promoter analysis using live-cell imaging clarified that Meox1 oscillated throughout the cell-cycle and specifically emerged in G2/M phase. Finally, we revealed that Meox1 heterogenously expressed in the interstitial fibrotic are of human ventricular heart tissues from patients with end-stage heart failure. Notably, Meox1 expression was significantly correlated with the fibrosis-related genes in diseased ventricular heart tissues (n=15), suggesting the pathological relevance in clinical settings.
Conclusion
Our findings identify a novel cell-cycle regulator and propose that Meox1 is a potential target for therapies aimed at preventing tissue fibrosis.

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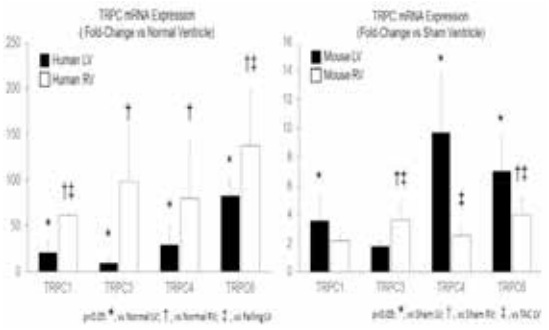
Distinct Profiles of Transient Receptor Potential Canonical (TRPC) Channel Expression in Biventricular Failure

Kevin J Morine, Vikram Paruchuri, Xiaoying Qiao, Duc Thinh Pham, Gordon S Huggins, David DeNofrio, Michael S Kiernan, Tufts Medical Ctr/MCRI, Boston, MA; Lixia Yue, Univ of Connecticut Health Ctr, Farmington, CT; Richard H Karas, Navin K Kapur, Tufts Medical Ctr/MCRI, Boston, MA

Heart failure is a major cause of morbidity and mortality. The transient receptor potential canonical (TRPC) family of channels mediate pathologic cardiac remodeling. In particular, TRPC6 participates in a self-propagating circuit that amplifies cardiac hypertrophy and fibrosis. The objective of this study was to explore biventricular expression of TRPCs in advanced heart failure.
Methods: Viable left (LV) and right (RV) ventricular free wall tissue was obtained from human subjects with end-stage heart failure (n=12) referred for transplantation or biventricular assist devices. Control LV and RV tissue was obtained from the National Disease Research Interchange (n=3/group). To explore TRPC expression in a murine model, adult male C57BL/6 mice underwent thoracic aortic constriction (TAC) for 10 weeks (n=6/group). Biventricular tissue was analyzed by real-time polymerase chain reaction.
Results: Compared to normal LV and RV, levels of TRPC 1, 3, 4 and 6 were increased in failing LV and RV samples, respectively. Levels of TRPC1 and TRPC6 were greater in failing RV than failing LV samples. TRPC 5 and 7 expression were not consistently detected in normal or failing tissue samples. Compared to sham LV, levels of TPRC 1, 4 and 6 increased in the LV after TAC. Compared to sham RV, levels of TRPC 3, 4, and 6 increased in the RV after TAC. Levels of TRPC3 were greater in the RV than LV after TAC.
Conclusions: Our results identify distinct profiles of TRPC expression in the RV versus LV in both human tissue and in a murine model of advanced biventricular failure. Levels of select TRPCs are higher in the failing RV compared to LV, suggesting a potentially important role for TRPCs in RV remodeling.



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Late Gadolinium Enhancement on Cardiac MRI Identifies Ventricular Dysfunction and Regional Myocardial Dyssynchrony in Patients with Univentricular Heart Diseases

Michihiro Okuyama, Shuta Ishigami, Daiki Ousaka, Junko Kobayashi, Shingo Kasahara, Shunji Sano, Hidemasa Oh, Okayama Univ Hosp, Okayama, Japan

Backgrounds- The impact of myocardial fibrosis on cardiac performance and clinical outcomes in patients with a functional single ventricle before stage-3 operation is unknown. Objective- The purpose of this study is to investigate the prognostic value of myocardial fibrosis identified by cardiac magnetic resonance imaging (cMRI) in patients with univentricular heart diseases. Methods- Consecutive 23 patients undergoing staged-3 surgical palliation with single ventricle physiology were prospectively scheduled to have cMRI study with late gadolinium enhancement (LGE) imaging and ventricle circumferential strain were examined. Results- Of 23 patients (mean age 3.3±0.9 years), 6 (26%) had positive late gadolinium enhancement (LGE+) in the ventricular myocardium, median percent LGE was 3.0% (interquartile range 3.0% to 14.0%). Pre-Fontan examinations revealed that patients with LGE+ had increased end-diastolic volume index (142.8 ml/BSA vs. 113.8 ml/BSA; P=0.02), increased end-systolic volume index (101.0 ml/BSA vs. 72.2 ml/BSA); P=0.01) compared with those without LGE (LGE-). Patients with LGE have shown to have lower ventricular circumferential strain compared with the area without LGE (basal: -1.9±1.9% vs. -4.0±3.0%, P=0.046; mid: -3.9±2.1% vs -8.0±3.9%, P=0.007; apical: -3.9±2.4% vs. -8.2±2.8%, P=0.004). In contrast to LGE- group, patients in LGE+ group had decreased right ventricular ejection fraction (27.7±8.8% vs. 38.2±8.4%; P=0.02) as well as higher levels of BNP (99.2±75.7 pg/ml vs. 32.6±44.3 pg/ml, P=0.02). In addition, patients with LGE+ had higher score of Ross classification (2.5±0.55 vs. 2.0±0, P=0.02) and New York University Pediatric Heart Failure Index (11.0±3.5 vs. 7.8±1.1, P=0.01) than in LGE- group. Age at stage-2 palliation was significantly older in patients with LGE+ group than LGE- subjects (16.8±16 months vs. 8.8±3.4 months, P=0.03). Conclusion- In this pre-stage-3 cMRI study, the age to stage-2 palliation may attribute to substantial myocardial fibrosis. The area of LGE was associated with impaired regional circumferential strain as well as disturbed ventricular performance. This novel strategy may provide a possible prognostic value of latent myocardial dysfunction after staged palliation.

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Mitochondrial Mechanisms of Structural and Electrical Remodeling in Atrial Fibrillation

Cevher Ozcan, Emily Battaglia, Rebeccah Young, Gen Suzuki, John M Canty, Univ at Buffalo Sch of Med and Biomedical Sciences, Buffalo, NY

Background: The mechanisms responsible for atrial electro-anatomic remodeling during the development of atrial fibrillation (AF) are not known. Mitochondria determine atrial myocyte cell survival and function by regulating cellular oxidative and metabolic stress. We therefore postulated that mitochondria play a central role in the remodeling of atrial myocardium and the pathogenesis of AF.
Methods: Our hypothesis was tested in a novel murine model (cardiac specific LKB1 knockout (KO)) where there is a high rate of sinus rhythm progressing to AF (95% of mice). Structural and electrophysiological properties of atria were studied in sinus rhythm and AF. Multiple parameters including the ECG, cardiac MRI, echocardiography, histopathology and immunoblotting in KO mice were compared with age- and gender-matched wild type controls. Results: LKB1 KO mice developed biatrial enlargement while in sinus rhythm that progressively increased as persistent AF developed. Apoptotic and necrotic myocyte death produced a substantial reduction in atrial cardiomyocytes (44%) that was replaced by fibrosis (34% vs 4.9%, $p<0.05$) and non-myocyte cells. In KO atria, there was a 6 fold increase in TUNEL positive nuclei and significant elevations of caspase 3 (10.4±1 vs 3.6±0.5 AU, $p<0.05$) and caspase 9 (1.4±0.1 vs 0.7±0.06 AU, $p<0.05$) activity. Mitochondrial reactive oxygen species were increased in KO atria (13.6±0.7 vs. 4.2±1 AU, $p<0.05$). In addition to extracellular matrix remodeling, cell-to-cell coupling was disrupted in KO atria with reduced gap junction proteins such as connexin 40 (0.24±0.03 vs 0.82±0.01 AU, $p<0.05$) and connexin 43 (1.4±0.1 vs 14.4±1 AU) ($p<0.05$). Electron microscopy showed impaired ultrastructure of mitochondrion and myofibrils in atrial myocytes.

Conclusions: We conclude that mitochondria play a pivotal role in the genesis of AF. Mitochondrial ROS generation and caspase activation lead to progressive cardiomyocyte loss and the transition of sinus rhythm to paroxysmal and persistent AF. These findings raise the possibility that therapies directed at maintaining atrial mitochondrial function may prevent the development of chronic AF.

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Cardiac Fibroblast GRK2 Deletion Enhances Contractility and Remodeling following Ischemia/Reperfusion Injury

Meryl C Woodall, Benjamin P Woodall, Erhe Gao, Xiying Shang, Ancai Yuan, Jessica Ibetti, Walter J Koch, Temple Univ, Philadelphia, PA

Decades of research have identified G Protein Coupled Receptor Kinase 2 (GRK2) as an important molecule that is upregulated in the cardiomyocyte after myocardial injury and during heart failure development. Research from our lab has convincingly demonstrated that myocyte-specific loss of GRK2 both before and after myocardial ischemic injury improves cardiac function and remodeling. Recent studies have reported that GRK2 is also upregulated in the cardiac fibroblast in the failing heart, suggesting a potential role for this molecule in the most abundant cell type in the heart. However, the in vivo implications of GRK2 expression in the fibroblast following cardiac stress remain a mystery. Tamoxifen inducible, fibroblast-specific GRK2 knockout mice (Col1α2CreER/GRK2flox) were treated with tamoxifen along with their control murine counterparts (GRK2flox alone) for 10 days to induce deletion of GRK2 in fibroblasts. Two weeks later mice were subjected to ischemia/reperfusion (I/R) injury via coronary artery occlusion for 30 minutes followed by periods of reperfusion. Fibroblast GRK2 knockout mice presented with preserved cardiac function 24 hours post-I/R compared to control mice as demonstrated by increased ejection fraction (58.1±1.8% vs. 48.7±1.2%, respectively, N=11-14, p=0.0005). GRK2 knockout mice also presented with decreased fibrosis in the infarcted area 72 hours following I/R injury as shown by Masson's Trichrome staining. In line with decreased fibrosis, these mice also expressed decreased amounts of TGFβ1 and Collagen I. Additionally, α-smooth muscle actin expression is significantly diminished, indicating reduced fibroblast to myofibroblast transformation. These data suggest that GRK2 plays a key role up-stream in fibroblast activation and function in the ischemic heart and indicate that, like in the cardiomyocyte, inhibition of GRK2 in the cardiac fibroblast is a potential therapeutic target to limit cardiac dysfunction and remodeling after ischemic injury.

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Sphingosine 1-phosphate Signaling Contributes To Cardiac Inflammation, Remodeling And Dysfunction Following Myocardial Infarction.

Fuyang Zhang, Yunlong Xia, Wei Wang, Wenjun Yan, Lu Yang, Yan Li, Fen Zhou, Ling Tao, Xijing Hosp, The Fourth Military Medical Univ, Xi'an, China

Sphingosine 1-phosphate (S1P) generated by sphingosine kinases (SphK) regulates multiple pathophysiological processes in cardiovascular system. The study was designed to investigate the role of SphK/S1P axis in myocardial infarction (MI) induced heart failure and the underlying mechanisms. Male C57BL/6J mice were subjected to permanent left coronary artery occlusion for 4 weeks. The infarcted heart showed increased SphK1 expression and increased S1P content. S1PR1, the predominant type of S1P receptors expressed in left ventricle, was upregulated by 3-fold in cardiac tissue following MI (n=6, all $P<0.05$). Further, we observed that S1P significantly activated NF-κB/STAT3 signaling and upregulated pro-inflammatory cytokines (TNF-α and IL-6) expression in a dose-dependent manner in cultured neonatal rat ventricular cardiomyocytes (n=3, all $P<0.01$), all of which were almost blocked by pretreatment with S1PR1 siRNA or FTY720, a functional S1PR1 inhibitor (n=3, all $P<0.01$). In vivo, administration of FTY720 (3mg/kg, i.p. daily for 4 weeks) decreased SphK1/S1P/S1PR1 axis, inhibited persistent NF-κB/STAT3 signaling activation and blocked pro-inflammatory cytokines production in post-MI

heart. Accordingly, FTY720 remarkably alleviated cardiac fibrosis and dysfunction (ejection fraction: 35.5±3.0% vs. 29.4±2.7% , P<0.05). Intriguingly, administration of SphK2 inhibitor ABC294640 (5mg/kg, i.p. daily for 4 weeks) amplified cardiac SphK1/S1P/S1PR1 axis, augmented NF-κB/STAT3 signaling activation and increased inflammatory cytokines expression. Consequently, SphK2 inhibition reduced survival rate (68.4% vs. 83.3%, P<0.05) and exacerbated cardiac maladaptive structural changes and ventricular dysfunction induced by MI (ejection fraction: 25.4±2.3% vs. 30.4±3.2%, P<0.05). Our results provide the evidence that upregulated SphK1/S1P/S1PR1 axis links persistent NF-κB/STAT3 activation, chronic cardiac inflammation and heart failure progression following MI, whereas SphK2 serves as an endogenous suppressor of pathological S1P signaling. Targeting SphK1/S1P/S1PR1 axis by FTY720 treatment or enhancing SphK2 activity might be a potential therapeutic strategy for ischemic heart failure.

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Regulation Of Frataxin By HIF-1 In The Ischemic Diabetic Heart

Raj H Amin, Abdullah AlAsmari, Gayani Nanayakkari, John Quindry, Shavanthi Mouli, Haitham Eldoumani, Graham McGinnis, Xiaoyu Fu, Auburn Univ, Auburn, AL

Background: Diabetes is at epidemic proportions, with the major form of fatality due to congestive heart failure triggered by myocardial infarction (MI). The impaired insulin signalling in the diabetic heart leads to myocardial energy dysregulation that compromises the cardioprotective mechanism against ischemic injury. Therefore understanding how mitochondrial energetics is altered in the diabetic ischemic heart would greatly advance the knowledge base for improving outcomes from heart failure in diabetic patients. Methods/ findings: We observed that db/db mice (leptin deficient, type 2 diabetic mice) have increased infarction size (>30%) compared to wild type mice after ischemia/reperfusion (IR) injury by TTC stain. We also found that activity of Hypoxia inducible factor-1 (HIF1) is involved in the cardioprotective response to ischemia, is impaired in db/db hearts. HIF1 is known to transcriptionally regulate genes involved in myocardial energetics. We recently found that HIF1 transcriptionally regulates the mitochondrial protein frataxin (Fxn) in cardiomyocytes as determined by luciferase assays (>3 fold). In vitro studies indicate that hypoxic conditions increase Fxn protein expression in cardiomyocytes as determined by western analysis (2 fold). Fxn plays an important role in the Fe-S cluster biogenesis required for aconitase, succinate dehydrogenase and complexes in the mitochondria. Interestingly, we observed decreased expression of Fxn in the ischemic diabetic heart. Conclusion: we postulate that attenuated HIF1-Fxn signalling in ischemic diabetic heart leads to abnormally enlarged infarction size in response to IR. The decline in HIF-1 activity in response to hypoxia was further validated in cardiomyocytes cultured in high glucose media. The significance for Fxn against hypoxic injury was confirmed by utilizing overexpressed Fxn cardiomyocytes via MTT, ATP and aconitase activity assays. Current and future work: currently we are attempting to identify the HIF response element (HRE) in Fxn promoter to further validate the transcriptional activity of HIF1. In addition, we are completing the IR surgeries on HIF1 KO mice to address the cardioprotective nature of HIF1-Fxn signalling against MI.

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β1 Vs β2 Adrenergic Signaling Differentially Regulates Mitochondrial Dynamics Through Alterations In Calcineurin And Drp1

Michael Coronado, Giovanni Fajardo, Mingming Zhao, Dongquing Hu, Stanford Univ, Stanford, CA; Aleksandr Stotland, Cedars-Sinai Medical Ctr, West Hollywood, CA; Sushma Reddy, Stanford Univ, Stanford, CA; Roberta Gottlieb, Cedars-Sinai Medical Ctr, West Hollywood, CA; Daniel Bernstein, Stanford Univ, Stanford, CA

Signal transduction through β1 and β2-adrenergic receptors (ARs) is considered a primary mechanism for regulating cardiovascular function and remodeling. Upon β-AR activation (i.e., physical activity, cardiac pathology) inotropy and chonotropy increase and mitochondria must quickly meet increased energy demand. This suggests that βARs and mitochondria are coupled mechanistically to rapidly respond to the functional and energetic needs of the heart. To investigate the role of β1 vs. β2-AR signaling on mitochondrial dynamics, we compared β1-/- and β2-/- to WT controls. β2-/- had increased mitochondrial fragmentation (increased number and decreased size) by electron microscopy vs. both WT and β1-/-. β2-/- showed altered regulation of mitochondrial fission: increased Drp1 translocation to the mitochondria vs. WT, whereas β1-/- had lower Drp1 translocation. These data suggest differential regulation of fission by βAR signaling, β1 activating and β2 suppressing fission. Since Ca2+-dependent calcineurin is known to activate Drp1 and [Ca2+]i is differentially regulated by β-AR signaling, we examined calcineurin as the bridge between β-AR signaling and Drp1 activation. In β2-/-, both Ca2+ transients and calcineurin activity were increased, suggesting β1-AR/Ca2+/calcinurin-mediated fission. To quantify mitochondrial fragmentation and biogenesis, mitotimer-transfected C2C12 cells were treated with the non-specific β-AR agonist isoproterenol resulting in mitochondrial fragmentation that was inhibited by the β1-antagonist CGP 12177 but not by the ß2-antagonist ICI 118551. Taken together, our data indicate that β1 and β2-AR signaling differentially regulate mitochondrial dynamics in the heart through alterations in [Ca2+]i, leading to calcineurin-induced translocation of Drp1.

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85

Long-term Monotherapy with Bendavia (MTP-131), A Novel Mitochondria-Targeting Peptide, Restores H11 Kinase Protein Levels in Mitochondrial Fractions of Left Ventricular Myocardium of Dogs with Advanced Heart Failure

Ramesh C. Gupta, Kristina Szekely, Mengjun Wang, Sharad Rastogi, Kefei Zhang, Paula Mohyi, Hani N. Sabbah, HENRY FORD HEALTH SYSTEM, Detroit, MI

Background: The heat shock protein H11 kinase (H11K) is expressed in the heart of dogs, rodents and humans and rapidly increases in response to increased contractile workload. Deletion of H11K in pressure overload transgenic mice accelerates LV dysfunction and remodeling by deactivating STAT3, a stress-inducible transcription factor. Deactivation of STAT3 has been shown to adversely impact mitochondrial respiration and consequently oxidative phosphorylation. We previously showed that chronic therapy (3 months) with Bendavia (BEN, MTP-131), a novel mitochondria-targeting peptide, improves LV systolic function in dogs with heart failure (HF) and normalizes mitochondrial respiration and rate of ATP synthesis in LV myocardium of dogs with HF. This study tested the hypothesis that H11K is reduced in mitochondria of the failing heart and that long-term therapy with BEN normalizes levels of this key heat shock protein. **Methods:** LV tissue was obtained from 12 dogs with microembolization-induced HF (LV ejection fraction ~30%) randomized to 3 months therapy with subcutaneous injections of BEN (0.5 mg/kg once daily, n=6) or saline (Control, n=6). LV tissue from 6 normal dogs was used for comparison. Tissue samples were used to extract mitochondrial fractions (MF). Protein levels of H11K and Porin, a mitochondrial protein not altered in HF, were determined in MF by Western blotting and protein band were quantified in densitometric units (du). **Results:** Protein level of Porin in MF was similar among NL, Control and BEN-treated dogs (0.24±0.0 vs. 0.22±0.01 vs. 0.23±0.01 du, respectively). Level of H11K was 0.45±0.03 du in NL, decreased to 0.17±0.02 in Controls ($p<0.05$vs. NL) and was normalized by BEN (0.37±0.05, $p<0.05$ vs. Control). H11K normalized to Porin was 1.93±0.23 du in MF of NL dogs, decreased to 0.78±0.0.09 du in Controls ($p<0.05$ vs. NL) and was restored to near normal levels after treatment with BEN (1.61±0.20 du, $p<0.05$ vs. Control). **Conclusions:** H11K protein levels are reduced in MF from LV myocardium of dogs with HF and normalized after chronic therapy with BEN. Restoring H11K with BEN likely contributed to the observed improvement in mitochondrial

respiration and rate of ATP synthesis previously reported after long-term therapy with BEN.

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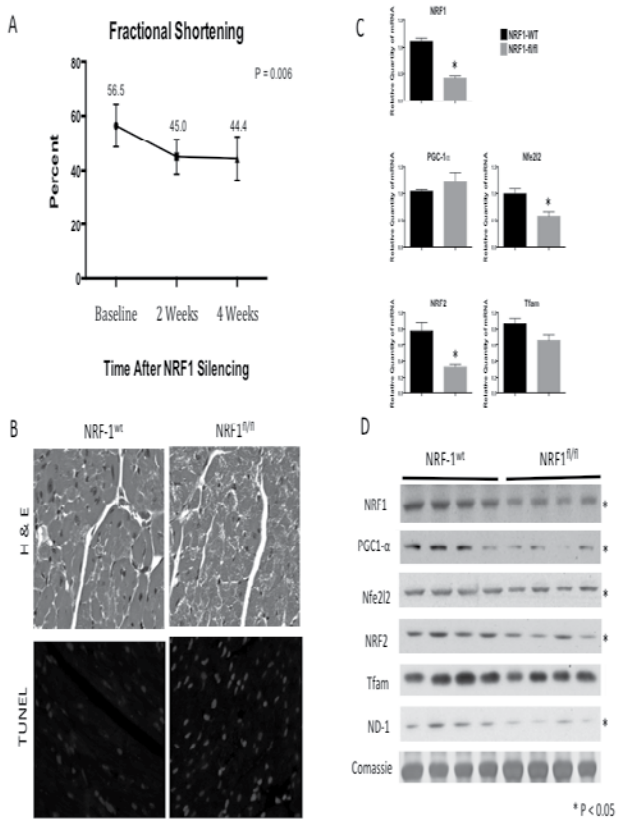
Cardiomyocyte-Specific Ablation of Nuclear Respiratory Factor 1 in the Mouse Leads to Dysregulation of Mitochondrial Biogenesis, Apoptosis, and Heart Failure

Jeffrey E Keenan, Hagir Sulliman, Allison Ulrich, Lan Mao, Claude A. Piantadosi, Duke Univ Medical Ctr, Durham, NC

The DNA-binding transcription factor Nuclear Respiratory Factor 1 (NRF1) regulates mitochondrial homeostasis. Its constitutive ablation in the mouse is embryonically lethal (~E3.5). This has limited our understanding of NRF1 functionality in the heart, where mitochondrial dysfunction is often a major pathogenic factor. Therefore, we generated conditional cardiomyocyte-specific NRF1 knockout mice (MYH6-mer-Cre-mer-NRF1fl/fl or NRFfl/fl) to elucidate the role of cardiac NRF1. Two weeks after NRF1 silencing, echocardiography of NRF1fl/fl hearts revealed significant reductions in left ventricular fractional shortening (Figure A). Histology demonstrated degradation of cellular structural integrity and nuclear condensation (Figure B), with a high number of TUNEL positive nuclei compared to littermate controls (MYH6-mer-Cre-mer-NRF-1wt), indicative of apoptosis (37.8% vs. 1.1%, $p < 0.001$). The mRNA and protein levels of key mediators of mitochondrial biogenesis were evaluated by real-time RT-PCR and immunoblotting (Figure C & D). Compared to littermate controls, there was down-regulation of the mitochondrial encoded NADH dehydrogenase 1, implying a reduction of functional mitochondrial mass. Key biogenesis regulators PGC1-α (protein only), Nfe2l2, and NRF2 were also reduced. In total, these data support that dysregulation of mitochondrial biogenesis after loss of NRF1 results in cardiomyocyte apoptosis and reduced left ventricular function. These findings and further delineation of the mechanisms involved should lay the foundation for the exploitation of NRF1 as a therapeutic target in heart failure.



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Sestrin2 Promotes LKB1-Mediated AMPK Activation in the Ischemic Heart

Ji Li, Alex Morrison, Yina Ma, Jinli Wang, Zhenling Liu, Univ at Buffalo-SUNY, Buffalo, NY; Andrei Budanov, Jun-Hee Lee, Michael Karin, UC San Diego, San Diego, CA

AMP-activated protein kinase (AMPK) has emerged as a pertinent stress-activated protein kinase shown to have substantial cardioprotective capabilities against myocardial ischemia/reperfusion injury. When activated

ABSTRACTS

during ischemia, AMPK produces effects that have been demonstrated to be beneficial to the myocardium by means of increasing GLUT4 translocation and glucose uptake, activating eNOS, decreasing apoptosis, and increasing autophagic flux. However, the molecular basis behind the regulation of AMPK activity in the ischemic and reperfused heart remains incompletely understood. Recent evidence implicates the role of Sestrin2 in the AMPK signaling pathway and it is hypothesized that Sestrin2 plays an influential role during myocardial ischemia in order to promote AMPK activation. Sestrin2 protein was found to be expressed in adult cardiomyocytes and accumulated in the heart during ischemic conditions. Sesn2 KO mice were used to determine the importance of Sestrin2 during I/R injury. Sesn2 KO cardiac phenotype analysis indicated no significant difference in left ventricular function as measured by a pressure-volume loop catheter. When wild type (WT) and Sesn2 KO mice were subjected to in vivo I/R, myocardial infarct size was significantly greater in Sesn2 KO compared to WT hearts. Similarly, Langendorff perfused hearts indicated exacerbated post-ischemic contractile function in Sesn2 KO hearts when compared to WT. Ischemic AMPK activation was found to be impaired in the Sesn2 KO hearts. Immunoprecipitation of Sestrin2 demonstrated an association with AMPK. Moreover, LKB1, a major AMPK upstream kinase was associated with the Sestrin2-AMPK complex in a time-dependent manner during ischemia while this interaction was nearly abolished in Sesn2 KO hearts. Thus, Sestrin2 plays an important role in cardioprotection against I/R injury by acting as an LKB1-AMPK scaffold to initiate AMPK activation during ischemic insults.

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AMP-activated Protein Kinase Signaling Mediates the Cardioprotective effect of Antithrombin against Myocardial Ischemia and Reperfusion Injury

Ji Li, Yina Ma, Jinli Wang, Hui Yang, Yanqing Wang, Univ at Buffalo-SUNY, Buffalo, NY; Chandrashekhara Masnithody, Alireza R Rezaie, St Louis Univ, St. Louis, MO

Background - Antithrombin (AT) is an endogenous protein of the serpin superfamily involved in regulation of the proteolytic activity of the serine proteases of the coagulation system. AT is known to exhibit anti-inflammatory and cardioprotective properties when it binds to distinct heparan sulfate proteoglycans (HSPGs) on vascular endothelial cells. The energy sensor AMP-activated protein kinase (AMPK) plays an important cardioprotective role during myocardial ischemia and reperfusion (I/R). The objective of this study was to investigate whether the cardioprotective signaling function of AT against I/R injury is mediated through the AMPK pathway.
Methods and Results - The cardioprotective activities of wild-type (WT) AT and its two recombinant derivatives, one having high affinity and the other no affinity for heparin, were evaluated in an acute I/R (20 min/4 h) injury model in which the left anterior descending coronary artery (LAD) was occluded. The serpin derivatives were given 5 min before reperfusion. The results showed that AT-WT can activate the protective AMPK signaling pathway in both in vivo and ex vivo conditions. Blocking AMPK activity abolished the cardioprotective function of AT against I/R injury. The AT derivative having high affinity for heparin was more effective in activating AMPK, but the derivative lacking any affinity for heparin was inactive in eliciting AMPK-dependent cardioprotective activity. The activation of AMPK by AT inhibited the inflammatory c-Jun N-terminal protein kinase (JNK) pathway during I/R. Further studies revealed that the AMPK activity of AT also modulates cardiac substrate metabolism by increasing glucose oxidation but inhibiting fatty acid oxidation during I/R.
Conclusions - These results suggest that AT binds to vascular HSPGs to invoke a cardioprotective function by triggering cardiac AMPK activation, thereby attenuating JNK inflammatory signaling pathways and modulating substrate metabolism during I/R.

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Mitochondrial Protein Acetylation in Aged Hearts in the Context of Ischemia-Reperfusion Injury

Sergiy M Nadtochiy, William R Urciuoli, Paul S Brookes, George A Porter Jr, Univ of Rochester Medical Ctr, Rochester, NY

Introduction: SIRT3 deficiency leads to increased mitochondrial acetylation, and enhances sensitivity of the heart to stress. Recently we demonstrated that a worse outcome of ischemia-reperfusion (IR) injury in aged hearts was associated with increased protein acetylation. The aim of this study was to investigate the mechanism of acetylation and the identity of acetylated targets in aged hearts in the context of IR injury. **Methods:** Mitochondria were isolated from WT adult (7 mo.), SIRT3$^{+/-}$ adult (7 mo.) and WT aged (18 mo.) hearts. SIRT3 protein expression and identification of acetylated proteins from SIRT3$^{+/-}$ adult and WT aged samples were investigated using proteomics (2D gel, western blot, peptide mass fingerprinting). **Results:** Mitochondrial acetylome analysis revealed that many hyperacetylated patterns were identical between adult SIRT3$^{+/-}$ and aged WT hearts, suggesting that increased protein acetylation in the aged WT heart might be due to SIRT3 inhibition. Several targets were indentified, including complex I NADH dehydrogenase flavoprotein 1, which is known to interact with SIRT3. In this regard, we demonstrated that respiratory complex I (CxI) activity was significantly inhibited in both SIRT3$^{+/-}$ adult and WT aged hearts. Analysis of SIRT3 protein revealed that negatively charged species of SIRT3 (relative to the bulk SIRT3 population), were lost in aged hearts. These alterations suggest post-translational modification of SIRT3 which may lead to loss of its deacetylase activity. Alternatively, we found that upregulation of the mitochondrial acetyltransferase GCN5L1 in aged hearts (examined by western blot) may also contribute to enhanced protein acetylation. **Conclusions:** These data support a connection between SIRT3 downregulation, mitochondrial protein acetylation, and exacerbation of IR injury in aging hearts. Furthermore, it is known that functional CxI is required for several modes of cardioprotection suggesting that downregulation of CxI in SIRT3$^{+/-}$ or WT aged hearts may underlie their poor recovery from IR injury.

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Bendavia (MTP-131), a Novel Mitochondria-Targeting Peptide, Improves ADP-Stimulated Mitochondrial Respiration in Cardiomyocytes Isolated From Dogs with Chronic Heart failure

Hani Sabbah, Ramesh C. Gupta, Sharad Rastogi, Paula Mohyi, Kristina Szekely, HENRY FORD HEALTH SYSTEM, Detroit, MI

Background: Mitochondria (MITO) of failed human hearts and hearts of dogs with experimental heart failure (HF) manifest structural and functional abnormalities characterized by hyperplasia and reduced organelle size and reduced respiration. These abnormalities lead to reduced ATP synthesis that adversely impacts LV function. We previously showed that chronic therapy (3 months) with Bendavia (MTP-131), a novel mitochondria-targeting peptide, improves LV systolic function in dogs with heart failure (HF), reverses MITO abnormalities and normalizes mitochondria ATP synthesis in myocardium from Bendavia-treated HF dogs. In the present study we examined the direct effects of Bendavia on mitochondria ADP-stimulated state 3 respiration in freshly isolated cardiomyocytes from dogs with advanced chronic HF. **Methods:** Cardiomyocytes were isolated from LV free wall of 3 untreated dogs with HF produced by intracoronary microembolizations (LV ejection fraction <30%). A standard collagenase-based enzymatic process was used for isolation that yielded ~70% viable rod-shaped cardiomyocytes that excluded trypan blue. Equal aliquotes of cardiomyocytes were incubated in 0, 0.01, 0.10, 1.0 and 10 μM concentration of Bendavia for one hour at 37ºC. At the end of incubation, ADP-stimulated state-3 respiration was measured using a Clark electrode system

and quatified in nAtom Oxygen/min/mg protein. **Results:** State-3 respiration in the absence of Bendavia (Vehicle-Control) was 248±9 nAtom Oxygen/min/mg protein. Compared to vehicle-control, incubation of failing cardiomyocytes with Bendavia significantly increased state-3 respiration to 303±33 at 0.01 μM, $p<0.05$; 405±39 at 0.10 μM, $p<0.05$; 371±28 at 1.0 μM, $p<0.05$; and 346±29 at 10.0 μM, $p<0.05$. **Conclusions:** Results of this study indicate that the effects of Bendavia on mitochondrial respiration in cardiomyocytes is direct and not a consequence of improved global LV structure or function. Furthermore, the results indicate that the improvement in mitochondrial respiration after treatment with Bendavia can occur early after initiation of therapy (within one hour) and is dose-dependent up to concentrations of 0.10 μM.

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Alteration of Myocardial GRK2 Produces a Global Metabolic Phenotype.

Benjamin P Woodall, Meryl A Castellini, Alessandro Cannavo, Walter J Koch, Temple U, Philadelphia, PA

A vast body of literature has established GRK2 as a key player in the development and progression of heart failure. GRK2 levels increase in the heart post injury in an attempt to normalize sympathetic overdrive. While initially beneficial, this response becomes maladaptive. Inhibition of GRK2 improves cardiac function post injury in numerous animal models. Recently, discovery of several non-canonical GRK2 targets has expanded our view of this kinase. Amongst these, our lab has identified GRK2 as a negative regulator of cardiac insulin signaling and this is through direct interaction and phosphorylation of the insulin receptor substrate-1 (IRS1). While continuing to study cardiac metabolic aspects of GRK2 manipulation we have uncovered the novel and exciting finding that cardiac GRK2 activity can regulate whole body metabolism. We found that transgenic mice with cardiac-specific overexpression of GRK2 (Tg-GRK2) show resistance to high fat diet (HFD) induced obesity. In contrast, Tg-βARKct mice with cardiac-specific expression of a peptide inhibitor of GRK2, known as the βARKct display an enhanced obesogenic phenotype when fed a HFD. We placed transgenic mice (and their non-transgenic littermate controls) on a high fat diet (60% kcal% fat) or a control diet (10% kcal% fat) for up to 16 weeks. Mice were housed in grouped cages at room temperature (18-22°C). HFD feeding increased white adipose mass in Tg-BARKct mice vs. NLC, whereas adipose mass in Tg-GRK2 mice is lower than NLC animals. Moreover, HFD Tg-βARKct mice exhibit elevated serum leptin levels relative to NLC, indicating development of leptin resistance. The novelty of this finding could bear great clinical significance, not least because these cardiac specific transgenes elicit a global metabolic phenotype implicating the heart as an endocrine organ and GRK2 activity is essential for this new and unexpected finding.

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Sirtuin 2 mediated-deacetylation regulates cellular iron homeostasis

Xiaoyan Yang, Athanassios Vassilopoulos, Seong-Hoon Park, David Gius, Hossein Ardehali, Northwestern Univ, Chicago, IL

Background: Sirtuins (SIRTs) are NAD+-dependent deacetylases, which regulate energy metabolism and response to oxidative stress in the heart. Iron is essential for these processes but is toxic when present in excess. However, whether SIRTs are involved in maintaining cellular iron homeostasis is not known. SIRT2 is among the least characterized SIRTs and is mainly present in the cytoplasm. We hypothesized that SIRT2 is required for cellular iron homeostasis.
Methods and Results: Iron content was significantly lower in SIRT2-/- mouse embryonic fibroblasts (MEFs) compared to SIRT2+/+ MEFs (non-heme iron: 0.073 vs. 0.060 nmol/μg protein, $p=0.02$), andlevels of ferroportin-1 (FPN1), the major cellular iron exporter, was significantly increased in SIRT2-/- MEFs. Similarly, silencing SIRT2 in HepG2 cells

decreased cellular iron levels and increased FPN1 expression, indicating that enhanced FPN1 with SIRT2 downregulation drove iron export and caused a reduction in cellular iron levels. Furthermore, iron export assays showed that iron export was increased in HepG2 cells with SIRT2 knockdown. To investigate the underlying mechanism, we focused our studies on nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a known regulator of FPN1. Our results demonstrated that Nrf2 is upregulated and translocates into the nucleus in SIRT2-/- MEFs and knocking down Nrf2 in SIRT2-/- MEFs reverses iron deficiency. Furthermore, Nrf2 is acetylated by P300/CBP and can be deacetylated by SIRT2. Finally, to confirm the role of SIRT2 in iron regulation, cellular heme and non-heme iron in the heart (major iron-consuming organ) and liver (major iron-storage organ) were measured in wild type (WT) and SIRT2-/- mice. Heme and non-heme iron content were significantly decreased in SIRT2-/- mouse livers compared to WT livers (heme: 2.25 vs. 1.65 nmol/mg protein, p=0.002; non-heme iron: 0.073 vs. 0.064 nmol/µg protein, p=0.03). Furthermore, heme levels were also significant decreased in the heart, while non-heme iron was not significantly altered.
Conclusions: Our results suggest that SIRT2 regulates cellular iron homeostasis by deacetylating NRF2 and altering iron export through FPN1.

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A Gain-of-Function Mutation in Cardiac Myosin Binding Protein-C Increases Viscoelastic Load and Slows Shortening Velocity in Myocytes from Transgenic Mice

Kristina L Bezold, Stanford Univ, Stanford, CA; Jaskiran K Khosa, Samantha P Harris, Univ of Arizona, Tucson, AZ

Cardiac myosin binding protein C (cMyBP-C) is a sarcomeric protein involved in the regulation of cardiac muscle contraction. Effects of cMyBP-C on contraction are thought to be mediated in part by limiting the interactions of actin and myosin to slow myocyte shortening velocity and power output. Although interactions with myosin S2 on the thick filament have been proposed as a way in which cMyBP-C could limit shortening velocity (e.g., by creating a drag force on myosin heads), interactions of cMyBP-C with actin could also account for slowed shortening velocity. For instance, cMyBP-C could create a drag that opposes filament sliding by transiently linking thick and thin filaments together. To explore this possibility we created transgenic mice that express a mutant cMyBP-C with a point mutation, L348P (human L352P), located in a conserved sequence within the regulatory M-domain that increases cMyBP-C binding to actin in vitro. We reasoned that if the mutation also enhanced binding to actin in sarcomeres then shortening velocity would be slowed in myocytes from L348P mice. Results show that transgenic mice expressing the L348P mutation are viable and that L348P cMyBP-C is expressed in sarcomeres. Permeabilized myocytes from transgenic mice showed altered force production including reduced maximal force and enhanced calcium sensitivity of tension. Shortening velocity and power output were significantly reduced whereas passive stiffness and myocyte visco-elasticity were significantly increased. Together these data are consistent with the idea that cMyBP-C creates an internal load in the sarcomere by binding to actin.

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Comparison of Ultrasound and Compact MRI for the Assessment of Cardiac Function in Mice

Tonya Coulthard, Aspect Imaging, Toronto, ON, Canada; Jun Wu, Princess Margaret Hosp, Toronto, ON, Canada

Introduction: Ultrasound (US) is a standard for the assessment of cardiovascular function. Pre-clinically, this is due the modality's translational potential, its high temporal resolution and the absence of infrastructure. Recent advancements in novel high-performance compact MRI has made cardiac MRI a more accessible technique for assessing a variety of pathologies in murine models of CVD while reducing the complexity and costs traditionally associated with

superconducting MRI systems. These developments may allow compact MRI to overcome some of the limitations of US meanwhile producing viable solutions for quantification along with new capabilities for cardiovascular biomarker assessment.
Hypothesis: This study explores the relative capabilities of novel compact high-performance MRI compared to HFU for phenotypic analysis of mouse models of CVD.
Methods: Four normal and one induced myocardial infarct C57BL/6 mice were imaged using both US and MRI. MI mice were prepared by ligation of the left anterior descending artery. In order to assess the ability of each modality to qualitatively and quantitatively characterize cardiac function, CINE loops of long axis and short axis slices were acquired with both systems and analyzed offline. In addition to traditional measures of cardiac function, strain analysis was performed using the HARP method for compact MRI and using speckle tracking for US. Finally, a cardiac MRI method for mapping and measuring infarct extent using gadolinium (Gd) contrast agents is described.
Results and Conclusions: Although compact MRI has lower temporal resolution, when compared with US, it still provides many advantages in pre-clinical CVD imaging. In particular, because MRI signal is not attenuated by dense tissue or air, compact MRI can successfully generate artefact-free imaging of pathologies difficult or impossible to image with US, such as clearly visualizing and quantifying endo and epicardial borders. It also provides a whole body image and a “whole heart” image, making pre-clinical CV imaging easier for biologists and helping to reduce inter-operator variability. Finally, high sensitivity to Gd-based contrast agent with compact MRI enable new applications such as infarct quantification.

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Longitudinal In Vivo Quantification of Infarct Size and Cardiac Function in a Murine Model of Myocardial Infarction using Novel Compact High-Performance MRI

Tonya Coulthard, Aspect Imaging, Toronto, ON, Canada; Jun Wu, Princess Margaret Hosp, Toronto, ON, Canada

Intro: Delayed Enhancement Imaging (DEI) characterizes MI and predicts therapeutic efficacy following coronary revascularization, stem cell transplantation, or other procedures. Typically gadolinium-based (Gd) contrast agents are injected intravenously and accumulate in the lesion, differentiating between normal and diseased myocardium. Traditionally, DEI has been explored using superconducting high-field MRI. Using a novel compact MRI at 1 Tesla, there is increased sensitivity to Gd-based contrast agents compared with higher-field MRI systems. Hypothesis: In this study, DEI on a compact MRI system was employed with spatial intensity analysis to measure infarct size. Tagged cine MRI assessed myocardial strain using the HARP method. Post-mortem histology of Masson Trichrome staining estimated infarct size. Correlation analysis determined relationships between infarct size measured via DEI and histology.
Methods:
C57BL/6 MI mice had ligation of the left anterior descending artery. Four sham control and five MI mice we imaged using compact MRI (ICON compact MRI, Billerica, MA) and monitored longitudinally over 21 days for infarct progression and cardiac function in vivo. DE imaging was performed 10-20 minutes post injection of 2 µL Gd contrast agent (ProHance, 0.5 mmol/ml). Images were acquired in short axis, 1 mm superior to the apex. Post-processing analysis (pcVirtue, Diagnosoft, Morrisville, NC) measured cardiac function and infarct size.
Results and Conclusions:
Analyses of DEI data indicate an average infarct size of 24.85 +/- 15.09 % in the left ventricle. One week after MI induction, a much lower EF was observed in MI mice with respect to control (35.3 +/- 7.567% vs. 67.3 +/- 1.6 %), with a slight recovery of cardiac function in MI mice by week 3 (EF = 45.5 +/- 3.1%). Strain analysis also indicated significantly lower average peak strain in MI vs control mice (6.9 +/- 4.3 vs. 21.5 +/- 0.4 %). Preliminary results demonstrate successful monitoring of cardiac function while performing DEI to visualize and quantify MI at 1 Tesla and leverage the benefits of the increased sensitivity of Gd-based contrast imaging with novel compact MRI. Such imaging and analysis techniques could be used to test efficacy of multiple therapeutic interventions.

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Epigenetic And Transcriptional Regulation Of Induced Pluripotent Stem Cells In Patients With Hypoplastic Left Heart Syndrome

Junko Kobayashi, Dept of Cardiovascular Surgery, Okayama Univ, Okayama, Japan; Masashi Yoshida, Dept of Cardiovascular Med, Okayama Univ, Okayama, Japan; Suguru Tarui, Shuta Ishigami, Michihiro Okuyama, Shingo Kasahara, Dept of Cardiovascular Surgery, Okayama Univ, Okayama, Japan; Hiroshi Ito, Dept of Cardiovascular Med, Okayama Univ, Okayama, Japan; Shunji Sano, Dept of Cardiovascular Surgery, Okayama Univ, Okayama, Japan; Hidemasa Oh, Ctr for Innovative Clinical Med, Okayama Univ Hosp, Okayama, Japan

Background- Although a number of studies have uncovered heterozygous mutations in cardiac regulatory genes caused hypoplastic left heart syndrome (HLHS), the identified genetic variants may not be directly correlated with the disease development. The aim of this study is to determine the epigenetic and transcriptional network responsible for myocardial patterning and morphogenesis during cardiac development in HLHS by using patient-derived induced pluripotent stem (iPS) cells. ***Methods-*** Five-independent iPS cell lines were generated from HLHS and biventricle (BV) heart-derived cardiac progenitor cells (CPCs). Global gene expression analysis, real-time RT-PCR, mutation analysis, ChIP assay, and cardiac-specific gene promoter activities were examined during differentiation. ***Results-*** We found one synonymous single nucleotide polymorphism in NKX2-5 and five in NOTCH1, respectively. Cardiac transcriptional factors such as NKX2-5, HAND1, and NOTCH/HEY, those are known to drive primary heart field and outflow tract development, were significantly reduced in HLHS-derived iPS cells after differentiation compared with BV- and control 201B7 iPS-derived cardiomyocytes. ChIP assay showed that a marked decrease in dimethylated histone H3-lysine 4 and acetylated histone H3 was found within the NKX2-5 promoter regions, whereas a significantly increased trimethylated H3-lysine 27 was identified in differentiated HLHS-derived iPS cells. To specify the target transcripts responsible for cardiac development of HLHS, cardiac troponin-T and natriuretic peptide A promoter analyses were performed. We found that both promoter activities were significantly suppressed in HLHS-derived CPCs and iPS cells compared with those from BV hearts. These repressed promoter activities could be fully restored by transient transfection of NKX2-5, HAND1, and NOTCH1 genes into these stem/progenitor cells by synergistic manner. ***Conclusions-*** These findings suggest that patient-specific iPS cells provide a potential to dissecting the complex cardiac malformations in human. The epigenetic and transcriptional regulation of NKX2-5, HAND1, and NOTCH1 may mutually participate in the development of myocardial growth, patterning, and morphogenesis in HLHS.

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High mobility group box-1 Promotes Restenosis Via Toll like receptor-4 Signal Pathway

Jingjing Cai, upmc, pittsburgh, PA; Hong Yuan, Ctr for Clinical Pharmacology, Third Xiangya Hosp, Changsha, China; Qingde Wang, Timothy R. Billiar, Alex F. Chen, upmc, pittsburgh, PA

Background: High-mobility group box 1 (HMGB1) is an endogenous molecule released during cell stress and death termed damage-associated molecular patterns (DAMPs). HMGB1 activates the pattern recognition receptor, toll-like receptor 4 (TLR4), and induces sterile inflammation. However, how HMGB1 and TLR4 affect restenosis, the major complication following balloon and stent intervention clinically, remains unknown. We tested the hypothesis that HMGB1 released following acute arterial injury promotes intimal hyperplasia (IH), a hallmark of restenosis, via TLR4 signaling pathway. Methods and Results: Wire injury of the carotid artery in C57BL/6 wild-type (WT) mice significantly increased intima-to-media ratio in 4 weeks. Global deletion of HMGB1 using an inducible knockout mouse strain prevented IH

and vessel remodeling. IH decreased by over 50% in WT mice treated with a HMGB1 neutralizing antibody. Of the mouse strains deficient in putative receptors and co-regulator for HMGB1 (TLR4-/-, TLR2-/-, RAGE-/- and CD14-/-), TLR4-/- mice showed the greatest inhibition of IH after injury. Both TLR4 adaptors MYD88 and TRIF synergistically participated in the inflammatory response to vascular injury. HMGB1 antibody-treated mice and TLR4-/- mice showed a marked decrease in monocytic recruitment following injury. Mice with selective depletion of TLR4 from macrophages (TLR4-/--Mø) exhibited similar level of IH inhibition and macrophage infiltration, compared to the global TLR4-/- mice. In vitro, disulfide HMGB1 concentration-dependently promoted smooth muscle cell (SMC) migration and MCP-1/CCR2 expression, which were abolished by treatment with TLR4 inhibitory peptide. Moreover, conditioned media from HMGB1-treated macrophage induced SMC proliferation, which was blunted by blocking TLR4 on macrophage, but not SMCs. Finally, HMGB1 increased cytokine (TNF-α and IL-6), chemokine (MCP-1) and mitogen (PDGF-A) levels in macrophage in a TLR4-dependent manner.
Conclusion: These findings demonstrate, for the first time, that HMGB1 released following acute arterial injury promotes restenosis via SMC migration and MCP-1/CCR2 production as well as macrophage-released TNF-α, IL-6, MCP-1 and PDGF-A in SMC through the TLR4-MyD88/TRIF signal cascade.

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Reducing Mitochondrial, But Not Cytosolic Iron, Protects The Heart Against Ischemia-reperfusion Injury

Hsiang-Chun J Chang, Rongxue Wu, Hossein Ardehali, Feinberg Cardiovascular Res Inst, Northwestern Univ, Chicago, IL

Introduction: Iron is essential for the activity of a large number of cellular proteins, but excess free iron can cause cellular damage through production of reactive oxygen species (ROS). Mitochondria are the major site of cellular iron homeostasis, and we recently showed the mitochondrial iron export is mediated by ATP-binding cassette protein-B8 (ABCB8). The role of mitochondrial iron in ischemia-reperfusion (I/R) injury in the heart has not been examined. We hypothesize that mitochondrial iron has a critical role in I/R damage and a reduction of mitochondrial iron is protective against I/R injury through a reduction in ROS.
Results: Cardiomyocyte-specific ABCB8 transgenic (TG) mice 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 indistinguishable from NTG littermates. To study the role of mitochondrial iron in I/R injury, we subjected ABCB8 TG mice to I/R. TG mice displayed significantly less apoptosis compared to NTG littermates (11.76% vs. 17.63%, $p<0.05$, n=4-6) and had significantly reduced lipid peroxidation products 48 hours after I/R.
To further confirm that our in vivo finding was due to reduced mitochondrial iron, we studied the effect of pharmacological reduction of mitochondrial iron in vitro. 2,2-bipyridyl (BPD) is a mitochondria-accessible iron chelator while deferoxamine (DFO) has poor penetrance into mitochondria. Treating rat cardiomyoblasts H9C2 with BPD but not DFO significantly reduced chelatable mitochondrial iron, as measured by staining cells with rhodamine B-[(1,10-phenanthrolin-5-yl)aminocarbonyl]benzyl ester. In addition, BPD but not DFO pretreatment protected cells against H2O2 induced cell death ($p<0.05$). BPD treatment in mice decreased baseline mitochondrial iron and significantly preserved cardiac function after I/R.
Conclusions: Our findings demonstrate that selective reduction in mitochondrial iron is protective in I/R injury, and show that mitochondrial iron is a source of ROS and cellular damage in I/R. Thus, targeting mitochondrial iron with selective iron chelators, as studied in our system, may provide a novel approach for treatment of ischemic heart disease.

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ABSTRACTS

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Hypotonic Swelling Promotes Nitric Oxide Release in Cardiac Ventricular Myocytes

Luis Gonano, Malena Morell, Juan I Burgos, Martin Vila Petroff, Ctr de Investigaciones Cardiovasculares, La Plata, Argentina

Cardiac myocyte swelling occurs in multiple pathological situations and in particular contributes to the deleterious effects of ischemia and reperfusion by promoting contractile dysfunction. We investigated whether hypotonic swelling promotes nitric oxide (NO) release in cardiac myocytes and if so, whether it impacts on swelling induced contractile dysfunction. Perfusing rat cardiac myocytes, loaded with the NO sensor DAF-FM, with a hypotonic solution (HS; 217 mOsm), increased cell volume, reduced myocyte contraction and Ca2+ transient amplitude and significantly increased DAF-FM fluorescence. When cells were exposed to the HS supplemented with 2.5 mM of the NO synthase inhibitor L-NAME, cell swelling occurred in the absence of NO release. Swelling-induced NO release was also prevented by the NOS1 inhibitor, Nitroguanidine. In addition, Colchicine (an inhibitor of microtubule polymerization) prevented the increase in DAF-FM fluorescence induced by HS indicating that microtubule integrity is necessary for swelling-induced NO release. The swelling-induced negative inotropic effect was exacerbated in the presence of either L-NAME, Nitroguandine or the guanylate cyclase inhibitor, ODQ, suggesting that NOS1-derived NO provides contractile support via a GMP-dependent mechanism. Indeed, ODQ reduced Ca2+ wave velocity and the HS-induced increment in ryanodine receptor (RyR2) phosphorylation at site Ser2808 suggesting that in the context of hypotonic swelling, cGMP may contribute to preserve contractile function by enhancing SR Ca2+ release. Our findings suggest a novel mechanism for NO release in cardiac myocytes with putative pathophysiological relevance in the context of ischemia and reperfusion, where it may be cardioprotective by reducing the extent of contractile dysfunction associated with hypotonic swelling.

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G-Protein Coupled Estrogen Receptor1 Prevents Mitochondria Inner Membrane Protein (Mitofilin) Degradation after Ischemia/Reperfusion

Tanoya L. Harris, UTHSCSA, San Antonio, TX; Bjorn Olde, Fredrik L.M. Leeb-Lundberg, Univ of Lund, Lund, Sweden; Jean C. Bopassa, UTHSCSA, San Antonio, TX

We recently found that estrogen treatment delays the mitochondrial permeability transition pore 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, we investigated whether estrogen effect on mitochondrial integrity after ischemia/reperfusion involved regulation of mitofilin via activation of G-Protein Coupled Estrogen Receptor1 (GPER1). Isolated hearts from male WT (C57BL/6NCrL), and GPER1-/- mice were perfused using Langendorff technique, with and without estrogen (40 nM). Hearts were subjected to 20 min global ischemia followed by 10 min reperfusion. Mitochondria were isolated, and 2D-DIGE followed by mass spectrometry was performed. Mitofilin expression level was confirmed using Western blot analysis in mitochondrial fractions. Mitofilin distribution was visualized using confocal microscopy in isolated cardiomyocytes, and its spatial organization in mitochondria was imaged using high resolution fluorescence microscopy. Electron microscopy was used to observe mitochondrial cristae morphology.

We obtained 52 unique proteins of interest, in which mitofilin was identified. Immunoblot analysis confirmed the increased in mitofilin level with estrogen treatment as compared to control in WT but not in GPER1 KO. Cardiomyocyte images revealed as observed in non-ischemic myocytes that estrogen treatment conserved mitofilin distribution in the peri-membrane and T-tubules, while only peri-membrane mitofilin was more visible in control group. High resolution microscopy showed a better spatial organization of mitofilin in single mitochondria with estrogen treatment compared to control, in which mitofilin was almost absent. Electron microscopy showed that mitochondrial morphology was conserved in estrogen-treated

group as cristae were well organized compared to control, in which cristae were disrupted. These data indicate that estrogen action induces regulation of mitofilin expression during ischemia/reperfusion. Estrogen effect on mitofilin may contribute to improved mitochondrial integrity and function.

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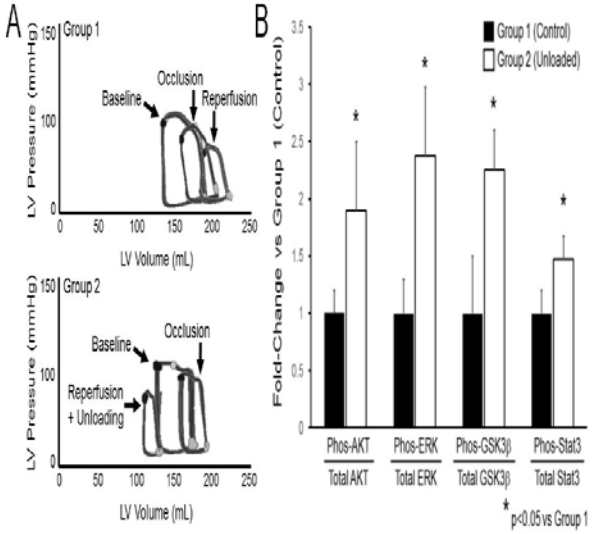
Acute Mechanical Unloading of the Left Ventricle Promotes Cardioprotective Signaling and Limits Myocardial Infarct Size

Navin K Kapur, Vikram Paruchuri, Xiaoying Qiao, Kevin Morine, Wajih Syed, Hamid Salehi, Sam Dow, Nimish Shah, Michele Esposito, Natesa Pandian, Richard H Karas, Tufts Medical Ctr MCRI, Boston, MA

Management of an acute myocardial infarction (AMI) focuses on restoring oxygen supply to limit myocardial damage, however ischemia-reperfusion injury (IRI) remains a major determinant of mortality in AMI. No studies have targeted initially reducing left ventricular stroke work (LVSW) to limit IRI in AMI. The Impella CP axial-flow pump reduces LVSW. We tested the hypothesis that first reducing myocardial work and delaying coronary reperfusion reduces infarct size by activating cardioprotective signaling pathways. **Methods:** AMI was induced by occlusion of the left anterior descending artery (LAD) via angioplasty for 90 minutes in 50kg male Yorkshire swine (n=5/group). In Group 1, the LAD was reperfused for 120 minutes. In Group 2, after 90 minutes of ischemia the Impella CP device was activated and the LAD left occluded for an additional 60 minutes (150 minutes of LAD occlusion total), followed by 120 minutes of reperfusion. The Impella CP was active throughout reperfusion. Western blot analysis quantified myocardial kinase activity. **Results:** Compared to Group 1, Group 2 had a reduced LVSW, LV end-diastolic volume and end-diastolic pressure after reperfusion [Fig A]. Group 2 showed increased myocardial phosphorylation of cardioprotective kinases: AKT, ERK, GSK3β and STAT-3 [Fig B]. Compared to Group 1, the percent myocardial infarct size normalized to the area at risk (AAR) was reduced in Group 2 (73+13% vs 42+15%, p=0.02). **Conclusion:** We report the potential benefit of primarily unloading the heart and delaying coronary reperfusion to salvage myocardium in AMI. This is the first report to examine the impact of the Impella CP on cardioprotective signaling in the heart.



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Interferon Regulatory Factor-1 Is Responsible For Cardiac Protective Effect Of 5-azacytidine On Macrophages In Myocardial Infarction

Yong Sook Kim, Hye-yun Jeong, Youngkeun Ahn, Chonnam Nat Univ Hosp, Gwangju, Korea, Republic of

Background: During progression of cardiac injury, macrophages with specialized phenotypes are actively involved in inflammatory responses. In this study, we investigated the effect of 5-azacytidine (5AZ) on macrophages in the damaged myocardium.
Methods: A mouse macrophage cell line RAW264.7 was stimulated with lipopolysaccharide (LPS, 100 ng/mL) with or

without 5AZ (10 μM). Nitric oxide was quantified by Griess's method. Expression patterns of inducible nitric oxide synthase (iNOS) and interferon regulatory factor 1 (IRF-1) were determined by Western blot analysis. For animal study, myocardial infarction (MI) was induced by ligation of left coronary artery in mice, and divided into four groups; non MI + saline, non-MI + 5AZ , MI + saline, and MI + 5AZ. Saline or 5AZ was injected (5 mg/kg/d) every other day. Cardiac fibrosis was evaluated by Masson's trichrome stain was performed and cardiac function was by echocardiography 2 weeks after MI.
Results: LPS-induced nitric oxide formation was reduced by 5AZ treatment in RAW264.7 cells. LPS-induced iNOS mRNA and protein inductions were blocked by 5AZ treatment. Next, the effect of 5AZ on IRF1, a regulator of iNOS, was examined. IRF-1 was dramatically increased by LPS to peak at 4 hr and then reduced to basal level. In the presence of MG132, a proteasome inhibitor, IRF-1 protein sustained maximal level without degradation. On the other hand, IRF-1 protein was significantly highly maintained by 5AZ treatment. In animal study, there were significant improvements in ejection fraction (53.25 ± 2.55 % vs. 62.50 ± 7.2 %, $p < 0.05$) and cardiac fibrosis (28.85 ± 5.44% vs. 16.57 ± 6.30%, $p < 0.05$). Collectively, 5AZ inhibited iNOS induction by modulation of IRF-1 in the activated macrophages to preserved cardiac function and fibrosis after MI.
Conclusion: 5AZ protected post-MI injury by regulation of IRF-1 kinetics to modulate macrophages in infarcted myocardium. This study suggested 5AZ as a novel therapeutic intervention for cardiac repair.

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Sex-specific Impact Of S-nitrosoglutathione Reductase (gsnor) On Ventricular Remodeling And Function Following Myocardial Infarction In Mice.

Shathiyah Kulandavelu, Ellena Paulino, Lauro Takeuchi, Rosemeire Kanashiro-Takeuchi, Wayne Balkan, Joshua M Hare, Interdisciplinary Stem Cell Inst, Miller Sch of Med, Miami, FL

Introduction: Female hearts are less susceptible to myocardial injury following myocardial infarction (MI) and estrogen working through the nitric oxide (NO) pathway is thought to play a role. S-nitrosylation of cysteine thiols is a major signaling pathway through which NO exerts its action and mice with targeted deletion of S-nitrosoglutathione reductase (GSNOR), a denitrosylase that regulates S-nitrosylation, show increased levels of nitrosylated proteins. Male $GSNOR^{-/-}$ mice show a more favorable outcome after MI as compared to wild-type (WT), including reduced myocardial infarct size, improved ejection fraction and preserved left ventricular volumes. Whether female $GSNOR^{-/-}$ mice show gender-related cardiac protection following MI was not known and thus investigated.

Methods & Results: MI was induced in male and female $GSNOR^{-/-}$ mice and their respective controls, C57Bl/6J, at 3-5 months via left anterior descending coronary artery occlusion. Serial echocardiography was performed prior to MI and after 1- and 4-weeks post-MI to assess ejection fraction (EF) and left ventricular volume both at diastole (end-diastolic volume, EDV) and systole (end-systolic volume, ESV). Compared to WT, $GSNOR^{-/-}$ males showed less dilation in both EDV (98.6 ± 9.3 mm vs. 140.6 ± 7.4 mm in WT, $P<0.001$) and ESV (73.1 ± 9.2 vs. 112.7 ± 7.3 mm in WT, $P<0.05$) at 4 weeks post-MI. Whereas, $GSNOR^{-/-}$ females showed greater dilation in both EDV (162.2 ± 13 vs. 83.8 ± 12 mm in WT, $P<0.001$) and ESV (141.8 ± 13 vs. 65.6 ± 12 mm in WT, $P<0.001$) as compared to WT females. EF decreased ($P<0.001$) in all groups post-MI, but at 4 weeks post-MI, it was significantly worse in $GSNOR^{-/-}$ females compared to $GSNOR^{-/-}$ males (14 ± 4% vs. 28 ± 3%, $P<0.05$).

Conclusion: $GSNOR^{-/-}$ females exhibit significantly lower EF and greater dilation of left ventricular volumes both at diastole and systole following MI than any of the other groups suggesting that S-nitrosylation plays an important role in gender-related cardiac protection following myocardial injury. These findings suggest the importance of taking gender into account when exploring novel therapeutic treatments for myocardial injury.

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Downregulation Of Cardiac Na+ Channel In Myocardial Infarction Is Prevented By A Mitochondria-targeted Antioxidant

Man Liu, Lifespan Cardiovascular Inst, the Providence VA Medical Ctr, and Brown Univ, Providence, RI; Cody A. Rutledge, Univ of Illinois at Chicago, Chicago, IL; Kai-Chien Yang, Samuel C. Dudley Jr., Lifespan Cardiovascular Inst, the Providence VA Medical Ctr, and Brown Univ, Providence, RI

Objectives: The aim of this study was to investigate the effect of ischemia on cardiac Na+ channel (Nav1.5) and possible treatments in a mouse model of myocardial infarction (MI). Methods: MI was induced in 12-week old C57BL/6 mice by coronary artery occlusion. Sham-operated mice were used as controls. Two weeks following surgery, MI mice were either given a mitochondria-targeted antioxidant, mitoTEMPO (0.7 mg/kg/day, intraperitoneally), or left untreated for two weeks. Cardiomyocytes isolated from the scar border of MI mice or from the left ventricular (LV) anterior wall of sham-operated mice were utilized for whole-cell patch clamp recording of Na+ currents (INa) and for measurements of mitochondrial reactive oxygen species (mitoROS) using flow cytometry. Nav1.5 protein expression levels were determined in the LV from MI and sham-operated mice. Echocardiography was performed 2- and 4-weeks following MI.
Results: The peak INa densities of the isolated LV cardiomyocytes were significantly lower ($P<0.05$) in MI (-14.3±1.4 pA/pF), compared to sham (-24.0±1.8 pA/pF). The mitoROS levels were elevated to 1.5±0.2 fold in MI mice ($P<0.05$). INa was increased (-19.4±0.8 pA/pF, $P<0.05$) and mitoROS was decreased to 1.2±0.2 fold ($P<0.05$) with mitoTEMPO treatment. The Nav1.5 channel protein level was not altered in the heart tissue of MI mice. There were no significant differences in echocardiography parameters between untreated and mitoTEMPO groups to explain the increase in INa.
Conclusions: Ischemic cardiomyopathy leads to downregulation of cardiac Nav1.5 currents and overproduction of mitochondrial ROS. The mitochondria-targeted antioxidant can mitigate these changes and may help reduce arrhythmic risk after myocardial infarction.

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GYY4137 Attenuates Post-Ischemic Adverse Remodeling and Modulates ANP-Mediated cGMP Signal Transduction

Eliana C Martinez, Shera Lilyanna, Meng T Peh, Oi W Liew, Philip K Moore, Arthur M Richards, Natl Univ of Singapore, Singapore, Singapore

Myocardial infarction is the most frequent proximate cause of heart failure, which remains a leading cause of death in the developed world. Hydrogen sulfide (H_2S) is emerging as an important endogenous modulator in diverse physiological and pathophysiological events. We hypothesized that the slow-releasing water-soluble H_2S donor GYY4137 (GYY) may exert cardioprotective effects through modulation of neurohormonal response to cardiac injury. We have found that treatment for 1 week with GYY (100mg/Kg/48hr, IP) after acute myocardial infarction in rats, provides powerful sustained preservation of left ventricular (LV) dimensions and function in vivo, compared to untreated infarcted (MI), placebo- and D-propargylglycine-(PAG, an inhibitor of endogenous H_2S synthesis) treated animals 2 and 7 days after infarct (n=6/group/time-point). LV dimensions and function in GYY-treated animals were comparable to healthy sham-operated rats. GYY-treated hearts displayed a significantly lower percentage of LV fibrosis than MI, placebo and PAG hearts, whereas PAG treated animals had significantly bigger scar size relative to GYY, at days 2 and 7 after MI. A higher density of blood vessels was found in the scar area of GYY-treated animals compared to all other infarcted groups at days 2 ($P<0.0002$) and 7 ($P<0.01$) post-MI. Furthermore, treatment with GYY resulted in increased levels of plasma atrial natriuretic peptide (ANP) compared to all groups at days 2 and 7 after MI, while cardiac mRNA expression of ANP was also significantly increased in GYY-treated rats compared to Sham ($P<0.001$), and all the infarcted groups ($P<0.05$) at day 2 after MI. Concordantly, ANP second messenger cGMP was increased in

plasma at day 7 in GYY rats compared to sham and all infarcted groups ($p<0.05$), paralleled by higher cGMP-dependent protein kinase type I (cGKI) protein levels than sham ($P<0.01$), and vasodilator-stimulated phosphoprotein phosphorylation (pVASP) at the cGKI preferred site (Ser239) compared to sham and PAG ($P<0.05$) at day 2 post-MI. In conclusion, our data suggest that H2S attenuates adverse remodeling and may exert post-ischemic cardioprotective (pro-angiogenic, anti-fibrotic) effects in part through modulation of ANP-mediated cGMP signal transduction.

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MicroRNA-31: A Novel Therapeutic Target for Ischemic Heart Disease

Eliana C Martinez, Shera Lilyanna, Natl Univ of Singapore, Singapore, Singapore; Leah A Vardy, A*STAR Inst of Medical Biology, Singapore, Singapore; Arunmozhiarasi Armugam, Kandiah Jeyaseelan, Arthur M Richards, Natl Univ of Singapore, Singapore, Singapore

MicroRNAs (miRNA), small sequences of non-coding RNA which interact with complementary sequences on the 3'untranslated region of target messenger RNAs to modulate translation, have a pivotal role in the development of the heart and its response to injury. Myocardial infarction (MI) triggers a dynamic miRNA response with the potential of yielding therapeutic targets. Following miRNA array profiling in rat hearts 2, 7 and 14 days after MI induced by coronary ligation, we identified a progressive time-dependent up-regulation of miR-31 compared to sham rats. Increase of miR-31 in heart tissue in the acute and subacute phases after MI (up to 90-fold) was also detected by Real-Time PCR ($P=0.02$ at day 2; $P<0.0001$ at days 7 and 14, vs. sham). We found that miR-31 has a repressive effect on tissue mRNA expression of cardiac troponin-T (TNNT2), E2F transcription factor 6 (E2F6) and mineralocorticoid receptor (NR3C2). Reporter gene assays showed that miR-31 targets the 3´UTR of these genes, with a marked repressive effect on TNNT2. In vitro, exposure to hypoxia significantly induced the expression of miR-31 in neonatal rat cardiomyocytes (nRCM), rat cardiac fibroblasts (nRCF) and cardiomyoblasts (H9C2) and suppressed the expression of TNNT2, E2F6 and NR3C2 in nRCM and H9C2 cells, and of E2F6 and NR3C2 in nRCF. LNA-based oligonucleotide inhibition of miR-31(miR-31i) in vitro reversed its repressive effect on translation from target genes. Therapeutic modulation of miR-31 expression in vivo after MI via subcutaneous administration of miR-31i (25mg/Kg/q2w) in rats, led to cardiac repression of miR-31 and subsequent enhanced expression of target genes. Also, miR-31i led to preservation of cardiac function and structure by day 14 after treatment. An absolute 10% improvement in left ventricular (LV) ejection fraction (EF) was observed in miR-31i-treated rats from day 2 to 16 after MI, while control rats that received scrambled LNA inhibitor or placebo displayed 23% deterioration in EF ($n=6$-8/group, $P<0.0001$). We conclude that miR-31 induction after MI is deleterious to cardiac function and plays an important role in adverse remodeling, while its therapeutic inhibition in vivo ameliorates cardiac dysfunction and prevents the development of post-ischemic heart failure.

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Interleukins 17 A and 22 in diabetic patients with stable coronary artery disease

Dinaldo C Oliveira, Elayne Heide, Moacyr Rego, Viviane R Gomes, Danielle G Oliveira, Maira G Pita, Federal Univ of Pernambuco, Recife, Brazil

Introduction: The role of the immune and inflammatory pathways in diabetic 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 A and 22 in diabetic patients with stable CAD.
Hypothesis: Interleukins 17 A and 22 are not increased in diabetic patients with stable CAD.
Methods: This is a cross-sectional, prospective, analytical study, conducted from August to December 2012. We included 15 diabetic

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 interleukins (IL). Interleukins were evaluated in serum of patients and after 48 hours of cells in culture with and without stimulus. IL concentrations were expressed in pg / ml. Statistical analysis was performed using the Mann-Whitney or Student t test. P ≤ 0,05 was considered statistically significant.
Results: There were 6 men and 9 women in the group of the diabetic patients and 12 men and 8 women in the controls. The age was similar between the groups (61.6 ± 6.7 years vs 57.9 ± 9.4, p = ns). The main CAD risk factors: hypertension 73%, smoking 60%, dyslipidemia 40%, prior myocardial infarction 33%. The comparison between the groups showed: IL 17 A: Serum: P = 3.91 (3.91 -- 3.91) vs C = 3.91 (3.91 -- 3.91), p = 0.82; culture 48 hours without stimulus: P = 3.91 (3.91 -- 3.91) vs C = 3.91 (3.91 -- 3.91), p = 0.06; culture 48 hours with stimulus: P = 199 (3.91 -- 520) vs C = 154 (3.91 -- 574), p = 0.90. IL 22: Serum: P = 15.63 (15.63 -- 41.09) vs C = 15.63 (15.63 -- 41.09), p = 0.34; culture 48 hours without stimulus: P = 7.81 (7.81 -- 7.81) vs C = 7.81 (7.81 -- 7.81), p = 0.09; culture 48 hours with stimulus: P = 113.79 (7.81 - 248.63) vs C = 322.87 (7.81 - 628.49), p = 0.14.
Conclusions: There were no differences in concentrations of IL 17 A and 22, does not matter in serum or cell in culture. In conclusion in diabetic patients with stable CAD the concentrations of interleukins 17 A and 22 were not increased.

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Interleukin 17 A, Coronary Artery Disease And Gender of The Patients

Dinaldo C Oliveira, Elayne Heide, Moacyr Rego, Danielle A Oliveira, Felipi W Sarinho, Viviane R Gomes, Maira G Pita, Federal Univ of Pernambuco, Recife, Brazil

Introduction: The effects of interleukins during development of coronary artery disease (CAD) have been studied by researchers and it may help to understand better this disease. The aim of this study was to evaluate if there are differences of concentrations of the IL 17 A between men and women with stable CAD. Hypothesis: There are no differences of concentrations of the IL 17 A according to gender of the patients.
Methods: This is a cross-sectional, prospective, analytical study, conducted from August to December 2012. We included 40 patients (24 men (MP) and 16 women (WP) 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 (12 men (MC) and 8 women (WC), to take up comparison of concentrations of interleukins (IL). Interleukins were evaluated in serum of patients and after 48 hours of cells in culture with and without stimulus. IL concentrations were expressed in pg / ml. Statistical analysis was performed using the Mann-Whitney or Student t test. P ≤ 0,05 was considered statistically significant.
Results: The comparison between the groups showed:
IL 17 A: Serum: MP = 3.91 (3.91 - 3.91) vs MC = 3.91 (3.91 - 9.28), p = 0.31; culture 48 hours without stimulus: MP = 3.91 (3.91 - 3.91) vs MC = 3.91 (3.91 - 3.91), p = 0.45; culture 48 hours with stimulus: MP = 451.67 (99.02 - 892.58) vs MC = 135 (3.91 -- 285), p = 0.04.
IL 17 A: Serum: WP = 3.91 (3.91 - 3.91) vs WC = 3.91 (3.91 -- 6.25), p = 0.90; culture 48 hours without stimulus: WP = 3.91 (3.91 - 3.91) vs WC = 3.91 (3.91 -- 3.91), p = 0.20; culture 48 hours with stimulus: WP = 131.21 (3.91 - 231.97) vs WC = 173.78 (3.91 - 642), p = 0.24.
IL 17 A: Serum: MP = 3.91 (3.91 - 3.91) vs WP = 3.91 (3.91 - 3.91), p = 0.17; culture 48 hours without stimulus: MP = 3.91 (3.91 - 3.91) vs WP = 3.91 (3.91 - 3.91), p = 0.47; culture 48 hours with stimulus: MP = 451.67 (99.02 -- 892.58) vs WP = 131.21 (3.91 - 231.97), p = 0.02.
Conclusions: There were no differences of concentrations of the IL 17 A between WP and WC. However, MP had higher concentrations of the IL 17 A than MC and WP. Therefore, it is possible that the IL 17 A take part in a pathway which determines different patterns of CAD according to gender.

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Anesthetic Preconditioning and Mitochondrial Slo K^+ Channel Activity Require *Slo2.1*

Andrew P Wojtovich, C Owen Smith, Sergiy M Nadtochiy, William R Urciuoli, Univ of Rochester Medical Ctr, Rochester, NY; Xiao-Ming Xia, Washington Univ Sch of Med, St Louis, MO; Elizabeth Jonas, Yale Univ Sch of Med, New Haven, CT; Christopher J Lingle, Washington Univ Sch of Med, St Louis, MO; Keith Nehrke, Paul S Brookes, Univ of Rochester Medical Ctr, Rochester, NY

Introduction: Volatile anesthetic preconditioning (APC) protects the heart from ischemia-reperfusion (IR) injury. APC elicits evolutionarily-conserved protective signaling pathways that converge at the mitochondrial level, where the *Slo* family of K^+ channels is thought to mediate protection through an unknown mechanism. Recent work in *C. elegans* has focused attention on the *Slo2* gene product as a transducer of APC effects on hypoxic survival. In mammals, *Slo2* has diverged into two paralogs, *Slo2.1* (KCNT2; Slick) and *Slo2.2* (KCNT1; Slack). These genes code for Na^+-activated K^+ channels and are highly expressed in brain, but their function in cardiomyocytes and/or mitochondria is unknown.
Methods: The contribution of *Slo* channels to cardiac physiology was characterized using knockout mice, including *Slo1* and two novel *Slo2.x* alleles. APC was assessed through *ex-vivo* cardiac IR injury. Isolated mitochondrial K^+ channel activity was assessed using a Tl^+ flux assay, and by patch-clamp of cardiac mitoplasts. Electron microscopy was used to assess mitochondrial morphology in primary cardiomyocytes and Seahorse extracellular flux analysis used to assess bioenergetics.
Results: The *Slo2.x* (*double KO*) and the *Slo2.1* single KO mice could not be protected from cardiac IR injury by APC. Physiologic approaches demonstrated that *Slo2.1* is present in mitochondria and *Slo2.1*-dependent mitochondrial K^+ transport can be triggered directly by volatile anesthetics. Cardiomyocytes from *Slo2.x dKO* mice exhibited profound metabolic remodeling and electron microscopy revealed that *Slo2.1* knockouts had enlarged circular mitochondria. In contrast, *Slo1* KO mice responded normally to APC and exhibited wild type mitochondrial physiology.
Conclusion: *Slo2.1* activation protects against cardiac IR, and is required for APC. *Slo2.1* also contributes to mitochondrial metabolic homeostasis. As a molecular target for APC, identification of *Slo2.1* may facilitate development of targeted therapeutic molecules that can protect the heart and minimize the side effects of volatile anesthetics.

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Paradoxical Exacerbation of Arrhythmias by the Cardioprotective Mitochondrial K-ATP Channel Agonist Diazoxide in Type 2 Diabetes Mellitus

Chaoqin Xie, Basil S Karam, Fadi G Akar, MOUNT SINAI SCHOOL OF MEDICINE, New York, NY

Type 2 diabetes mellitus (T2DM) is a major risk factor for cardiovascular complications including ischemia reperfusion injury (IR). Activation of mitochondrial KATP channels by Diazoxide (DZX) promotes beta cell rest and suppresses glucose production in patients. We hypothesized that DZX prevents IR arrhythmias in T2DM owing to its dual cardioprotective & antidiabetic property.
Methods: Obese Zucker Diabetic Fatty (ZDF) rats (n=17) with established T2DM were studied. Control groups consisted of lean ZDF (n=6) and normal Sprague Dawley (n=10) rats. High resolution optical action potential (AP) mapping was performed in hearts before and after challenge with no flow ischemia for 12min followed by reperfusion.
Results: Basal properties including rate dependence of conduction velocity (CV) and AP duration (APD) were not significantly (p=NS) altered in T2DM. Remarkably, ischemia uncovered major differences between groups as APD in T2DM failed to adapt to the ischemic

challenge. Unlike APD, CV was reduced in all groups. DZX paradoxically promoted arrhythmias as all DZX (30uM) treated T2DM hearts exhibited ischemia related VT. In contrast, untreated T2DM (0/5) and control (0/9) hearts did not exhibit VT during ischemia. Underlying arrhythmic vulnerability of DZX treated T2DM hearts was a pronounced reduction (by 75% $p<0.01$) of the cardiac wavelength (WL) caused by accelerated APD shortening in response to ischemia. Upon reperfusion, T2DM and control groups exhibited a high (60% T2DM vs 44% control, p=NS) rate of VT, although the VT cycle length in T2DM was significantly longer (72 vs 44ms, $p<0.01$) suggesting a more adverse substrate modification by IR injury. Blocking the inner membrane anion channel (IMAC) fully abrogated reperfusion VT in T2DM.
Conclusion: Ischemia uncovers a paradoxical resistance of T2DM hearts to APD adaptation. DZX reverses this property resulting in accelerated APD & WL shortening. This promotes reentrant VT during ischemia. Therefore, the anti-diabetic agent DZX should be avoided in T2DM patients at risk of ischemic events. Instead IMAC is an effective antiarrhythmic target for these patients.

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Targeted Metabolomics Profiling of Heart Failure Patients Undergoing Mechanical Circulatory Support

X'avia Chan, J.H. Howard Choi, Chelsea J.-T. Ju, Wei Wang, Jun Zhang, Jason Tabaraki, David Liem, Martin Cadeiras, Mario Deng, Peipei Ping, UCLA, Los Angeles, CA

Metabolomics investigations hold promise for the characterization of small molecules, metabolites, which govern the ultimate manifestation of cardiac phenotypes. In this study, we employed a mass spectrometry-based metabolomics approach to identify metabolic marker(s), which dynamically reflect the cardiac performance of heart failure patients amid the implantation of mechanical circulatory support. Using the MRM-based and triple quadrupole technology platform, we have quantified 266 metabolites native to human plasma and collected from thirteen heart failure patients. The temporal profile of these metabolites was sampled from 1 day prior to the implantation of mechanical circulatory support, as well as 1-, 3-, 5-, and 7-day following their surgical interventions. We identified subgroups of these metabolites with coordinated behaviors that are interesting to their diseased phenotypes. In a pair-wise correlation analysis, 36.8% (98 out of 266) of metabolites were significantly correlated. Intriguingly, majority of which (65 out of 98) are representing the functional groups of phosphatidylcholines; several of them are known to have close associations with the pathogenesis of cardiovascular diseases. In addition, there are 33 metabolites contributing to multiple functional groups, including twelve of them belong to sphingomyelines, ten of them in the family of lysophosphatidylcholines, eight amino acids (Gln, Ser, Ala, His, Lys, Gly, Thr, and Arg), as well as three fatty acids (eicosapentaenoic acid, pentadecenoic acid, and heptadecenoic acid). The behaviors of these 266 metabolites have constituted individualized metabolic fingerprints. Delineation of the intrinsic relationships among alterations in distinct metabolite groups and their reflected cardiac function will enable us to identify new metabolic markers aiding stratification and/or prediction on the clinical outcome of each individual patient undergoing the treatment of mechanical circulatory support. This personalized metabolic fingerprint will offer unique prognostic utilities, supporting clinical decision-making process to deliver intervention that is most effective and beneficial to an individual.

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Mast Cell Plays Pivotal Role in Nicotine-Induced Atherosclerotic Plaque Progress and Instability

Han Chen, Chen Wang, Yinchuan Xu, Xinyang Hu, Jian-an Wang, Second Affiliated Hosp, Zhejiang Univ Sch of Med, HANGZHOU, China

Objectives: Nicotine has been identified to promote atherosclerosis. But the mechanism of nicotine induced atherogenesis has not been well elucidated. This study focus on the role of mast cell in nicotine induced atherogenesis and plaque instability. Methods: Peritoneal administration of 100mM disodium cromoglicate (DSCG) was introduced to inhibit mast cell degranulation. 45 ApoE deficient mice were divided into 3 groups: high-fat diet, high-fat diet + nicotine, and high-fat diet + nicotine + DSCG. After 12 weeks of treatments, atherosclerotic lesion size of the aortas were quantified. Toluidine blue and tryptase staining identified mast cell count and activation at the lesion. Immuno-staining were used to evaluate the inflammatory filtration, smooth muscle cell proliferation and collagen content in the lesion. In vitro , bone marrow-derived mast cells (BMMCs) were harvested and treated with PBS as a negative control, compound 48/80 as a positive control,100μg/ml nicotine, nicotine with 100mM DSCG pretreatment and nicotine with 10μg/ml mecamylamine pretreatment. At 0.5hr,1hr and 2hrs, supernatants were harvested to analyze the mast cell degranulation. Futhermore, conditioned medium were also used to induce the macrophage migration and foam cell formation. Results: Nicotine increases plaque size, and macrophage infiltration, decreases smooth muscle collagen content along with the increases in mast cells count and activation ratio at the lesion, which could be inhibited by DSCG.Nicotine induced mast cell degranulation at 2 hours comparing to PBS (43.60% vs 2.3%) , which could be inhibited by mast cell stablizer DSCG (23.7%) and nAChR blocker mecamylamine (20.35%). Macrophage migration ability in the compound 48/80 and nicotine conditional medium group were significantly higher comparing to PBS, DSCG and mecamylamine group. Foam cell formation ratio in the compound 48/80 and nicotine conditional group were significantly higher comparing to PBS, DSCG and mecamylamine group. Conclusions: Nicotine induces mast cell degranulation through nAChR and then increases macrophages function, which leads to plaque instability. Administration of mast cell stabilizer showed potential of preventing nicotine induced atherogenesis.

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Cytokine Response to Diet and Exercise Affects Atheromatous Matrix Metalloproteinase-2/9 Activity in Mice

Dong-Eog Kim, DONGGUK UNIV INTERNAT HOSP, Goyang, Korea, Democratic People's Republic of

Objective: To identify the principal circulating factors that modulate atheromatous matrix metalloproteinase (MMP) activity in response to diet and exercise.
Methods and Results: Apolipoprotein-E knock-out mice (n=56) with preexisting plaque, fed either Western diet (WD) or normal diet (ND), underwent either 10-week treadmill exercise or not. In vivo atheromatous MMP activity was visualized using molecular imaging with an MMP-2/9 activatable near-infrared-fluorescent probe. We measured atherosclerosis-related cytokines, lipid levels, visceral fat, and correlated these outcome measures to atheromatous MMP activity. Body weight, visceral fat, and plaque size were all higher in WD-fed animals than in ND-fed animals. Exercise training did not significantly affect these parameters in either WD-fed animals or ND-fed animals. However, atheromatous MMP activity was different: ND animals with and without exercise had similar low MMP activities, WD animals without exercise had high MMP activity, and WD animals with exercise had reduced levels of MMP activity, close to the levels of ND animals. Factor analysis and path analysis showed that soluble vascular cell adhesion molecule (sVCAM)-1 was directly positively related to atheromatous MMP activity. Adiponectin was indirectly negatively related to atheromatous MMP activity by way of sVCAM-1. Resistin was indirectly positively related to atheromatous MMP activity by way of sVCAM-1. In addition, visceral fat amount was indirectly positively associated with atheromatous MMP activity, by way of adiponectin reduction and resistin elevation.
Conclusion: Diet and exercise affects atheromatous MMP activity by modulating the systemic inflammatory milieu, with sVCAM-1, resistin, and adiponectin closely interacting with each other and with visceral fat.

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ABSTRACTS

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S100A6 Regulates Endothelial Cell Cycle Progression via Modulation of the STAT1 Signaling Pathway

Carolin Lerchenmüller, Beth Israel Deaconess Medical Ctr, Cardiovascular Inst, Boston, MA; Julian Heissenberg, Univ Hosp Heidelberg, Heidelberg, Germany; Karsten Peppel, Jefferson Medical Coll, Philadelphia, PA; Hugo A. Katus, Univ Hosp Heidelberg, Heidelberg, Germany; Hauke Busch, Melanie Börries, Albert-Ludwigs-Univ Freiburg, Freiburg, Germany; Patrick Most, Heidelberg Univ Hosp, Heidelberg, Germany

S100A6, also known as Calcyclin, is a 10.5kDa Ca^{2+}-binding protein that belongs to the S100 protein family. We have found S100A6 to be highly expressed throughout all vascular layers during the remodeling process in pig coronary arteries after stenting and rat carotid arteries post balloon dilatation injury. It was the objective of this study to decipher S100A6's function in vascular cells.
Abundant S100A6 expression was confirmed *in vitro* in human umbilical vein endothelial cells (HUVEC) and human umbilical artery smooth muscle cells (SMC). Upon serum stimulation, a significant increase of S100A6 protein levels was observed in both cell types. S100A6 depletion due to siRNA transfection lead to a profoundly reduced proliferation rate in response to growth factors (e.g. VEGF-A), shown by stagnating cell counts and decreased EdU incorporation in HUVEC and SMC. Of note, S100A6 depletion caused a significant migration deficit of SMC. Also, reduced S100A6 levels in HUVEC lead to an increase in cellular senescence, as measured by the expression of senescence associated β-galactosidase expression. To decipher the molecular mechanisms that lead to the phenotype of S100A6 depleted vascular cells, a time-resolved gene expression analysis was carried out in HUVECs and revealed S100A6 to be in control of antiproliferative signaling pathways, while typical pro-proliferative signaling, e.g. the MAPK pathway, was not disrupted. S100A6 depletion caused increased expression and activation of the STAT1 signaling pathway. With increased STAT1 activity, interferon-inducible protein (e.g. IFITM1) expression was upregulated and lead to stabilization and increased expression of the p53 pathway, specifically p21. We propose that S100A6, found to co-localize with the VEGFR2 in a Ca^{2+}-dependent manner, controls antiproliferative signaling by inhibition of the STAT1-phosphorylation by the VEGFR2 and suppression of the STAT1-p53 signaling cascade in human endothelial cells. Endothelialization is a hallmark of vascular healing after stenting and balloon dilatation, for example, while it is of utmost importance to block neointima formation. Understanding these signaling pathways is critical to better direct optimization of interventional therapies.

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Arterial Endothelial Cell Has Stronger Angiogenic Potential Compared To Venous Endothelial Cell Through Up-regulation Of Endogenous FGF2 And FGF5 Expression In 3D Microfluidic System

Ha-Rim Seo, Korea Univ Anam Hosp, Seoul, Korea, Republic of; Hyo Eun Jeong, Korea Univ Dept of Mechanical Engineering, Seoul, Korea, Republic of; Hyung Joon Joo, Seung-Cheol Choi, Jong-Ho Kim, Chi-Yeon Park, Ji-Hyun Choi, Long-Hui Cui, Korea Univ Anam Hosp, Seoul, Korea, Republic of; Seok Chung, Korea Univ Dept of Mechanical Engineering, Seoul, Korea, Republic of; Do-Sun Lim, Korea Univ Anam Hosp, Seoul, Korea, Republic of

Background: Human body contains many kinds of different type of endothelial cells (EC). However, cellular difference of their angiogenic potential has been hardly understood. We compared *in vitro* angiogenic potential between arterial EC and venous EC and investigated its underlying molecular mechanisms.
Method: Used human aortic endothelial cells (HAEC) which was indicated from arterial EC and human umbilical vein endothelial cells (HUVEC) indicated from venous EC. To explore angiogenic potential in detail, we adopted a novel 3D microfluidic angiogenesis assay system, which closely mimic in vivo angiogenesis.
Results: In 3D microfluidic angiogenesis assay system, HAEC demonstrated stronger angiogenic potential compared to HUVEC. HAEC maintained its profound angiogenic

property under different biophysical conditions. In mRNA microarray sorted on up- regulated or down-regulated genes, HAEC demonstrated significantly higher expression of gastrulation brain homeobox 2 (GBX2), fibroblast grow factor 2 (FGF2), FGF5 and collagen 8a1. Angiogenesis-related protein assay revealed that HAEC has higher secretion of endogenous FGF2 than HUVEC**.** HAEC has only up-regulated FGF2 and FGF5 in this part of FGF family. Furthermore, FGF5 expression under vascular endothelial growth factor-A (VEGF-A) stimulation was higher in HAEC compared to HUVEC although VEGF-A augmented FGF5 expression in both HAEC and HUVEC. Those data suggested that FGF5 expression in both HAEC and HUVEC is partially dependent to VEGF-A stimulate. HUVEC and HAEC reduced vascular density after FGF2 and FGF5 siRNA treat.

Conclusion: HAEC has stronger angiogenic potential than HUVEC through up-regulation of endogenous FGF2 and FGF5 expression

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RhoC Regulates VEGF-induced Signaling in Endothelial Cells

Luke Hoeppner, Sutapa Sinha, Ying Wang, Resham Bhattacharya, Shamit Dutta, Xun Gong, Victoria Bedell, Sandip Suresh, Mayo Clinic, Rochester, MN; Chang zoon Chun, Ramani Ramchandran, Medical Coll of Wisconsin, Wauwatosa, WI; Stephen Ekker, Debabrata Mukhopadhyay, Mayo Clinic, Rochester, MN

Vascular permeability factor/vascular endothelial growth factor A (VEGF) is a central regulator of angiogenesis and potently promotes vascular permeability. VEGF plays a key role in the pathologies of heart disease, stroke, and cancer. Therefore, understanding the molecular regulation of VEGF signaling is an important pursuit. Rho GTPase proteins play various roles in vasculogenesis and angiogenesis. While the functions of RhoA and RhoB in these processes have been well defined, little is known about the role of RhoC in VEGF-mediated signaling in endothelial cells and vascular development. Here, we describe how RhoC modulates VEGF signaling to regulate endothelial cell proliferation, migration and permeability. We found VEGF stimulation activates RhoC in human umbilical vein endothelial cells (HUVECs), which was completely blocked after VEGF receptor 2 (VEGFR-2) knockdown indicating that VEGF activates RhoC through VEGFR-2 signaling. Interestingly, RhoC knockdown delayed the degradation of VEGFR-2 compared to control siRNA treated HUVECs, thus implicating RhoC in VEGFR-2 trafficking. In light of our results suggesting VEGF activates RhoC through VEGFR-2, we sought to determine whether RhoC regulates vascular permeability through the VEGFR-2/phospholipase Cγ (PLCγ) /Ca^{2+}/eNOS cascade. We found RhoC knockdown in VEGF-stimulated HUVECs significantly increased PLC-γ1 phosphorylation at tyrosine 783, promoted basal and VEGF-stimulated eNOS phophorylation at serine 1177, and increased calcium flux compared with control siRNA transfected HUVECs. Taken together, our findings suggest RhoC negatively regulates VEGF-induced vascular permeability. We confirmed this finding through a VEGF-inducible zebrafish model of vascular permeability by observing significantly greater vascular permeability in RhoC morpholino (MO)-injected zebrafish than control MO-injected zebrafish. Furthermore, we showed that RhoC promotes endothelial cell proliferation and negatively regulates endothelial cell migration. Our data suggests a scenario in which RhoC promotes proliferation by upregulating -catenin in a Wnt signaling-independent manner, which in turn, promotes Cyclin D1 expression and subsequently drives cell cycle progression.

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ABSTRACTS

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Epigenetic Regulation of Ventricular Development

Youngsook Lee, Eunjin Cho, Matthew Mysliwiec, Univ of Wisconsin-Madison, Madison, WI

Perturbations of the epigenetic machinery can lead to the deregulation of cardiac gene expression, resulting in defective cardiac development and cardiac hypertrophy. Due to a groundbreaking discovery of histone demethylases such as Jumonji (Jmj) family factors, histone methylation is now considered as a reversible epigenetic mark. Jarid2/Jumonji is the founding member of the JMJ family. Jarid2 is enzymatically inactive, but functions as a transcriptional regulator. Jarid2 critically regulates cardiovascular development as well as ES cell differentiation and generation of iPS cells. Jarid2 knockout (KO) mice exhibit cardiac defects including hyper-trabeculation with noncompaction of the ventricular wall. Although Jarid2 interacts with Polycomb Repressor Complex in ES cells, the precise function of Jarid2 in cardiac development remains to be determined. Therefore, we set out to determine molecular mechanisms of Jarid2 critical for cardiac development. To identify cardiac-specific roles of Jarid2, we generated deletion of Jarid2 in early cardiac progenitors using Nkx2.5-Cre Knock-in mice (Jarid2Nkx-KI). Jarid2Nkx-KI mice recapitulate partial phenotypic defects observed in Jarid2 KO including hyper-trabeculation, thin myocardium and ventricular septal defects. By overlapping ChIP-chip and microarray analyses, we have identified potential transcriptional targets of Jarid2, which are occupied by Jarid2, SETDB1, H3K9me3 or H3K27me3, and upregulated in Jarid2 mutant hearts. 174 genes including Isl1 were identified as dysregulated genes that showed accumulation of Jarid2 and H3K27me3. 172 genes including Bmp10 were identified as dysregulated genes and were occupied by Jarid2, SETDB1 and H3K9me3. Isl1, Bmp10/p-Smad1/5/8, and Ifgbp2 were upregulated in Jarid2Nkx-KI hearts by qRT-PCR and Western blotting. Islet1 plays crucial roles in early cardiac development and a marker for cardiac progenitors. Jarid2 occupancy was observed at the Isl1 promoter region by ChIP assays in WT hearts, which was reduced in Jarid2Nkx-KI. All together, our data indicate that Jarid2 regulates target gene expression by interacting with different histone modifiers depending on the cell/promoter context, which is critical for ventricular wall maturation.

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Cardiac Transcription Factor EB Sumoylation Deficiency Exacerbates Age-associated Reduction In Autophagy

Heng Ma, Fourth Military Medical Univ, Xi'an,Shaanxi, China; Le Zhang, Dept of Cardiology, Xijing Hosp, Fourth Military Medical Univ, Xi'an,Shaanxi, China; Lu Yu, Dept of Pathology, Xijing Hosp, Fourth Military Medical Univ, Xi'an,Shaanxi, China; Yan Li, Dept of Cardiology, Xijing Hosp, Fourth Military Medical Univ, Xi'an,Shaanxi, China

Background_Aging-dependent decline of autophagy contributes to cardiac dysfunction and ischemic intolerance. Transcription factor EB (TFEB) is a master transcriptional regulator of the autophagy-lysosome pathway. The present study aimed to characterize the role of TFEB in the autophagic decrease with aging. Methods and Results_We analyzed age-associated autophagic changes in male C57BL/6 young (4-6 mo) and aged (22-24 mo) mice. The results demonstrated that TFEB expressed predominantly as a SUMOylated form in cardiomyocyte nuclei and this SUMOylation of TFEB declined in aged heart associated with autophagy reduction. Interestingly, SUMOylation of TFEB was unaffected by rapamycin. Rapamycin induced translocation of TFEB into nucleus but lower level of nuclear TFEB in aged hearts than that seen in young hearts ($P<0.05$). SUMO1 downregulation by adeno-associated-virus-mediated small hairpin RNA (rAAV9-shSUMO1) significantly reduced nuclear TFEB levels ($P<0.05$), depressed cardiac autophagy and accelerated cardiomyocyte contractile dysfunction with worse hypoxia/reoxygenation (H/R) injury (all $P<0.05$). Therefore, impaired SUMOylation decreased nuclear TFEB during aging. By contrast, SUMO1 restitution

significantly augmented nuclear SUMOylated TFEB with enhanced autophagy and ultimately reduced infarct size in aged heart. However, knockdown of cardiac TFEB blocked the protective effect of upregulation of SUMO1 in aged hearts, resulted in decline of autophagy and worse in vivo I/R injury.
Conclusions_The present study newly demonstrates that SUMOylation is a critical post-translational modification that regulates cardiac TFEB. Impaired SUMOylation of TFEB aggravates decline of autophagy in the senescent heart. Targeting SUMO1 may provide a novel therapeutic strategy for the treatment of aging-related loss of cardioprotection.

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Genome-wide DNA Methylation Analysis Reveals Dynamic Changes in the Cardiac Methylome During Post-natal Heart Development

Choon Boon Sim, The Univ of Queensland, Brisbane, Australia; Mark Ziemann, K.N. Harikrishnan, Antony Kaspi, Jenny Ooi, Lisa Chang, Ishant Khurana, Baker IDI Heart and Diabetes Inst, Melbourne, Australia; Eric N. Olson, UT Southwestern Medical Ctr, Dallas, TX; Assam El-Osta, Baker IDI Heart and Diabetes Inst, Melbourne, Australia; **Enzo R. Porrello**, The Univ of Queensland, Brisbane, Australia

Epigenetic modifications have emerged as central players in the coordination of gene expression networks during cardiac development. Much attention has focused on the role of histone modifications during embryonic heart development, but relatively little is known about the epigenetic control mechanisms that guide post-natal heart maturation. Furthermore, few studies have investigated the role of DNA methylation during cardiac development, despite the fundamental importance of this biological process for transcriptional regulation. The purpose of the current study was to determine whether DNA methylation plays an important role in guiding transcription during the neonatal period, which is an important developmental window for cardiac maturation, including cardiomyocyte cell cycle withdrawal and loss of endogenous regenerative capacity. Here, we interrogated genome-wide changes in transcription and CpG methylation during post-natal cardiac maturation in the mouse (P1 vs. P14). CpG sequencing (CpG-seq) identified 2251 differentially methylated regions (DMRs) between P1 and P14. Intersection of DMRs with different genomic features revealed that 1248 DMRs were associated with promoter regions and transcription start sites. Interestingly, increased methylation of genes associated with well-known signalling pathways for muscle development and differentiation, such as the bone morphogenetic protein, fibroblast growth factor, Wnt and Notch signalling pathways, was associated with transcriptional repression of these regulatory networks at P14. To determine the functional significance of these dynamic changes in the cardiac methylome, we inhibited DNA methylation in vivo by administration of the DNA hypomethylating agent 5-azacytidine from P2 to P12. Post-natal inhibition of DNA methylation caused a marked increase in heart size and was associated with increased cardiomyocyte proliferation. This study provides evidence for widespread alterations in DNA methylation during post-natal heart maturation and suggests that DNA methylation may play an important role in the transcriptional silencing of key regulatory networks for muscle development and cardiomyocyte proliferation during neonatal life.

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Combinatorial Tailored Polymers Enhanced Maturation of Human iPSC-CMs

Young Wook Chun, Tromondae K Feaster, Timothy Boire, Calvin C Sheng, Hak-Joon Sung, Charles C Hong, Vanderbilt Univ, Nashville, TN

There is a tremendous interest in human cardiomyocytes generated from patient-derived induced pluripotent stem cells (iPSC-CMs) for the study and possible treatment of human heart diseases. Despite their vast potential, a significant impediment to a broader application of iPSC-CMs to study human myocyte biology is the structural and functional immaturity of iPSC-CMs. Growing evidence indicates that synthetic polymers utilized as extracellular substrates can exert significant effects on in vitro tissue

generation, although the underlying mechanisms remain largely unknown. Based on the profound impact of the extracellular matrix of developing embryos on in vivo organogenesis, we hypothesize that engineered polymer substrates will likewise influence in vitro maturation of iPSC-CMs. A subset of combinatorial polymers was synthesized by polymerizing poly(ε-caprolacton) (PCL), polyethylene glycol (PEG), and carboxylated PCL (cPCL), abbreviated as x%PEG-y%PCL-z%cPCL (x, y, and z: molar %). We investigated effects of the polymer composition on maturation of iPSC-CMs with respect to the beating behavior, mitochondrial function and molecular profiles after 30 days in culture on polymer scaffolds. Results showed the 4%PEG-96%PCL scaffold promoted the most active beating in iPSC-CMs at 30 days and further, that the mitochondrial function, as assessed by tetramethyl rhodamine methylester (TMRM) was significantly increased in the iPSC-CMs cultured on 4%PEG-96%PCL over other polymers. Molecular profiling analysis indicates 4%PEG-96%PCL scaffolds enhanced the expression of MYL2 (a commonly accepted marker of mature ventricular myocytes) as well as of components of the intermediate filaments linking the plasma membrane to the myofilament. In summary, although the polymers we used here exhibit similar physicochemical properties, they have divergent effects on iPSC-CM differentiation. Thus, specific chemical compositions of synthetic substrates can exert profound influence on in vitro maturation of hiPSC-CMs. Our work exploring the effects of synthetic biomaterials on human stem cell differentiation could pave the way for a successful translation of ongoing advances in tissue engineering to new treatments for human heart diseases.

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A Novel Mutation in a X-linked Gene Causes Human Congenital Dilated Cardiomyopathy

Young Wook Chun, Tromondae K Feaster, Charles H Williams, Calvin C Sheng, Audrey Y Frist, Yan Ru Su, David P Bichell, Charles C Hong, Vanderbilt Univ, Nashville, TN

Congenital dilated cardiomyopathy (cDCM) is a rare but often fatal disease. In most cases, there is no family history, and its etiology is unknown. A major hurdle to elucidating a mechanistic understanding of congenital cardiomyopathy, and primary cardiomyopathies in general, has been a lack of access to diseased human cardiac tissues. Recent advances in patient-derived induced pluripotent stem cells (iPSCs) now enable production of human cardiomyocytes (iPSC-CMs) and allows for a systematic study of normal and diseased cardiomyocytes. We hypothesize that cardiomyocytes generated from iPSCs derived from cDCM patients will exhibit cellular and molecular differences from those generated from healthy donor iPSCs and that a rare genetic mutation, or a collection of mutations, plays a critical role in cDCM pathogenesis. To test these hypotheses, we generated cardiomyocytes from iPSCs derived from a 7-month old male with cDCM using a robust cardiac induction protocol based on the “matrigel sandwich” method of Kamp and colleagues. With this remarkably robust induction method, iPSC-CMs from the cDCM patient and a healthy control donor exhibited proteomic profiles that were 99.7% superimposable. Despite the close similarity at the global proteome level, iPSC-CMs from the cDCM patient showed greatly reduced contractility and dramatic structural defects in the sarcomere and the mitochondria. Finally, bioinformatics analyses of the RNAseq data of the patient’s iPSC-CMs discovered a putative causal mutation in an evolutionarily conserved site in a X-linked gene with unknown function. In summary, our work demonstrates that iPSC-based approaches are particularly useful for the study of human congenital heart diseases. We plan to confirm the causality of this mutation using gene editing techniques such as CRISPR/Cas9 and explore the role of this novel gene in cardiomyocyte structure and function.

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Optical Mapping of Host and Human Embryonic Stem Cell-Derived Cardiomyocyte Graft Electrical Activity in Injured Hearts

Dominic Filice, Wei-Zhong Zhu, Benjamin Van Biber, Kip D Hauch, Michael A Laflamme, Univ of Washington, Seattle, WA

Human embryonic stem cell-derived cardiomyocytes (hESC-CMs) show tremendous promise for cardiac repair, but more information is required as to their electrical behavior in vivo. hESC-CMs expressing the protein calcium sensor GCaMP3 provide a graft autonomous reporter of activation, which we have used to show hESC-CM grafts can couple with host myocardium in injured hearts. When we sought to assess host-graft electrical interactions by optical voltage mapping, we found the commonly used lipophilic voltage dyes RH237 and di-4-ANEPPS label host but not graft tissue. We hypothesized the water-soluble voltage dye di-2-ANEPEQ could overcome this limitation and efficiently label both host and graft. After confirming good spectral separation of GCaMP3 and di-2-ANEPEQ signals by spectrofluorimetry and confocal spectral imaging, we transplanted 1x10^8 GCaMP3+ hESC-CMs into guinea pig hearts (n=6) at 4 weeks following cardiac injury and then imaged engrafted hearts at 2 weeks post-transplantation with a dual-channel CCD-based system. We found a differential time-course of di-2-ANEPEQ labeling between host and graft tissues, with stable optical action potentials (APs) obtained in host and graft tissue after ~4 and ~13 minutes of dye perfusion, respectively. This differential labeling kinetics, which was also observed on washout, presumably reflects sluggish graft perfusion and provides another tool for distinguishing host and graft signals. No regions of 1:1 host-graft coupling were identified, and graft tissue had spontaneous rates from 0.1-2 Hz and long optical AP durations from 500-1090 ms. Activation maps based on di-2-ANEPEQ and GCaMP3 signals indicated relatively slow conduction velocities in graft tissue, as well as patterns of propagation that commonly occurred along a vector distinct from that in host tissue. These imaging studies reveal multiple potentially pro-arrhythmic properties in hESC-CM graft tissue including slow propagation, ultralong AP duration, as well as aberrant patterns of activation that can vary from beat to beat. We conclude the electrical behavior of both graft and host myocardium can be reliably assessed by the simultaneous imaging of a graft-autonomous fluorescent reporter of activation and a water-soluble voltage dye.

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Parthenogenetic Stem Cell-derived Cardiomyocytes Express Major Histocompatibility Complex-I only after Inflammatory Stimulation

Satish Galla, Michael Didie, Vijayakumar Muppala, Inst of pharmacology, Goettingen, Germany; Ralf Dressel, Inst for cellular and molecular immunology, Goettingen, Germany; Wolfram Hubertus Zimmermann, Inst of pharmacology, Goettingen, Germany

Background: Pluripotent parthenogenetic stem cells (PSCs) can be directed towards a cardiac fate and utilized in tissue engineered heart repair. In vivo applications of tissue engineered allografts are compromised by expression of mismatching major histocompatibility complex proteins (MHC; encoded in the murine H2 locus). Here we investigated whether PSC-derived cardiomyocytes (CM) express MHC-I. **Methods:** Mouse PSCs (A3-line from B6D2F1 strain with haploidentical $H2K^d$-locus) expressing a CM-specific neomycin-resistance and GFP were differentiated and purified for CM by addition of G418 (85% purity by FACS for actinin). To simulate heart muscle biology in vitro, we made use of engineered heart muscle (EHM) constructed from PSC-derived CM (75%), growth-inhibited murine embryonic fibroblasts (MEF (25%); NMRI mice), and collagen type I. MHC class-I $H2K^d$ (MHC-I) expression was assessed on CM and Non myocytes before EHM assembly and from enzymatically digested EHMs (cultured for 10 days) by FACS. Interferon gamma (IFNγ) was added for 48 h to stimulate MHC-I expression. As a reference, we investigated MHC-I expression in CM from neonatal mice and adult mouse hearts by FACS and by immunofluorescence staining. **Results:** EHM showed a positive ionotropic response to beta-adrenergic stimulation which could be reduced by muscarinergic stimulation. A3-CM, in contrast to Non myocytes, showed negligible expression of MHC-I (1±0.5% vs. 60±10% positive cells; n=3). EHM culture did not change MHC-I expression in CM. IFNγ treatment resulted in a marked increase of MHC-

I-expression in CM monolayer culture (40±6%; n=3) and in EHM (30±8%; n=3). For comparison, 30% (n=2) neonatal CM expressed MHC-I while MHC–I was not detectable in adult CM.
Conclusion: PSC-derived CM show a similarly low expression of MHC-I as adult CM and respond with MHC-I upregulation to IFNγ stimulation. This suggests a mature immunological response in PSC-CM with important implications for in vivo applications, i.e., MHC-I matching will likely be a prerequisite for successful allografting of PSC-EHM.

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Role for β-Arrestins in Cardiac Inflammation and Regenerative Repair

Anna M Gumpert, Temple Univ, Philadelphia, PA; Mai Chen, Henriette Brinks, Karsten Peppel, Thomas Jefferson Univ, Philadelphia, PA; Erhe Gao, Walter J Koch, Temple Univ, Philadelphia, PA

Myocardial ischemic injury is characterized by an extensive loss of myocytes due to apoptosis/necrosis followed by an immune/inflammatory response and reestablishment of tissue integrity by a fibrotic scar. Prevention of rupture due to scar formation however, compromises contractility and reduces cardiac function. As bone marrow derived stem/precursor cells (BMSCs) have the ability to trans-differentiate and aid in the regeneration of injured myocardium, we studied BMSCs and their response to inflammation. BM cell mobilization, egress from the BM and recruitment to the site of injury can be regulated by signals through G protein-coupled receptors (GPCRs). β-arrestins are known for their GPCR signaling termination and scaffolding functions. Here, we explored the potential role for β-arrestins expressed in total BM in mediation of the initiation and progression of inflammation. We investigated the role β-arrestin1 (βArr1) and β-arrestin2 (βArr2) with respect to modulation of regenerative competence of BMSCs and inflammatory cells and their contribution to cardiac repair following ischemic injury. We carried out BM transplants to determine whether βArrs may be involved in cardiac repair. WT mice were irradiated and received BM transplants from WT, βArr1 or βArr2 KO donor mice. Subsequent to BM reconstitution, mice underwent MI and their recovery and progress were followed. Interestingly, βArr1 and βArr2 KO chimeras had inferior outcomes than mice receiving WT BM. This included significantly decreased post-MI survival with βArr2 KO BM and both βArr chimeras had significantly lower cardiac function post-MI compared to WT BM recipients. Histology shows that both chimeras had larger infarcts, accelerated rate of hypertrophy, quicker induction of fibrosis, exacerbated cell infiltration and increased number of apoptotic cells. At 3d post-MI immune cell intrusion of βArr2KO chimeric hearts was highest and the blood plasma content of TNFα and IL-6 was increased. We conclude that βArrs play a novel role downstream of GPCR desensitization in cardiac progenitor/inflammatory cells in the BM and are critical in the heart's response to ischemic injury via cardiac repair and regeneration.

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Pregnancy Hormones Induce An Increase Of Capillary Density In The Mouse Heart

Michael Hesse, Daniel Korzus, Kristina Thaben, Bernd K Fleischmann, Univ Clinic Bonn, Bonn, Germany

During pregnancy the maternal organism undergoes a variety of adaptive physiological changes. The cardiovascular system is strongly affected and its response is characterized by cardiac hypertrophy. The molecular mechanisms underlying these adaptations are still poorly understood. The recent postulation of resident organotypic stem cells in the cardiovascular system prompted us to explore the potential involvement of these cells and the related signaling cascades involved in this adaptation process in the heart.
We have analyzed cell proliferation using immunostainings in mouse hearts at different stages during pregnancy. The average percentage of proliferating cells in cardiac sections increased from virgin controls to

gestational day 3 (GD3), peaked at GD14, and immediately stopped after delivery. Co-staining revealed that the majority of proliferating cells were fibroblasts and endothelial cells (ECs). The lack of cardiomyocyte proliferation was corroborated using BrdU pulse chase experiments analyzed in single cells upon Langendorff perfusion. The proliferation of fibroblasts and ECs reflects angiogenesis, which was underscored by an increase of capillary density, and extracellular matrix remodeling known to occur during pregnancy induced hypertrophy. To mimic the action of pregnancy hormones we implanted pellets with constant release of hormone for 21 days subcutaneously in ovariectomized mice. Capillary density was significantly increased in mice receiving progesterone-pellets alone or in combination with estrogen-pellets without induction of hypertrophy. Thus, increase of capillary density is directly induced by pregnancy hormones. Assessment of differentially expressed genes after hormone treatment revealed induction of angiogenetic factors and their receptors. We are currently investigating the mechanisms underlying the pregnancy hormone-induced proliferation in cardiac ECs and fibroblasts.

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Collagen Nanopatterning Modulates Endothelial Cell Atheroprotective Function, Survival, and Angiogenesis

Ngan F. Huang, Stanford Univ, Palo Alto, CA; Edwina Lai, Stanford Univ, Stanford, CA; Tatiana Zaitseva, Michael V Paukshto, Fibralign Corp, Union City, CA; John P Cooke, Houston Method Res Inst, Houston, TX

Vascular endothelial cells (ECs) are longitudinally oriented in regions of laminar flow, but randomly distributed in regions of disturbed flow. The unaligned ECs in disturbed flow fields manifest altered function and reduced survival that promote atherosclerotic lesion formation. We hypothesized that the alignment of the ECs may directly influence their biology, independent of fluid flow. We fabricated parallel-aligned nanofibrillar collagen scaffolds with that mimic the native structure of collagen extracellular matrix within blood vessels, and examined the effects of aligned collagen nanopatterning on EC alignment, function, and in vivo survival. The 30-nm diameter aligned nanofibrils reoriented F-actin assembly along the nanofibril direction. ECs cultured on aligned nanofibrils were also 50% less adhesive for monocytes than the ECs grown on randomly oriented fibrils. To test the efficacy of the aligned nanofibrillar scaffolds in improving neovascularization in vivo, we induced unilateral hindlimb ischemia in SCID mice by excising the superficial femoral artery. The mice received one of the following treatments at the site of the excised femoral artery: 1) aligned nanofibrillar scaffold seeded with human ECs; 2) non-patterned scaffold seeded with ECs; 3) EC delivery in saline; or 4) no treatment (n>4). After 14 days, laser Doppler blood spectroscopy demonstrated significant improvement in blood perfusion in the group treated with cell-seeded aligned nanofibrillar scaffolds, in comparison to the groups with no treatment or EC delivery in saline. Furthermore, based on non-invasive bioluminescence imaging, the transplanted ECs persisted for longer periods of time when cultured on aligned nanofibrillar scaffolds, in comparison to non-patterned scaffolds. Together, these studies demonstrate that 30-nm aligned nanofibrillar collagen scaffolds guide cellular organization, modulate endothelial inflammatory response, and enhance cell survival and angiogenesis after implantation in the ischemic hind limb. These results have important implications for therapeutic cell delivery approaches.

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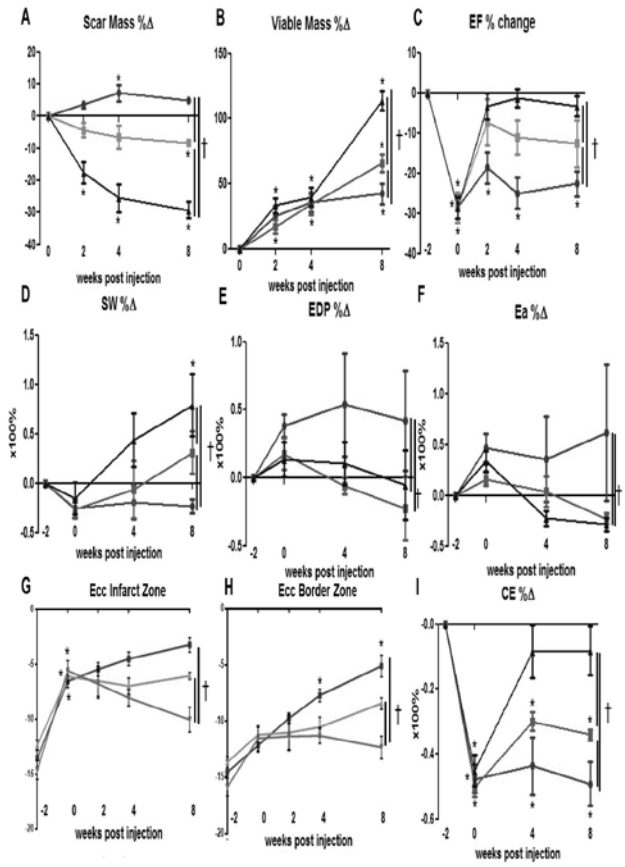
Pim1 kinase Overexpression Enhances ckit+ Cardiac Stem Cells Cardioreparative Ability After Intramyocardial Delivery

Vasileios Karantalis, Viky Y Suncion, Frederic C McCall III, Luiza L Bagno, Bo Wang, Samuel Golpanian, Univ of Miami Miller Sch of Med, Miami, FL; Sadia Mohsin, San Diego State Univ, San Diego, CA; Jose E Rodriguez, David Valdes, Marcos Rosado, Wayne Balkan, Muzammil Mushtaq, Univ of Miami Miller Sch of Med, Miami, FL; Mark A Susman, San Diego State Univ, San Diego, CA; Joshua M Hare, Univ of Miami Miller Sch of Med, Miami, FL

Background: Pim-1 kinase plays an important role in cell division, survival and commitment towards myocardial lineage. We hypothesized that Pim-1 overexpression in ckit+ cardiac stem cell (CSCs) enhances cardioreparative effects. Methods: Immunosuppressed Yorkshire swine (n=31) received human ckit+ CSCs (n=9), Pim1 modified human ckit+ CSCs (n=9) or PBS (n=13) two weeks after myocardial infarction. Cardiac MRI and PV loops were obtained before and after cell administration. Results: At 8 weeks post transplantation, scar mass (Fig. 1A), viable tissue (Fig. 1B), ejection fraction (Fig. 1C) and stroke work (Fig. 1D) was significantly improved in Pim-1 modified ckit+ CSC compared to control ckit+, while both cell groups showed partial recovery compared to placebo (two way ANOVA, $p<0.05$). Both cell types similarly reduced preload (end diastolic pressure; Fig. 1E) and afterload (Arterial elastance; Fig 1F) compared to placebo, while only administration of Pim-1 CPCs improved regional contractility at both the infarct (Fig. 1G) and border zones (Fig. 1H). Collectively, mechanoenergetic recoupling was superior in the Pim-1 group compared to ckit+ controls (Cardiac Efficiency; Fig. 1I). Conclusions: Cardioreparative potential of CSCs delivered by intramyocardial injection to infarcted porcine hearts is significantly enhanced by overexpress Pim1, supporting translational development of Pim-1 as a validated genetic modification of CSCs for incorporation into clinical trials.



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Embryonic Stem Cell Derived Exosomes Revive Endogenous Repair Mechanisms In Failing Heart

Mohsin Khan, Suresh K Verma, Alexander R Mackie, Erin Vaughan, Srikanth Garikipati, Erin Lambers, Veronica Ramirez, Kenneth Ellingson, Tatiana Abramova, Aiko Ito, Prasanna Krishnamurthy, Sol Misener, Gangjian Qin, Walter J Koch, Raj Kishore, Temple Univ, Philadelphia, PA

Rationale: Embryonic stem cells (ESCs) hold great promise for cardiac regeneration but are susceptible to ethical concerns, lack of autologous donors and teratoma formation. Recently, it has been observed that beneficial effects of stem cells are mediated by exosomes secreted out under various physiological conditions. ESCs have the ability to produce exosomes however their effect in the context of the heart is unknown.
Objective: Determine the effect of ESC derived exosomes for cardiac repair and modulation of CPCs functions in the heart following myocardial infarction.
Methods and Results: Exosomes were isolated from murine ESCs (mES Ex) or embryonic fibroblasts (MEFs) by ultracentrifugation and verified by Flotillin-1 immunoblot analysis. Induction of pluripotent markers, survival and in vitro tube formation was enhanced in target cells receiving ESC exosomes indicating therapeutic potential of mES Ex. mES Ex administration resulted in enhanced neovascularization, cardiomyocyte survival and reduced fibrosis post infarction consistent with resurgence of cardiac proliferative response. Importantly, mES Ex mediated considerable enhancement of cardiac progenitor cell (CPC) survival, proliferation and cardiac commitment concurrent with increased c-kit+ CPCs in vivo 4 weeks after mES Ex transfer. miRNA Array analysis of ESC and MEF exosomes revealed significantly high expression of miR290-295 cluster in the ESC exosomes compared to MEF exosomes. The underlying beneficial effect of mES Ex was tied to delivery of ESC miR-294 to the heart and in particular CPCs thereby promoting CPC survival and proliferation as analyzed by FACS based cell death analysis and CyQuant assay respectively. Interestingly, enhanced G1/S transition was observed in CPCs treated with miR-294 in conjunction with significant reduction of G1 phase.
Conclusion: In conclusion, mES Ex provide a novel cell free system for cardiac regeneration with the ability to modulate both cardiomyocyte and CPC based repair programs in the heart thereby avoiding the risk of teratoma formation associated with ESCs.

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Ox-LDL Impairs The Survival Of Bone Marrow Stem Cells Partially Through Membrane Damage Independent Of ROS Production In Vitro

Xin Li, Yuan Xiao, Yuqi Cui, Hua Zhu, Chandrakala A Narasimhulu, Jia Zhang, Hong Hao, Lingjuan Liu, Xiaoyun Xie, Guanglong He, Jianjie Ma, Catherine M Verfaillie, Sampath Parthasarathy, Zhenguo Liu, The Ohio State Univ Medical Ctr, Columbus, OH

Aims: cell-based therapy with bone marrow stem cells (MSCs) remains a viable option for tissue repair and regeneration. One of the major challenges for cell-based therapy is the limited survival of the cells after in vivo administration. The exact mechanism(s) for impaired in vivo survival of the implanted MSCs remains to be defined. Oxidized low-density lipid protein (ox-LDL) is a natural product in human blood, and the major contributor to the development of atherosclerosis. The present study was to investigate the effect of ox-LDL on the survival of bone marrow stem cells and the mechanisms in vitro. **Methods and Results:** Rat bone marrow multipotent adult progenitor cells (MAPCs) were treated with ox-LDL (with the final concentration of 10 and 20 ug/ml) for up to 48 hours. Exposure to ox-LDL resulted in significant cell death and apoptosis of MAPCs in association with a significant increase in LDH release in the conditioned media in a dose- and time-dependent manner, indicating significant cell membrane damage. The membrane damage was further confirmed with the rapid entry of the small fluorescent dye FM1-43 as detected using confocal microscope. Ox-LDL generated a significant amount of reactive oxygen species (ROS) in the culture system as measured with electron paramagnetic resonance spectroscopy. The antioxidant N-acetylcysteine (NAC, 0.1 mM) completely inhibited the production of ROS from

ox-LDL. However, it didn't prevent ox-LDL-induced cell death or apoptosis. However, pre-treatment of the cells with the specific membrane protective recombinant human MG53 protein (rhMG53)(66 ug/ml, final concentration) significantly, reduced LDH release and the entry of FM1-43 dye into the cells exposed to ox-LDL. **Conclusion:** Ox-LDL enhanced cell death and apoptosis of MAPCs with a mechanism independent of ROS generation in vitro. Ox-LDL impaired the survival of MAPCs partially through cell membrane damage in vitro.

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Age dependent regenrative ability of ckit+ cells Derived from Neonatal and adult Hearts

Rachana Mishra, Rachana Mishra, Sudhish Sharma, Savitha Desmukh, Keerti Balachandaran, Ling Chen, Sunjay Kaushal, Univ of Maryland, Baltimore, MD

Background—Human adult c-kit$^+$ cardiac stem cells (CSCs) alleviates post-myocardial infarction left ventricle dysfunction in animal models and a Phase I clinical study. The regenerative capacity of CSCs in the very young patients with non-ischemic congenital heart defects has not been explored, even in the most surgically challenging Hypoplastic Left Heart Syndrome (HLHS) patients. We hypothesized that isolated neonatal-derived ckit$^+$ CSCs have higher regenerative abilities than adult-derived CSCs and might address the anatomical deficiency of HLHS myocardium. ***Methods and Results***—Human specimens were obtained during routine cardiac surgical procedures from right atrial appendage tissue discarded from 2 age groups: neonates and adults patients. We developed a reproducible isolation method that generated c-kit$^+$ cells using immune-activated magnetic bead selection, regardless of the initial weight or age. More than 85% ckit+ cells were isolated from both groups and these cells were negative for tryptase, collagen, CD45, CD34 and CD31. Single cell proliferation assay showed neonatal ckit+ cells are significantly more proliferative compare to adult ckit$^+$ cells. Neonatal c-kit$^+$ cells showed significantly higher telomere length (p=0.0286) compare to Adult. The neonatal-derived c-kit$^+$ cells secreted higher levels of VEGF-A, ANG, and SDF-1α when compared to adult-derived c-kit$^+$ cells. When transplanted into infarcted myocardium, neonatal-derived c-kit$^+$ cells had a significantly higher ability to preserve myocardial function, prevent adverse remodeling, and enhance blood vessel preservation when compared to adult-derived c-kit$^+$ CSCs. . Treatment with Neonatal-derived ckit$^+$ cells also augmented the preservation/formation of neovessels (isolectin B4) and arterioles (α-SMA) compared to adult-derived ckit$^+$ cells ***Conclusions***—Neonatal-derived c-kit$^+$ cells have a strong regenerative ability when compared with adult-derived c-kit$^+$ cells that may depend on angiogenic cytokines. This has important implications in the potential use of CSCs in future HLHS clinical trials.

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Cardiomyocyte Specific Conditional Overexpression Of Stromal Cell Derived Factor 1 Facilitates Cardiac Regeneration After Permanent Coronary Artery Ligation In Mice.

Detlef Obal, Kenneth Brittian, Michael Book, Aruni Bhatnagar, Yiru Guo, Roberto Bolli, Gregg Rokosh, Univ of Louisville, Louisville, KY

Background: Interruption of cardiac stromal cell derived factor 1 (SDF1)-CXCR4 axis by chronic AMD3100 administration increased myocardial injury after permanent coronary artery ligation demonstrating the important role of this chemokine in cardiac regeneration. Hypothesis: Cardiomyocyte specific conditional overexpression of SDF1 prevents heart failure after permanent coronary ligation and facilitates cardiac regeneration. Methods and Results: Tetracycline-controlled, αMyHC promoter directed overexpression of cardiac SDF1, resulted in a significant increase of SDF1 expression (SDF1: 8.1 ng/mg protein) compared to littermate WT mice (0.02 ng/mg protein) four weeks after doxycycline withdraw.

SDF1 overexpression increased AKT and casein kinase 1 levels in the heart. Although there was no difference in cardiac function and scar size 1 week after infarction, SDF1 overexpression improved left ventricular (LV) ejection fraction (SDF1 [n=13]: 47±5% [mean±SEM] vs. WT [n=15]: 29±4%, p<0.05) decreased end-diastolic volume (78±10 vs. 158±30, p<0.05) and reduced infarct size measured by trichrome staining (13±3% vs. 23±3% of LV wall, p<0.05) 4 weeks after permanent ligation. Bromodeoxyuridine (BrdU) staining revealed increased regeneration indicated by a 5-fold increase in $BrdU^+$cardiomyocyte (CM) nuclei in the borderzone of the infarct (22±3% vs. 5±1% CM nuclei, p<0.01). Increased proliferation in SDF1 mice was confirmed by a higher number of $KI67^+$ cells compared to WT mice. Cardiomyocyte cross sectional area in the border zone was significantly reduced in SDF1 mice (365±13 μm^2 vs. 434±10 μm^2, p<0.001) while capillary density was unchanged (2348±151/ mm^2 vs. 2498±153/ mm^2) compared to WT mice. Conclusion: This study demonstrates for the first time that cardiac specific overexpression of SDF1 increases myocardial regeneration and improves LV function 4 weeks after permanent coronary ligation.

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Novel Transgenic Mouse Lines for Identification of Cardiomyocyte Nuclei and Visualization of Their Cell Cycle Status In-vitro and In-vivo

Alexandra Raulf, Hannes Horder, Caroline Geisen, Sabine Grünberg, Patricia Freitag, Bernd K. Fleischmann, Michael Hesse, Univ of Bonn, Bonn, Germany

The typical remodelling process after cardiac injury is scarring and compensatory hypertrophy. The limited regeneration potential of the adult heart is due to the post-mitotic status of cardiomyocytes (CMs), which are mostly binucleated. Nevertheless, there is evidence for CM turnover in the adult heart, but its extent is still under debate. One technical limitation of quantitations is the unequivocal identification of In order to enable a clear identification of CM nuclei in-vivo, we have developed a transgenic mouse line in which a fusion protein of the human histone 2B and the red fluorescence protein mCherry is specifically and persistently expressed in CM nuclei (αMHC-H2B-mCh). The fluorescence label allowed the investigation of CM percentages in native tissue slices, which properly reflect the cellular composition of the heart. We focused on regional (atrium versus left and right ventricle, apical versus basal slices) and developmental-stage dependent changes in the percentage of CM nuclei and binuclearity. We therefore analyzed time-points before/during and after terminal differentiation of CMs (postnatal day 3 (P3), P7 and 9 weeks). In addition, we also investigated endoreduplication and acytokinetic mitosis using time lapse microscopy in postnatal CMs to better understand cell biological mechanisms leading to terminal differentiation.
As currently huge efforts are invested for the search of substances that increase the regeneration potential of the heart, we established a novel screening assay for cell-cycle modifying substances in isolated, postnatal CMs. We crossed the αMHC-H2B-mCh with the CAG-eGFPanillin mouse line, which marks cell-cycle activity with a high resolution of M-phase. Analysis of binuclearity and of different eGFPanillin subcellular localizations will be helpful to understand, whether CMs complete cytokinesis. As a proof of principle we investigated the effects of cell cycle activating micro RNAs199 and 590.
Thus, our double transgenic mouse line will be useful to examine the plasticity of mono- and binuclear CMs and to unravel cell biological mechanisms leading to terminal differentiation of CMs.

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Low Dose Particle Radiation Affects Long-Term Survival of Bone Marrow Progenitor Cell Populations

Sharath Sasi, GeneSys Res Inst, Boston, MA; Daniel Park, Genesys Res Inst, Boston, MA;

ABSTRACTS

BOSTON UNIVERSITY SCHOOL OF MEDICINE, Boston, MA; Xinhua Yan, GenSys Res Inst, TUFTS UNIVERSITY SCHOOL OF MEDICINE, Boston, MA; David A Goukassian, GeneSys Res Inst, TUFTS UNIVESITY SCHOOL OF MEDICINE, Boston, MA

Radiation-induced decreases in the number of bone marrow (BM)-derived endothelial progenitor cell (BM-EPCs) and their lineage precursors which include Early- and Late-Multi-Potent Progenitor cells (E-MPP and L-MPP) could contribute to the pathogenesis of ischemic and vascular diseases. We examined the effect of full-body single dose of proton (1H) at 0.5 Gy, 1 GeV and 0.15 Gy, 1 GeV/nucleon of iron (56Fe) - ionizing radiation (IR) on survival and proliferation of BM-EPCs. The survival of E-MPPs and L-MPPs in the BM after particle IR in C57BL/6 mice were determined at 1, 2, 4, 8, 12, 28 and 40 weeks post-IR. BM-derived mononuclear cells were triple-stained with RAM34 (CD34, c-kit, and Sca1), AC133, and hematopoietic lineage negative cocktail, then sorted by FASC for E- and L-MPP. BM EPCs ex-vivo - There was a transient 2.5-3.5-fold increase in BM-EPC apoptosis, with 3.5-fold increases for 56Fe and 1H at 5hrs and 24hrs, respectively that was no longer detected by day 7. Subsequently, there was a 3-fold increase in BM-EPC apoptosis on day 28 for both ion-IR mice. Compared to 24 hrs, there was a ~20% (1H) and ~45% (56Fe) increase in the rate of EPC proliferation on day 14 that returned to control levels on day 28. BM E-MPP and L-MPP in vivo - Compared to control mice, 1H-IR increased the number of both E-MPPs (665%) and L-MPPs (203%), whereas 56Fe-IR decreased E-MPP (74%) and L-MPPs (65%) at 1 week post-IR, suggesting stimulation by 1H but overt damage by 56Fe in the BM milieu. In 56Fe-IR mice, E-MPPs recovered between 4 and 12 weeks, followed by declines at later time points. In 1H-IR mice, E-MPPs were near control levels up to 4 weeks, but declined at later time points. The long-lasting and cyclical effects of IR on the BM E- and L-MPPs after a single 1H or 56Fe IR dose suggests the presence of prolonged and non-targeted effects in BM milieu, that occur in cells that were not traversed by IR, rather induced by signals from IR cells. Our studies showed that, both 1H- and 56Fe-IR has profound and long-lasting (28-40 months) negative effects on the number of E- and L-MPPs. Future longitudinal studies are necessary to determine whether BM progenitor cells may be affected after terrestrial IR exposure, such as cancer radiotherapy, CT and PET scans, and in astronauts after exploration-type space missions.

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Reprogramming of Pig Cardiac Fibroblasts to Cardiomyocyte Fate: Implications for Gene Therapy to Treat Myocardial Infarction

Vivek P Singh, Megumi Mathison, Xueping Xu, Vivek K Patel, Narasimhaswamy S Belaguli, Brian W Gibson, Austin J Cooney, Todd K Rosengart, Baylor Coll of Med, Houston, TX

There is an urgent clinical need to develop new therapeutic approaches to treat heart failure, but the biology of cardiovascular regeneration is complex. A new generation of reprogramming technology involves trans-differentiating one adult somatic cell type directly into another. We reported previously that administration of gene transfer vectors encoding Gata4 (G), Mef 2c (M) and Tbx5 (T), reprograms rat cardiac fibroblasts into induced cardiomyocytes (iCMs) in vitro and improves cardiac function in myocardial infarction models. Previous cardiac reprogramming studies were restricted to rodent and human fibroblasts. However studies in large animal models relevant for pre-clinical studies is lacking.

The Aim of the present study was to determine the optimal combination of factors necessary for direct reprogramming of porcine fibroblasts towards a cardiomyocyte lineage for the first time. Here we have used human lentiviral system to express various cardiomyocyte enriched transcriptional regulators such as GMT, Hand2, myocardin, and two microRNAs, miR-590 and mir-199, in porcine cardiac fibroblasts. Reprogramming of fibroblasts into iCMs was determined 4 weeks post-virus transduction by FACS analysis for the activation of endogenous cardiac troponin T (cTnT). GMT alone was not sufficient to reprogram porcine fibroblasts although this combination

was shown previously to be sufficient to convert rodent fibroblasts into iCMs. Addition of mir-199 to GMT was also not sufficient to promote reprogramming. However, adding miR-590 or Hand2, and myocardin (HM) to GMT resulted in reprogramming, although the efficiency remained low. Together, our results show that combination of 5 distinct transcription regulators (GMTHM) or GMT plus mir-590 is necessary for reprogramming porcine fibroblast. These findings demonstrate that pig fibroblasts can be directly reprogrammed toward the cardiomyocyte lineage, and represent a step toward possible therapeutic application of this reprogramming approach in a pre-clinical setting.

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Gel Stiffness-tuned, Nanostructure-templated Alignment and Maturation of Cardiomyocytes For Cardiac Tissue Engineering

Eduard Sleep, Jason R Mantei, Mark T McClendon, Samuel I Stupp, Northwestern Univ, Chicago, IL

Objective: To investigate how the supramolecular structure and mechanical properties of hydrogels made of chemically-defined peptide amphiphiles (PAs) could influence i) the alignment of cardiomyocytes seeded in them and ii) the functional maturation of the cell construct. **Methods**: We generated a series of PAs with different peptide sequences that allowed us to make PA gels with stiffness values ranging three orders of magnitude. The nature of the gelation process provides these gels with same-direction oriented nanofibers along an elongated PA gel. We seeded these PA gels with either HL-1 cardiomyocytes or iPSC-derived cardiomyocytes and cultured them for up to two weeks. We then measured cell survival, proliferation and alignment over time and also the electrophysiological properties of the cell construct as a whole. **Results**: We found that cardiomyocytes responded to the alignment of the nanofibers in the gel by aligning to them and that this process can be tuned by changing the stiffness of the PA gel. This in turn influenced the investigated electrophysiological parameters of the cell construct suggesting a functional maturation induced by the PA gel properties. **Conclusions**: We believe this study shows for the first time that the nanostructural and mechanical properties of hydrogels can be exploited to influence the maturation of cardiomyocytes/cell construct. This can have important implications for strategies that aim to use ESC- or iPSC-derived cardiomyocytes in tissue engineering, as there is a need for their proper maturation and function.

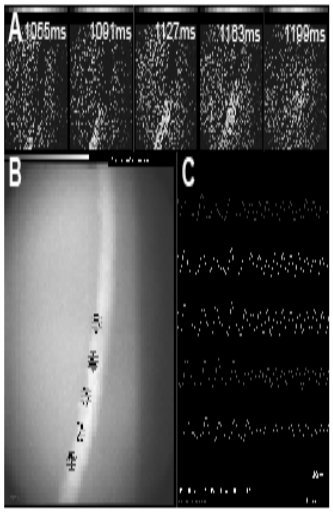

Figure 1. Calcium imaging of cells constructs at day 10 in culture. Cell constructs were incubated with Calcium Green-1 and imaged under fluorescence using a high-speed camera. A) Snapshots of the recorded calcium transients propagation. B) Macroscopic image of the recorded cell construct depicting the areas chosen to show the fluorescence read in C). C) Spikes of fluorescence appear at ~2Hz due to the intrinsic pacing activity of HL-1 cardiomyocytes but once externally paced, the spikes appear at a frequency of 3Hz

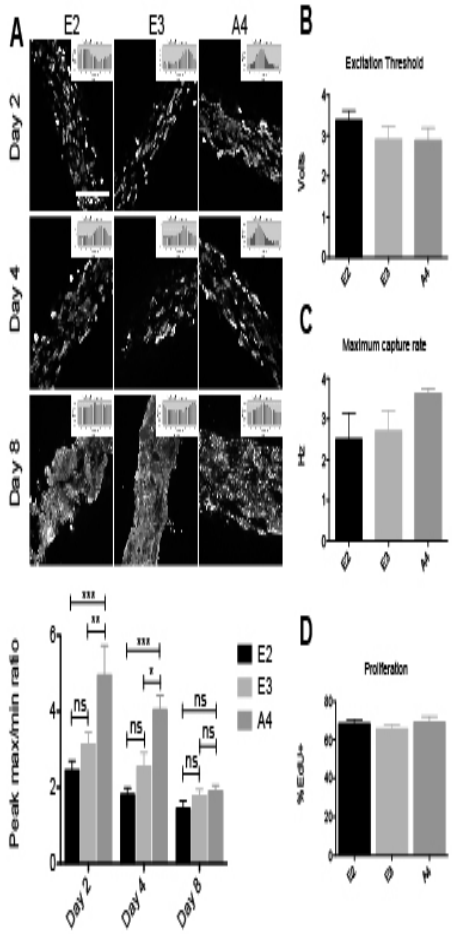

Figure 2. The behavior of the cell constructs is modified by the PA gels' mechanical properties. A) Confocal fluorescent images of Calcein AM-stained cell constructs at different times in culture and its subsequent alignment analysis. E2, E3 and A4 denote different peptide composition of the PAs. *P<0.05, **P<0.01, ***P<0.001. B) and C) graphs of the analysis of the excitation threshold (ET) and maximum capture rate of the cell constructs with varying PA gel stiffness after 10 days in culture. There is a trend of lower ET and higher MCR as the stiffness of the PA gel augments. D) Quantification of the percentage of EdU-positive cells of day 8 cell constructs showing no difference in their proliferative behavior.

E. Sleep: None. **J.R. Mantei:** None. **M.T. McClendon:** None. **S.I. Stupp:** None.

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Pharmacologically Controlled IGF-1 Release For Paracrine Support Of Engineered Heart Muscle

POH LOONG SOONG, Malte Tiburcy, Inst of Pharmacology, Univ Medical Ctr Göttingen, Goettingen, Germany; Jan Christoph, Stefan Luther, Biomedical Physics Group, Max Planck Inst for Dynamics and Self Organization, Goettingen, Germany; Fawad Jebran, Fritz Schoendube, Clinic for Cardiovascular Surgery, Univ Medical Ctr Göttingen, Goettingen, Germany; Wolfram H Zimmermann, Inst of Pharmacology, Univ Medical Ctr Göttingen, Goettingen, Germany

Tissue engineered heart repair is developing rapidly, but needs refinement before clinical translation. We tested the hypothesis that force generating human engineered heart muscle (EHM) can be enhanced by integration of insulin-like growth factor-1 (IGF-1) secreting fibroblasts.
Methods: TetOn lentiviral particles encoding for IGF-1 and the Tet-transactivator (tTA) were cloned and used to stably transduce human foreskin fibroblasts (HFF). Baseline and doxycycline induced IGF-1 release from HFFTetOn+IGF1 was quantified by ELISA. HFFwt and HFFTetOn+IGF1 conditioned medium was layered over human embryonic stem cell (HES2) derived cardiomyocytes followed by an analysis of AKT-phosphorylation. EHMs were assembled from HES2-derived cardiomyocytes and HFF (HFFwt or HFFTetOn+IGF1) at 70:30 ratio. Transgene activation was induced by addition of doxycycline (10 ng/ml) for 7 days. Twitch forces and response to pharmacological stimuli were measured to assess the functional consequences of IGF-1 release. EHMs were subsequently subjected to morphological analysis or dissociated into single cells to assess cellular composition of EHMs. Results: HFFTetOn+IGF1 released IGF-1 upon doxycycline stimulation (3.3x10-6 vs 8.3x10-8 [HFFwt] ng/ml/cell/day. Secreted IGF-1 from HFFTetOn+IGF1 induced Akt phosphorylation in HES2-derived cardiomyocytes (2.4±0.6 fold increase of [HFFwt]; n=3). EHMs with HFFTetOn+IGF1 developed significantly higher twitch forces than EHMs with HFFwt (0.24±0.03 vs 0.15±0.02 mN; n=10) under baseline conditions (IGF-1 leak). Doxycycline induced

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IGF-1 release further enhanced (P<0.05) EHM twitch force (0.26±0.03 mN; n=10). Single cell analysis from EHMs demonstrated cardiomyocyte hypertrophy in response to paracrine IGF-1 release (154±11% of [HFFwt] control; n=4). Histological analyses demonstrated that HFFTetOn+IGF1 supplemented EHMs contained thicker muscle bundles and enlarged cardiomyocytes. Conclusion: EHM can be functionally enhanced by integration of drug-controllable IGF-1 release. Drug controllable, cell based paracrine release of protective factors may not only be exploited to enhance tissue engineered myocardium in vitro but also to achieve better survival and integration of EHM grafts in vivo.

P. Soong: None. **M. Tiburcy:** None. **J. Christoph:** None. **S. Luther:** None. **F. Jebran:** None. **F. Schoendube:** None. **W.H. Zimmermann:** None.

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The Use of β-1.3/1.6-D-Glucan as Complementary Therapy for Cardiovascular Diseases

Peter Sugita, Sahabat Lingkungan Hidup, Surabaya, Indonesia

Cardiovascular disease nowadays is still the one silent killer over the world.So far, current standard therapies for cardiovascular diseases do not reach expected results such as the improving heart function by rehabilitating the damaged cardiomyocytes
For thousands of years *Ganoderma lucidum* extract has been used as herbal therapy for various types of diseases, including cardiovascular diseases. An International Publication No. WO 2005/084692 A2 with the title ***Effect of Glucan on Stem Cell Recruitment and Tissue Repair*** mentions that "*the orally administered glucan enhances proliferation, activation and differentiation of committed stem progenitor cells by functioning with the complement system by providing a second signal for CR3 activation*." Furthermore, it is also discussed that "*the committed stem cells are selected from the group consisting of committed stem cells from the liver, heart, muscle, kidney and neural tissue."*
In 1995 we began a research on β-D-Glucan derived from the purified extract of East-Java Indonesian *Ganoderma lucidum* mycelia.At the end of 2009 we have the first clinical experience the use of our β-1.3/1.6-D-Glucan for cardiovascular diseases patient. A bypass surgery patient who was also a candidate for heart transplant experienced a good clinical result after 8 months use of our beta glucan with dose of 3 x 200 mg beta glucan a day. A significant improvement of Ejection Fraction (EF) was reached from 22% to 44%. Previously, when he only took standard drug therapy for cardiovascular disease after bypass surgery, the improvement of EF was not significant (from 13% as baseline to 22%).This result also applied to 4 other patients.
We presumed that this specific *Ganoderma lucidum* β-D-Glucan has similar mechanism of actions as mentioned in the above publication and also in a journal ***Modulating Toll-like receptor mediated signaling by(1-3)-β-D-glucan rapidly induces cardioprotection***.
The conclusion is β-D-Glucan could serve as a complementary therapy in cardiovascular diseases but it still needs further investigations through in vitro / in vivo assays and clinical trials.

P. Sugita: None.

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Alterations to the Extracellular Matrix Composition Following Myocardial Infarction Impact Cardiac Progenitor Cell Fate In Vitro

Kelly E Sullivan, Sharada Sant, Laura Burns, Lauren D Black III, Tufts Univ, Medford, MA

Limitations associated with cardiac progenitor cell (CPC) therapy of myocardial infarction (MI) including poor engraftment, cell death and incomplete cardiac differentiation have hindered the efficacy of treatment in pre-clinical trials. Given that the extracellular environment plays an important role in regulating cell function and that it is significantly remodeled following MI, it is critical to understand how these changes impact the therapeutic potential of CPCs. In this study, we investigated how the alterations to the extracellular matrix (ECM) following MI impacted the regenerative potential of CPCs in vitro. Hearts were decellularized with 1% SDS prior to MI and 1 and 4 weeks post-MI (Fig A) and the composition of the left ventricle or scar was characterized through LC-MS/MS. While Periostin and Collagen I increased post-MI,

Laminin decreased (Fig B). c-kit+ CPCs isolated from rat hearts 1 week post-MI were cultured on tissue culture plastic (TCP) coated with pepsin-solubilized ECM. Our results demonstrated that the healthy matrix promoted the expression of pro-angiogenic growth factors, while maintaining the cells in an undifferentiated state (Fig D,E). Alternatively, 1 week ECM promoted cell adherence (Fig C) and the expression of pro-survival growth factors (Fig D) and GATA-4 (Fig E). Cells cultured on 4 week ECM demonstrated significant differentiation towards vascular lineages through their expression of smooth muscle (TAGLN) and endothelial (VWF) markers. By characterizing how the changing ECM composition following MI impacts CPC fate, we may be able to develop therapeutic strategies that modulate cell fate/ function in vivo following implantation.

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Effect of Transendocardial Autologous Cardiac Stem Cells and Bone Marrow Mesenchymal Stem Cells to Reduce Infarct Size and Restore Cardiac Function in a Heart Failure Swine Model

Viky Y Suncion, Vasileios Karantalis, Luiza Lima Bagno, Frederic McCall, Bo Wang, Samuel Golpanian, Jose E Rodriguez, Marcos Rosado, David Valdes, Samirah Gomes, Samirah Gomes, Jose Da Silva, Courtney Premer, Azorides Morales, Azorides Morales, Wayne Balkan, Muzammil Mushtaq, Juan Pablo Zambrano, Alan W Heldman, Joshua M Hare, Univ of Miami, Miami, FL

Background: A cell combination of human mesenchymal stem cells (MSCs) and c-kit+ cardiac stem cells (CSCs) improves left ventricular (LV) performance to a greater degree than MSCs alone in post myocardial infarction swine. To advance the development of cell combination therapy, we administered autologous swine cells, and tested the hypothesis that transendocardial autologous CSCs/MSCs produces greater improvement of performance than MSCs in a rigorous model of heart failure due to post infarct LV remodeling. **Methods:** Gottingen mini-swine (n=28) underwent LAD coronary artery occlusion followed by reperfusion, and allowed to undergo LV remodeling for 90 days. Autologous MSCs were amplified from bone marrow and CSCs from right ventricular biopsies in each swine, and injections of either CSC/MSC combo (1M/200M, n=7), MSCs (200M, n=7), or placebo (Plasmalyte, n=6) were injected to the infarct-border zone via the NOGA system. Cardiac MRI and pressure volume loops were obtained before and after therapy. **Results:** Both cell groups had substantially reduced scar size (Combo –37.2.9± 5.4% vs MSCs –38.8±7.5% vs placebo -7.2±6.3, P=0.0001) and increased viable tissue (Combo +30.9±7% vs MSCs +41.8±10.5% vs placebo +7.7±4.5, P<0.0001) relative to placebo. Ejection Fraction (EF) improved only in the Combo group (Combo +7.0±2.8 vs MSCs +3.4±1.3 vs placebo +1.2±1.6 EF units, P=0.04). Accompanying this EF restoration was a substantial improvement in the Combo group in stroke volume (Combo +47.2±11.1% vs MSCs +32.6±12.0% vs placebo +10.8±4.5, P<0.0001), cardiac output (Combo +35.9±7.6% vs MSCs 41.9±26.5% vs placebo -16.4±6.6%, P=0.01) and diastolic strain rate (Combo +18.9±8.6% vs MSCs 14.0±8.8% vs placebo -14.9±9.5%, P=0.03). **Conclusions:** Combination cell therapy and MSCs alone dramatically reduce scar size in a swine model of chronic ischemic cardiomyopathy. In contrast, combination therapy has much greater impact on functional recovery, increasing EF to [near normal] levels. These findings illustrate that interactions between ckit+ CSCs and MSCs result in substantial enhancement in cardiac performance, establish the safety of autologous cell combination strategies, and support the development of an advanced second generation cell therapeutic product.

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Influence of the Collagen Processing Heat Shock Protein 47 on Cardiomyocyte Homeostasis and Maturation

Sumon Sur, Peter Christalla, Angelica Roa, Wolfram Hubertus Zimmermann, Inst of Pharmacology, Goettingen, Germany

PURPOSE: Heat shock protein 47 (Hsp47) is a collagen-specific molecular chaperone required for maturation of collagen type 1. Little is known about the role of Hsp47 in the heart. This study aims to investigate whether Hsp47 is important for cardiomyocyte maturation and assembly into functional syncytia.
METHODS: We made use of recently developed 2D and 3D culture platforms to scrutinize the role of Hsp47 for cardiomyocyte maturation and function, i.e., (1) 2D model: culture of embryonic stem cell-derived cardiomyocytes (ESC-CM) on ECMs derived from both mouse embryonic wild type (WT)- and Hsp47 (-/-) fibroblasts and (2) 3D model: construction of engineered heart muscle (EHM) consisting of purified ESC-CMs with WT- or Hsp47 (-/-) fibroblasts. Cultures were investigated using confocal microscopy and isometric force measurements.
RESULTS: ESC-CMs showed mainly immature polygonal shaped morphology and disorganized sarcomeric structures when cultured on ECM secreted by Hsp47 (-/-) fibroblasts; this morphology was in clear contrast to the mostly anisotropic cardiomyocyte appearance in control conditions. EHM with Hsp47 (-/-) fibroblasts did not develop measurable contractile forces. In contrast, WT fibroblast-supplemented EHMs contracted regularly (WT: 0.14±0.1 mN at 2.4 mM Ca2+; n=8/group). Histological analysis of Hsp47 (-/-) EHMs showed mainly rounded and underdeveloped cardiomyocytes comparable to the cardiomyocyte phenotype observed in EHMs containing only ESC-CMs, without fibroblasts.
CONCLUSIONS: Hsp47 influences the deposition of ECM-collagen and affects cardiomyocyte morphology and functionality in our 2D and 3D culture models. Our data collectively suggest that Hsp47 may be an attractive target for the regulation of cardiac tissue homeostasis.

S. Sur: None. **P. Christalla:** None. **A. Roa:** None. **W.H. Zimmermann:** None.

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Defining the Non-Myocyte Compartment is Key for Enhanced Maturation of Human Engineered Heart Muscle

Malte Tiburcy, James E Hudson, Dirk Ziebolz, Wolfram H Zimmermann, Univ Med Ctr Goettingen, Goettingen, Germany

Background: Tissue engineering of heart muscle from human pluripotent stem cells holds great potential for in vitro studies, disease modeling, and cardiac replacement therapy. A number of variables may however affect maturation and function of human cardiomyocytes (CM) in tissue engineered heart muscle (EHM). Here, we hypothesized that defined non-myocyte (NM) populations support structural and functional maturation of EHM.
Methods and Results: To investigate the role of non-myocytes (NM) for heart muscle assembly in vitro we generated EHM from purified CM (93±1.5% actinin+) and a mixture of CM and NM (70/30%). Notably, only the NM-supplemented EHM generated measurable forces (0.8±0.1 mN, n=9) with anisotropically aligned cardiomyocytes. Depending on pluripotent stem cell line and differentiation protocol the NM compartment may vary considerably. To further define the influence of the NM compartment we generated EHM from HES2-derived CM with undefined NM, i.e the NM typically derived during cardiac differentiation, and defined NM (fibroblasts). Defined EHM were more mature with higher forces and lower variability between experimental series (defined: 9.8±0.9 nN/CM, undefined: 4.7±1.4 nN/CM, n=10/9), higher EC50 for calcium, and enhanced inotropic response to isoprenaline despite comparable CM:NM composition of 1:1. Increased actinin protein per CM, a reduction of MLC2V/2A double positive CM, and evidence of CM cycle withdrawal indicated enhanced ventricular maturation in defined EHM. Next, we tested whether defining cell composition and NM in iPS-derived EHM will yield a comparable functional phenotype to HES2-EHM. In agreement with the above data, defined iPS-EHM displayed advanced functional maturation with high specific forces, comparable calcium EC50, and inotropic response to isoprenaline.
Summary and Conclusions: Here we demonstrate that defining the NM compartment is essential for optimized human heart muscle formation and maturation in vitro. Moreover, our

data provide (1) evidence for the applicability of EHM in modelling of heart muscle development and (2) a strong rationale for the need to define CM and NM compartments in tissue engineered myocardium to reduce variability in applications such as disease modelling.

M. Tiburcy: None. **J.E. Hudson:** None. **D. Ziebolz:** None. **W.H. Zimmermann:** None.

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Evaluation of the Paracrine Effect of Encapsulated Mononuclear Stem Cells in the Acute Myocardial Rat Model

Santiago Alonso L Tobar Sr., Virgílio Olsen Sr., Amanda P. Phaelante, Daiane Silvello, Andréia Biolo, Luis Eduardo Rohde, Nadine O Clausell, Michael Andrades, Hosp de Clinicas de Porto Alegre, Porto Alegre, Brazil

Many studies have considered stem cell-based therapy as a treatment option for acute myocardial infarction (AMI). Paracrine signaling has been proposed as an underlying mechanism, in which released cytokines and chemokines may promote angiogenesis and activation of resident stem cells. In order to characterize this paracrine action, bone marrow mononuclear stem cells (BM-MNC) collected from femur of adults GFP+ Wistar rats were encapsulated in 1.5% of sodium alginate. Animals were randomized in three groups: SHAM (n=3); Empty capsules (n=8); BM-MNC capsules (n=8). AMI was induced by permanent occlusion of the left anterior descending artery after anesthesia with 100mg/kg ketamin and 10 mg/kg xylazine. Soon after the AMI, capsules (empty or BM-MNC) were delivered intra-thoracically. SHAM group was submitted to the same surgical procedures without permanent artery occlusion or treatment given. Troponin I (cTnI) was measured 24h after AMI in order to evaluate the success of the procedures; echocardiography was also performed to assess heart morphofunctional parameters 48h and 7 days after AMI. At day 7, after echocardiography, the animals were euthanized under profound anesthesia (isoflurane 5%) and their hearts were withdrawn for biochemical analysis. Plasma and tissue levels of TNF-α and IL-6 were measured by ELISA. All technical procedures were performed by blinded operators. Statistical comparisons were made using ANOVA analysis followed by Tukey post-test or using t-Student test when appropriated. BM-MNC were viable after day 7 since GFP+ cells were detected by fluorescence microscopy. Nevertheless, the empty capsules groups showed lower levels cTnI compared to BM-MNC group (25 vs. 40 ng/mL, respectively; p=0.03). There was no difference in the shortening fraction (24% VS. 18%, respectively; p=0.08) and in the infarcted area (32% vs. 43%, respectively; p=0.10) when both AMI groups (Empty and BM-MNC) were compared. Also TNF-α and IL-6 levels showed no difference between all groups. We concluded that paracrine effects of cell-based therapy with BM-MNC was unable to modulate events associated to AMI in rat in spite of cell viability after 7 days of implantation.

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Post-myocardial Infarction Therapeutics Using Cardiovascular Progenitor Cells (PC) Derived from Induced Pluripotent Stem Cells (iPSC)

Yigang Wang, Dept. of Path. & Lab Med., Univ of Cincinnati, Cincinnati, OH

Objective: We sought to assess the cardiac protective effects after MI of (1) PC differentiated directly into cardiomyocytes (CM) and endothelial cells (EC) to the site of injury, or (2) paracrine factors released from PC. **Methods:** These concepts were evaluated by using iPSC-derived PC genetically modified to express the herpes simplex virus thymidine kinase (TK) under the control of cardiomyocyte (NCX1) or endothelial cell (VE-cadherin) specific promoters. PC expressing the TK permitted ablation at the first week or the third week by iv ganciclovir (GCV). If GCV applied at the first week, but not at the third week, altered cardiac function, we would conclude that myocardial contractile recovery depends on CM and EC-derived from iPSC. If the beneficial effects on cardiac function persisted after GCV was given at the third week, we would surmise that the PC effect was via by a paracrine action. MI created by ligation of LAD, the cell patch with PC was

applied to the scarred myocardium. Rats were treated with GCV at 1 or 3 weeks to ablate implanted PC. Echocardiography, vessel density, and histological analysis were used to obtain endpoints for this study. **Result: *In vivo*:** The levels of IGF-1α and VEGF released from ischemic tissues were significant higher in the cell patch group. Heart function, infarction size, and vessel density were significantly improved after cell patch treatment. However, this beneficial effect on cardiac function was completely abolished in the group given GCV at week 1, but only partially abolished in the group given GCV at week 3 compared to the untreated cell patch group. **Conclusions:** Taken together, these data support our conclusion that iPSC-derived cardiovascular lineages (CM and EC) contribute directly to an improved cardiac performance and attenuated remodeling, and that paracrine factors also play a supporting role in the restoration of heart function after MI.

Y. Wang: None.

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Mitophagy Protects Against Statin-Induced Cell Death in Muscle Cells

Allen Andres, Cedars-Sinai Medical Ctr, Los Angeles, CA; Mridula Ramesh, San Diego State Univ, San Diego, CA; Genaro Hernandez, Univ of Texas Southwestern, Dallas, TX; Michael Gurney, Phyllis-Jean Linton, Moutusee Islam, San Diego State Univ, San Diego, CA; Roberta Gottlieb, Cedars-Sinai Medical Ctr, Los Angeles, CA

Statins are a family of cholesterol lowering drugs associated with undisputable cardiovascular benefits. However, their use is associated with the risk of developing skeletal muscle myopathy which remains a significant barrier to maximizing the efficacy of statin therapy. Many recognize that the instances of statin-associated myopathy reported grossly underestimate the actual frequency of these events. Statin myopathy is a major reason for why many patients are unable to comply with their prescribed statin treatments. Identifying and understanding the molecular events involved in the development of statin-induced myopathy will pave the way to mitigating the unwanted effect of statin-myopathy. We have identified that statins induce mitochondrial loss in both C2C12 myotubes, and in gastrocnemius muscle of mice evidenced by loss of mitochondrial markers Tom70 and Cox4 by western blot analysis. C2C12 myotubes were treated with 2μM simvastatin for 24 hours, and wild-type C57BL/6 mice were given a daily i.p. dose of 20mg/kg simvastatin for 2 weeks. Pretreatment of C2C12 cells with 100nM bafilomycin for 2 hours before statin treatment attenuated autophagic flux, and prevents the observed loss of mitochondria through Tom70 and Cox4 mitochondrial markers. Thus, the loss of mitochondria is attributable to autophagy (mitophagy). Statin treatment increases the expression of Bnip3 and p62/SQSTM1 (sequestosome 1) which have been identified as important facilitators of mitophagy. We found increased Bnip3 dimerization via western blot under non-reducing conditions which implies increased activity. Furthermore, we observed increased mitochondrial translocation of p62/SQSTM1. Based on our findings, we hypothesized that statin-induced mitophagy may play a key role in the development of muscle myopathy.

To investigate the role of mitophagy in statin myopathy, we silenced p62/SQSTM1 via RNAi prior to statin treatment. Surprisingly, reducing mitophagic flux via p62/SQSTM1 silencing resulted in increased destruction of myotubes. This was associated with increased levels of cytosolic cytochrome c. Our data combined suggests that mitophagy protects against statin-mediated cell death by sequestering and eliminating decrepit mitochondria via autophagy.

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Biphasic Effect Of Cyclic Amp Axis On Cardiomyocyte Survival

Morihiko Aoyama, Yasuko K Bando, Haruya Kawase, Akio Monji, Toyoaki Murohara, NAGOYA UNIV, Nagoya Aichi, Japan

[Background] Persistent cardiac hypertrophy in response to pathological stimuli is results in maladaptive myocardial remodeling and cell death. Clinical evidence revealed that one of the most significantly beneficial medications for

targeting heart failure with pathologic myocardial hypertrophy is beta1-adrenergic receptor (β1R) blockers. The molecular pathway of β1R is mediated by the second messenger cyclic AMP (cAMP). However, there are some debate regarding the role of cAMP in myocardial survival. We hypothesized whether there may be threshold concentration of cAMP in cell susceptibility to cardiomyocyte cell death. [Methods]
Male 14-week-old C57BL6 mice were subjected to the surgery of thoracic aortic constriction (TAC) to induce pressure overload. Changes in apoptosis were evaluated in each heart section and in vitro culture of neonatal cardiomyocytes using TUNEL. To elucidate the concentration-dependent distinct effect of cAMP on myocardial cell death, we tested the different concentration of cell-permeable cAMP (8-br-cAMP) at low (60 μM) and high concentration (6 mM), and receptor-mediated cAMP-stimulators (Ex4; exendin-4, ISO; isoproterenol). [Results]
In vitro analysis revealed that the high-cAMP and ISO exhibited marked increase in TUNEL-positivity (15.46%±3.09% for high-cAMP versus 6.71%±0.33% for ISO), which was reversed by Rp-cAMP (1.80%±0.17% and 2.05%±0.25%, respectively). Unexpectedly, the 8-p-Methoxyphenylthon-2-O-methyl-cAMP (pMe-cAMP, 50 μM), the specific activator of another cAMP-sensitive target Epac, reversed the high-cAMP-induced cell death even at a less extent compared to that observed by PKA-inhibitor Rp-cAMP (3.73%±0.70%). Serum depletion induced 3.22±0.24% of TUNEL-positive cell count of NRVM, which was reversed by pMe-cAMP, (50 μM) and Ex4 (1.74±0.18%, n=6, P<0.01), which was insensitive to PKA inhibition by Rp-cAMP (100 μM).
TAC increased myocardial apoptosis. TAC-CON heart exhibited 1.66-fold decrease in cardiac cAMP concentration compared to sham-CON. Ex4 ameliorated the TAC-induced cardiac dysfunction and apoptosis by increase in cAMP. [Conclusions]
The cAMP-related cell death was mediated by PKA activation, which were reversed by Epac activation.

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δ-opioid Receptor Activation Increases Nrf2 Nuclear Translocation And Stabilizes Mitochondrial Membrane Potential Of Hypoxic Cells

Shan Cao, Univ of Texas Medical Sch at Houston, Houston, TX; Tao Chen, Hainan General Hosp, Haikou, Hainan, China; Dongman Chao, Univ of Texas Medical Sch at Houston, Houston, TX; Guoqiang Wen, Hainan General Hosp, Haikou, Haikou, Hainan, China; Gianfranco Balboni, Univ of Cagliari, Cagliari, Italy; Ying Xia, Univ of Texas Medical Sch at Houston, Houston, TX

Conditions that limit O2 supply for a prolonged period lead to cardiac injury. However, current strategies against hypoxic cardiac injury are limited in clinical settings. It is of utmost importance to find novel clues for protecting cells from hypoxic injury. Our work has recently shown that δ-opioid receptor (DOR) activation strongly regulates microRNA expression in the heart exposed to prolonged hypoxia, suggesting a role of DOR in cardiac adaptation to hypoxic stress. To further determine DOR's action on cell survival under hypoxia and the underlying mechanisms, we exposed HEK293t cells to hypoxia for a prolonged period and then investigated cellular viability and changes in mitochondrial membrane potential and the nuclear factor erythroid 2-related factor 2 (Nrf2), which are thought to be involved in the regulation of cell survival under hypoxia. Our data show that prolonged hypoxia (0.5% of O2 for 16-48 hours) caused serious cellular injury. DOR activation with UFP-512, a potent and specific DOR agonist, prevented the collapse of mitochondrial membrane potential and markedly attenuated cell injury in the hypoxic condition. The DOR-mediated protection was largely reversed by DOR antagonism with naltrindole. Furthermore, we observed that DOR activation increased Nrf2 translocation from cytoplasm to nucleus. The DOR-mediated cytoprotection was largely abolished by the "knock-down" of Nrf2. Our results suggest that DOR activation increases Nrf2 nuclear translocation and stabilizes mitochondrial membrane potential during the process of cellular protection against hypoxic stress.
SC and TC are equal contributors.
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Parkin Mediates Clearance of Dysfunctional Mitochondria via Multiple Pathways

Babette C Hammerling, Melissa Q Cortez, Rita A Hanna, Rebecca E Jimenez, Åsa B Gustafsson, UC San Diego, La Jolla, CA

The ability to clear damaged mitochondria is critical to prevent unnecessary death. Studies have found that dysfunctional mitochondria are rapidly sequestered by autophagosomes and subsequently delivered to lysosomes for degradation. The E3 ubiquitin ligase Parkin has been identified as an important regulator of mitochondrial autophagy. We have previously shown that Parkin plays an important role in clearing dysfunctional mitochondria via autophagy in the heart after myocardial infarction. In this study, we have discovered that Parkin also induces clearance of mitochondria via an autophagy-independent pathway. We found that Parkin mediated clearance of damaged mitochondria in both wild type (WT) and autophagy-deficient Atg5 knockout mouse embryonic fibroblasts (MEFs) treated with the mitochondria uncoupler FCCP. Interestingly, mitochondrial clearance in both cell types was dependent on the presence of Parkin, suggesting that Parkin represents a rate-limiting step. Immunofluorescence analysis revealed that FCCP-treatment resulted in activation of the Vps34-Rab5 complex with subsequent sequestration of mitochondria inside Rab5-positive endosomes and LAMP2-positive lysosomes in Atg5-/- MEFs. The presence of mitochondria inside endosomes in Atg5-/- MEFs was confirmed by transmission electron microscopy (TEM). Pharmacological inhibition of the endosomal-lysosomal pathway with 3-methyladenine or Bafilomycin A1 caused a significant increase in FCCP-mediated cell death in Atg5-/- MEFs. Also, although BNIP3 functions as an autophagy receptor on mitochondria by interacting with LC3II on the autophagosome, it induced mitochondrial clearance in Atg5-/- MEFs via activation of the endosomal pathway. Finally, we confirmed that mitochondrial clearance occurs via both the autophagy and endosomal pathways in neonatal cardiomyocytes subjected to simulated ischemia/reperfusion (I/R). TEM analysis revealed the presence of mitochondria inside endosomes rat hearts subjected to ex vivo I/R. These data demonstrate that both autophagy and endosomal pathways contribute to clearance of damaged mitochondria in cells, and represent potential future therapeutic targets to enhance or preserve mitochondrial clearance in patients after MI.

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Parkin Translocation And Mitophagy Occur Independently of PINK1 in the Myocardium

Dieter A. Kubli, Univ of California, San Diego, San Diego, CA; Youngil Lee, Univ of West Florida, Pensacola, FL; Alexandra G. Moyzis, Åsa B. Gustafsson, Univ of California, San Diego, San Diego, CA

We recently reported that Parkin plays a crucial role in preserving myocardial tissue following a myocardial infarction (MI) by promoting removal of damaged mitochondria via autophagy (mitophagy). Parkin is thought to function downstream of the serine/threonine kinase PINK1 for mitophagy of mitochondria that have lost membrane potential ($\Delta\Psi m$). Here, we describe the ability of Parkin to translocate to dysfunctional mitochondria in the absence of PINK1. We discovered that after 4 hours of MI, Parkin accumulated on mitochondria in the infarct border zone in both wild type (WT) and PINK1 knockout mice ($PINK1^{-/-}$). Additionally, *ex vivo* perfusion of $PINK1^{-/-}$ hearts with the mitochondrial uncoupler FCCP caused rapid translocation of Parkin to mitochondria, equivalent to the response in WT hearts. We further confirmed Parkin translocation and subsequent mitophagy by fluorescence microscopy in isolated $PINK1^{-/-}$ adult cardiac myocytes treated with the mitochondrial complex I inhibitor rotenone. Rotenone-stimulated Parkin translocation resulted in increased co-localization of GFP-LC3-labeled autophagosomes with mitochondria in both WT and $PINK1^{-/-}$ myocytes. Thus, PINK1 is dispensable for Parkin translocation and

mitophagy in cardiac myocytes. In contrast, we found that Drp1-mediated mitochondrial fission was a prerequisite for Parkin translocation and mitophagy activation. Drp1 translocation to mitochondria preceded Parkin translocation in WT and PINK1$^{-/-}$ hearts perfused with FCCP. Moreover, overexpression of the dominant negative Drp1-K38E mutant prevented Parkin translocation in rotenone-treated isolated adult rat cardiac myocytes. Interestingly, while Drp1 was able to rapidly translocate to mitochondria in FCCP-perfused Parkin$^{-/-}$ hearts, Drp1 translocation to border zone mitochondria was only observed in WT hearts after MI and not Parkin$^{-/-}$. This suggests that Parkin stabilizes Drp1 that has translocated to mitochondria upon damage. We conclude that while Parkin translocation to cardiac mitochondria is independent of PINK1 activity, Drp1-mediated fission is required for Parkin translocation. Parkin and PINK1 are therefore components of distinct but overlapping pathways for mitochondrial maintenance in the heart.

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S-nitrosylation Of Thioredoxin1 At Cysteine 73 Promotes Trans-nitrosylation, Autophagy And Cell Survival During Glucose Deprivation.

Narayani Nagarajan, Sebastiano Sciarretta, Junichi Sadoshima, Rutgers Univ, NEWARK, NJ

Thioredoxin-1 (Trx1) is a key antioxidant protein that is known to play a protective role in the heart during oxidative stress mainly through its oxidoreductase activity. Trx1 can be S-nitrosylated and, in turn, can trans-nitrosylate other proteins. However, the role of Trx1-dependent S-nitrosylation in cardiomyocytes (CMs) is not known. Here, we investigated the role of Trx1-mediated protein S-nitrosylation in the regulation of CM survival in response to stress. Using the biotin-switch assay, we found that wild-type Trx1 (Trx1WT) was S-nitrosylated, whereas the extent of S-nitrosylation was attenuated in Trx1C73S, suggesting that Trx1 is S-nitrosylated at Cys73. Also, we observed that the redox activity of Trx1 was intact in the Trx1C73S mutant. Overall protein S-nitrosylation in rat neonatal CMs was increased in response to 4 hrs of glucose deprivation (GD). Using biotin-switch assay and immunocytochemistry (fluorescent staining of s-nitrosylated cysteines), we observed that overexpression of Trx1WT increased, whereas short-hairpin RNA-mediated knockdown of Trx1 (shTrx1) or overexpression of Trx1C73S decreased, total protein S-nitrosylation in response to GD. These results suggest that Trx1Cys73 plays a key role in the regulation of protein S-nitrosylation in CMs during GD. Overexpression of Trx1 increased CM survival after 24 hrs of GD (Trx1WT vs. LacZ: propidium iodide assay, 0.5 ± 0.08-fold, $p<0.01$). Conversely, shTrx1 or overexpression of Trx1C73S increased cell death during GD (Trx1C73S vs. LacZ: 1.7 ± 0.034-fold, $p<0.05$). Autophagy is a pro-survival mechanism during GD. Therefore, we tested the effect of Trx1 on autophagy. After 4 hrs of GD, knockdown of Trx1 or overexpression of Trx1C73S decreased autophagy compared to control cells (LC3-II/LC3-I, 0.7-fold; autophagosomes, 0.83 ± 0.16-fold; autolysosomes, 0.62 ± 0.13-fold, $p<0.005$). Taken altogether, our results suggest that Trx1 promotes autophagy during GD through a trans-nitrosylation dependent mechanism. S-nitrosylation of Trx1 at Cys73 is associated with an overall increase in protein S-nitrosylation in CMs and promotes autophagy and thus, cell survival during GD.

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MCL-1 Promotes Mitochondrial Fusion and Survival in Cardiac Myocytes

Robert L Thomas, Jennifer Kuo, Åsa B Gustafsson, Univ of California San Diego, San Diego, CA

Myeloid cell leukemia-1 (MCL-1) is an anti-apoptotic BCL-2 protein that is highly expressed in myocardium, but little is known about its function in myocytes. Recently, we reported that MCL-1 is essential for myocardial homeostasis and autophagy. Cardiac-specific deletion of MCL-1 in mice led to rapid mitochondrial dysfunction, hypertrophy, and lethal cardiomyopathy. Despite extensive mitochondrial damage, MCL-1 deficient hearts failed to activate mitochondrial autophagy. Parkin, an E3 ubiquitin ligase, normally translocates to damaged mitochondria to promote mitochondrial autophagy, but loss of

MCL-1 resulted in cytosolic accumulation of Parkin. However, we found no evidence that MCL-1 functions as a mitochondrial Parkin receptor or substrate. Instead, loss of MCL-1 reduced mitochondrial accumulation of PINK1, which is involved in Parkin recruitment. Additionally, we identified mitochondrial outer membrane (OM) and matrix isoforms of MCL-1 in mouse hearts and found that the two forms respond differently to ischemic injury. Four hours after myocardial infarction, MCL-1_{OM} levels were reduced by 40% in border zone tissue. After 24 hours, MCL-1_{OM} levels returned to baseline. Meanwhile, MCL-1_{Matrix} levels were preserved at four hours, and increased significantly compared to control 24 hours after infarction. These changes correlated with increased expression of HSP70, a chaperone protein that stabilizes MCL-1 and participates in import of mitochondrial proteins. Overexpression of MCL-1_{Matrix} promoted mitochondrial fusion in fibroblasts under baseline conditions and protected cells against FCCP-mediated mitochondrial fission. While 94.1% (1269 of 1349) of control cells exhibited fragmented mitochondria after two hours of FCCP treatment, mitochondria were only fragmented in 41.3% (391 of 947) of cells overexpressing MCL-1_{Matrix}. These data suggest MCL-1 isoforms play different roles in the cellular stress response. MCL-1_{OM} protects against apoptosis, whereas MCL-1_{Matrix} protects mitochondria by promoting fusion. In addition, upregulation of HSP70 may preserve mitochondrial function and cell viability in damaged cardiac myocytes by increasing the stability and mitochondrial import of MCL-1.

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IL-10 Inhibits Angiotensin II-induced Pathological Autophagy in Myocardium.

Suresh K Verma, Ctr for Transnational Med, Philadelphia, PA; Prasanna Krishnamurthy, Tatiana Abramova, Feinberg Cardiovascular Res Inst, Chicago, IL; Garikipati Srikanth, Mohsin Khan, Ctr for Transnational Med, Philadelphia, PA; Alexander R Mackie, Aiko Klingler, Veronica Ramirez, Sol Misener, Gangjian Qin, Feinberg Cardiovascular Res Inst, Chicago, IL; Raj Kishore, Ctr for Transnational Med, Philadelphia, PA

Background: In heart, persistent pressure overload causes pathological autophagy leading to cardiac cell death and heart failure. The role IL-10, a pleiotropic anti-inflammatory cytokine, on pathological autophagy is largely unknown. Here we hypothesized that IL-10 inhibits stress-induced pathological autophagy and therefore attenuates cardiac cells death and improve heart function. Method and Results: Cardiac stress was induced in C57 BL/6 mice by Angiotensin II treatment (Ang II-1.2mg.kg b.wt/day for 28 days) using mini osmotic pumps. Ang II treatment markedly induced autophagy in mice as measured by electron microscopy (autophagosome numbers) and Western blotting (Becline1 and LC3II proteins expression). Interestingly, systemic recombinant mouse IL-10 administration markedly inhibited Ang II-induced autophagy. To further understand the mechanism of IL-10 protection, neonatal rat ventricular myocytes (NRCM) were transfected with monomeric Red Fluorescent Protein-Enhanced Green Fluorescent Protein (mRFP-EGFP) tandem fluorescent-tagged LC3 (tfLC3) adenovirus (to measure autophagic flux) and then treated with AngII (1μM) and/or IL-10 (20ng/mL), in vitro. Ang II treatment significantly increased the numbers of both yellow (merged EGFP and mRFP signals) and red puncta, indicating active formation of both autophagosomes and autolysosomes, however, this flux was strongly inhibited by IL-10. Furthermore, Ang II significantly increased the Beclin1 and LC3II proteins expression, which was markedly reduced by IL-10 as measured by Western blot analysis. In addition, Ang II-inhibited AKT signaling (anti-autophagic signaling component) was strongly enhanced by IL-10. Ang II-induced autophagic signaling was mimicked by AKT inhibitor, suggesting AKT as the downstream target of IL-10 effects. Conclusion: Inhibition of pathological autophagy is a novel mechanism for cardio-protective effects of IL-10.

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IL-10 Accelerates Re-Endothelialization and Inhibits Post-injury Intimal Hyperplasia following Carotid Artery Denudation by Attenuating TNF-alpha-induced Endothelial Cell Dysfunction

Suresh K Verma, Ctr for Transnational Med, Philadelphia, PA; Prasanna Krishnamurthy, Alexander R Mackie, Erin E Vaughan, Feinberg Cardiovascular Res Inst, Chicago, IL; Mohsin Khan, Garikipathy Venkat Srikanth, Ctr for Transnational Med, Philadelphia, PA; Veronica Ramirez, Tatiana Abramova, Sol Misener, Gangjian Qin, Feinberg Cardiovascular Res Inst, Chicago, IL; Raj Kishore, Ctr for Transnational Med, Philadelphia, PA

The association of inflammation with atherosclerosis and restenosis is now fairly well established. Restenosis, a persistent complication of percutaneous vascular interventions, is thought to be a complex response to injury, which includes early thrombus formation, neointimal growth and acute inflammation. Mononuclear phagocytes are likely participants in the host response to vascular injury, via the secretion of cytokines and chemokines, including TNF-alpha (TNF). Others and we have previously shown that IL-10 inhibits TNF and other inflammatory mediators produced in response to cardiovascular injuries. The specific effect of IL-10 on endothelial cell (EC) biology is not well elucidated. Here we report that in a mouse model of carotid denudation, IL-10 knock-out mice (IL10KO) displayed significantly delayed ReEndothelialization and enhanced neointimal growth compared to their WT counterparts. Exogenous treatment of recombinant IL-10 dramatically blunted the inflammatory cell infiltration and neointimal thickening while significantly accelerating the recovery of the injured endothelium both WT and IL10KO mice. In vitro, IL10 co-treatment reversed TNF-mediated growth arrest, EC cell cycle inhibition, EC-monocyte adhesion and EC apoptosis. At signaling level, IL-10 reduced TNF-induced activation of JNK MAP kinase while simultaneously activating PI3K/Akt pathway. Because IL-10 function and signaling are important components for control of inflammatory responses, these results may provide insights necessary to develop strategies for modulating vascular repair and other accelerated arteriopathies, including transplant vasculopathy and vein graft hyperplasia.

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Interaction Between Nitroso-Redox Balance and Temperature Influences SR Ca^{2+} Leak In Cardiomyocytes: Role of Neuronal NOS Signaling

Raul A Dulce, Vera Mayo, Erika B Rangel, Wayne Balkan, Joshua M Hare, Univ of Miami Miller Sch of Med, Miami, FL

Sarcomeric reticulum (SR) Ca^{2+} leak contributes to impaired Ca^{2+} cycling and contractile dysfunction of the heart. Leak is thought to be regulated by post-translational modification of RyR2 associated with the nitroso-redox state. Ca^{2+} cycling is affected by both nitric oxide (NO) signaling and temperature. We studied the effect of temperature and nitroso-redox state on Ca^{2+} leak. We hypothesized that nitroso-redox balance modulates leak in a temperature-dependent manner. Tetracaine was used to estimate the leak in cardiomyocytes (CMs) in mouse models of nitroso-redox imbalance ($NOS1^{-/-}$), hyper S-nitrosylation ($GSNOR^{-/-}$) and in WT mice. Reactive oxygen species (ROS) were measured. Experiments were carried out at 23ºC - 37ºC. In WT CMs, Ca^{2+} leak was inversely proportional to temperature whereas, in $NOS1^{-/-}$ CMs, the leak suddenly increased when the temperature surpassed 30ºC. Supplementing the media with NO (1 microM SNAP), reduced leak in $NOS1^{-/-}$ CMs at physiologic temperature while at low temperatures, there was a trend toward an increase, a pattern similar to that of $GSNOR^{-/-}$ rather than WT CMs. The xanthine oxidase inhibitor, oxypurinol (100 microM), diminished the leak in $NOS1^{-/-}$ CMs. SR Ca^{2+} content in $NOS1^{-/-}$ CMs was reduced as the temperature decreased, whereas it was not affected in WT cells. Cooling from 37ºC to 23 ºC increased ROS generation in WT CMs; however there was an opposite effect observed in $NOS1^{-/-}$ CMs, which was further reduced by oxypurinol. Expression of

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the NOS isoforms (by RT-PCR) was unaffected by temperature in WT. Therefore, Ca^{2+} leak is temperature dependent, is affected by the absence of NOS1 activity and increases with the rise of ROS production. Defective denitrosylation activity keeps leak low, suggesting a protective effect of S-nitrosylation against the cooling-induced increase in SR Ca^{2+} leak. These results suggest that Ca^{2+} leak from the SR is regulated by a temperature-dependent balance between NO and ROS.

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Cardiac Function And Calcium Handling in Obesity-Resistant rats

Andre Leopoldo, Felipe de Sá, Wagner Estevam, Bruno Jacobsen, Artur Ferron, Márcia da Cunha, Edson Castardeli, Univ of Espirito Santo, Vitoria, Brazil; Dijon Campos, UNESP-Univ. Estadual Paulista, Botucatu, Brazil; Antonio Cicogna, UNESP - Univ. Estadual Paulista, Botucatu, Brazil; Ana Paula Lima Leopoldo, Univ of Espirito Santo, Vitoria, Brazil

Experimental studies have demonstrated that obesity induced by different types of high-fat and/or high-energy diets also lead to myocardial dysfunction. Nevertheless, few studies have evaluated the myocardial function in obesity-resistant rats. Moreover, the mechanisms underlying the participation of calcium (Ca^{2+}) handling on cardiac function in this model remain unknown. The aim of this study was to investigate in rodent model of obesity-resistant. In addition, as obesity-resistant group is fed a high-fat diet but did not become obese, this study tested whether obesity-resistant model develops cardiac abnormalities and impairment of calcium handling as obesity-prone. Male 30-day-old *Wistar* rats were fed standard (C) and alternately four palatable unsaturated high-fat diets (Ob) for 15 weeks. After experimental protocol, Ob rats consuming the unsaturated high-fat diets were ranked based on adiposity index. Rats on the unsaturated high-fat diets exhibiting the greatest adiposity index were referred to as OP, whereas those exhibiting the lowest adiposity index were referred to as OR. Obesity was determined by adiposity index and comorbidities were evaluated. Myocardial function was evaluated in isolated left ventricle papillary muscles under basal conditions and after inotropic and lusitropic maneuvers. After 15 weeks, final body weight, total body fat, adiposity index, triglycerides levels and heart weight were significantly greater in OP rats than C and OR rats, however, there was no change in systolic blood pressure between groups. The C, OP and OR muscles developed similar baseline data, but myocardial responsiveness to post-rest contraction stimulus was compromised in OP rats. In conclusion, obesity-resistant model by unsaturated high-fat diet, after 15 weeks, does not display nutritional and metabolic characteristics of obesity-prone. Furthermore, obesity-resistant does not promote cardiac abnormalities and impairment of calcium handling visualized in obesity-prone.

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Involvement of β-adrenergic System in Myocardial Dysfunction Induced by Obesity

Ana Paula Lima-Leopoldo, Artur Ferron, Bruno Jacobsen, Federal Univ of Espirito Santo, Vitoria, Brazil; Dijon Campos, Renata Luvizotto, UNESP- Univ. Estadual Paulista, Botucatu, Brazil; Márcia Regina Cunha, Federal Univ of Espirito Santo, Vitoria, Brazil; Antonio Cicogna, UNESP- Univ. Estadual Paulista, Botucatu, Brazil; Andre Leopoldo, Federal Univ of Espirito Santo, Vitoria, Brazil

Several structural and functional changes of the heart have often been associated with human and experimental models of obesity. Some factors have been suggested as responsible for possible cardiac abnormalities in models of obesity, among them β-adrenergic system, an important mechanism of regulation of myocardial contraction and relaxation. The objetive of present study was to evaluate the . Thirty-day-old male Wistar rats were assigned to one of two groups: control (C) and obese (Ob). The C group was fed a standard diet and Ob group was fed cycles of four unsaturated high-fat diets for 15

weeks. The body fat was measured from the sum of the individual fat pad weights and the obesity was defined by adiposity index. Isolated papillary muscle preparation was performed under basal conditions and after inotropic and lusitropic maneuvers. β-adrenergic system was evaluated by using cumulative concentrations of isoproterenol and Western Blot. After 15 weeks, the Ob rats developed higher adiposity index than C rats. Obesity promoted comorbities such as glucose intolerance, insulin resistance, hyperleptinemia, and dyslipidemia; however, were not associated with changes in systolic blood pressure. The cardiac structure results post-death showed that obesity caused cardiac hypertrophy. Furthermore, Ob muscles developed similar baseline data, but myocardial responsiveness to post-rest contraction stimulus and increased extracellular Ca2+ was compromised. There were no changes in cardiac function between groups after β-adrenergic stimulation. The obesity was not accompanied by changes in protein expression of Gsα, β1 and β2 adrenergic receptors. In conclusion, the myorcardial dysfunction caused by unsaturated high-fat diet-induced obesity, after 15 weeks, is not related to β-adrenergic system impairment.

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Toll like Receptor 4 Activation Promotes Cardiac Arrhythmias By Decreasing The Transient Outward Potassium Current (ito) Through An Irf3 dependent And Myd88 independent Pathway

Emiliano Medei Sr., Gustavo Monnerat-Cahli, Lab de Eletrofisiologia Cardiaca, Rio de Janeiro, Brazil; Hiart Alonso, Facultad de Farmacia, Vitoria, Spain; Monica Gallego, Dept de Fisiología - Facultad de Farmacia, Vitoria, Spain; Micaela Lopez Alarcon, Lab de Eletrofisiologia Cardiaca, Rio de Janeiro, Brazil; Rosana A Bassani, Dept of Biomedical Engineering/FEEC, Campinas, Brazil; Oscar Casis, Dept de Fisiologia - Facultad de Farmacia, Vitoria, Spain

Cardiac arrhythmias are one of the main causes of death worldwide. Several studies have shown that inflammation plays a key role in different cardiac diseases and Toll like receptors (TLR's) play an important role in cardiac complications. In the present study, we investigated whether the activation of TLR4 induces cardiac electrical remodeling and arrhythmias. Also the signaling pathway involved in these phenomena was studied. Action potentials, the presence of cardiac arrhythmias and transient outward K^+ current (I_{to}) were recorded in Wistar rat's hearts after 24 h exposure to the TLR4 agonist ultrapure Lipopolysaccharide (LPS - 1μg/ml). TLR4 stimulation *in vitro* promotes a cardiac electrical remodeling that leads to cardiac action potential prolongation which evokes arrhythmic events such as delayed after depolarization (DAD's) and triggered activity. The perfusion of LPS (1μg/ml) during 30 minutes did not modify I_{to}. Conversely, after 24 h of LPS incubation I_{to} was reduced, with no changes in the biophysical properties of the current. Major changes in Ca^{2+} cycling were not observed in ventricular myocytes after 24 h exposure to LPS; however, extrasystolic activity was present in a considerable number of cells (25%). Neither the blockade of Interleulink-1 receptor-associated kinase 4 nor nuclear factor kappa B (NF-kB) prevented the LPS effect on I_{to}. However, interferon regulatory factor 3 (IRF3) inhibition prevented the effect of TLR4 activation on I_{to}. Activation of TLR4 induced extrasystolic activity, longer AP duration and evoked DAD's and triggered activity because of a reduction in I_{to}. The mechanism involved is MyD88-independent and IRF3-dependent.

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Ultrasound-Induced Inhibition and Modulation of Neonatal Ventricular Cardiomyocyte Depolarization

Natasha Mehta, J Yasha Kresh, Steven P. Kutalek MD, Drexel Coll of Med, Philadelphia, PA; Peter A. Lewin, Sch of Biomedical Engineering, Science & Health System, Drexel Univ, Philadelphia, PA; Andrew R Kohut, Drexel Coll of Med, Philadelphia, PA

Background: Ultrasound can interact with tissue through either thermal or non-thermal

physical mechanisms. Radiation force has been shown to stimulate cardiac and neural tissue in vivo. Ultrasound might hold clinical potential as a noninvasive therapeutic tool via specific bioeffects on cardiomyocytes. This study aims to assess the effect of ultrasound on cardiomyocyte depolarization in a tissue culture model.
Methods: Cardiomyocytes were isolated from neonatal rat ventricular tissue and plated directly on microelectrode arrays to record depolarization patterns. A custom 2.5 MHz unfocused ultrasound transducer was directed at the cardiomyocytes in a tissue culture model. A function generator, with an amplified signal +50 dB, delivered acoustic energy at variable settings of 0.1, 0.3, 0.5 and 1.0 Vpp, pulse durations of 2, 5 and 10 ms, and burst periods of 100, 250 and 300 ms. Five trials were conducted at each setting (36 total trials) with 30s of continuous ultrasound exposure followed by an off interval of 1 minute.
Results: The R-R interval durations (ID) were measured throughout the recording period. Prior to ultrasound delivery, the IDs were highly irregular, ID range = 0.3-2.7 s. As ultrasound was delivered in an asynchronous manner, using 0.1 and 0.3 Vpp and PD = 2 and 5 ms, there was suppression/inhibition of cellular depolarization for the first 5-10 s. Then 10-15 s after the start of ultrasound delivery, the depolarization rate increased and demonstrated less R-R interval variability (ID=0.88-1.03 s, P value<0.05), even after the ultrasound exposure.
Conclusion: Ultrasound can inhibit and modify the frequency of spontaneous electrical depolarizations of neonatal ventricular cardiomyocytes in a tissue culture model. Our observations could be due to conditioning via stretch and compression-mediated mechanosensitive pathways, by modifying intracellular calcium handling or altering cell signaling.

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Ablation of the Hypertension Candidate Gene *ATP2B1* Leads To Deficient Calcium Cycling, Systolic Dysfunction and Heart Failure Following Pressure Overload

Nicholas P Stafford, Min Zi, Univ of Manchester, Manchester, United Kingdom; Ludwig Neyses, Univ of Luxembourg, Luxembourg, Luxembourg; Elizabeth J Cartwright, Univ of Manchester, Manchester, United Kingdom

Mutations in *ATP2B1* encoding the ubiquitous calcium extrusion pump Plasma Membrane Calcium ATPase 1 (PMCA1) have recently identified it as having the strongest association of any gene to hypertension, yet the role of PMCA1 in the pressure-overloaded heart is not known. To investigate this we generated a novel mouse line carrying cardiomyocyte-specific deletion of PMCA1 ($PMCA1^{cko}$) and challenged them with transverse aortic constriction (TAC) alongside littermate 'floxed' controls ($PMCA1^{f/f}$). After two weeks, echocardiographic analysis revealed signs of systolic dysfunction and left ventricular (LV) dilation in $PMCA1^{cko}$ hearts as evidenced by reduced fractional shortening and increased diastolic diameter (both $p<0.05$), whilst function in $PMCA1^{f/f}$ TAC controls remained preserved. This was accompanied by an increase in normalised lung weight in $PMCA1^{cko}$ mice compared to sham operated and TAC controls ($p<0.05$) indicative of pulmonary congestion and a progression into LV failure, despite comparable hypertrophic growth amongst the two TAC cohorts. Hemodynamic analysis following LV catheterisation revealed contractility, as measured by left ventricular elastance (E_{es}), to be increased in controls after TAC ($PMCA1^{f/f}$ TAC 12.69 ± 1.63 vs sham 7.02 ± 1.11 mmHg/µl, $p<0.05$), a change which was not reciprocated in knockout hearts ($PMCA1^{cko}$ TAC 7.70 ± 1.19 vs sham 7.22 ± 1.55 mmHg/µl). To examine whether altered calcium handling could be the underlying cause of the observed phenotype, cardiomyocytes were isolated following one week TAC and loaded with Indo-1, prior to the onset of failure in $PMCA1^{cko}$ hearts. Compatible with an increase in E_{es}, systolic calcium levels were higher in $PMCA1^{f/f}$ myocytes following pressure overload compared to sham controls ($p<0.05$), whilst $PMCA1^{cko}$ TAC myocytes displayed equivalent peak calcium levels to their respective sham controls. These results suggest that PMCA1 may play a necessary role in enhancing calcium cycling during the early response to pressure overload, and that disrupting this gene may increase the susceptibility to heart failure under these conditions. This may provide first evidence of a

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novel genetic basis for the development of heart failure in a proportion of hypertensive patients.

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Tuberosis Sclerosis Complex 1 Mediated Neointima Formation and Arterial Thrombosis Following Vascular Injury

Eo Jin Kim, Dept of Pathology, Ilsan, Korea, Republic of; Yong-Joo Ahn, Maya Hwewon Kim, Hyung-Hwan Kim, Massachusetts General Hosp, Charlestown, MA

Objectives: Vascular injury and thrombosis are main leading causes of cardiovascular diseases. Tuberous sclerosis complex (TSC) is a genetic disorder caused by heterozygous mutations in either of two genes, TSC1 and TSC2. Although role of TSCs has been implicated in cardiovascular diseases, the tissue- and isoform-specific roles of TSCs in the vascular response to injury are not known. Methods and Results: To determine the role of TSC1 in arterial injury and thrombosis, we generated vascular smooth muscle cell-specific TSC1 conditional knockout mice (TSC1SM22-/-) by crossing vascular smooth muscle cell-specific Cre (SM22Cre) mice with TSC1flox/flox mice and performed carotid artery ligation in haploinsufficient TSC1 conditional knockout mice (TSC1SM22+/-) compared with that of WT or haploinsufficient TSC2 knockout mice (TSC2+/-). Acute carotid artery occlusion was investigated by 5% ferric chloride injury. Arterial thrombosis and neointima formation were measured at 14 days after arterial ligation. Expression of proteins was observed by immunoblot analysis. The neointima formation was significantly increased in TSC1SM22+/- mice (intimal thickness/medial thickness ratio; 1.14 ± 0.14, $p<0.001$) compared with that of WT mice (0.13 ± 0.03). Two weeks after arterial injury, arterial thrombus area was increased in TSC1SM22+/- mice (thrombus area/luminal area ratio; 72.1 ± 4.4, $p<0.001$) compared with that of WT mice (0.1 ± 0.0) but there are no significant effect in acute arterial thrombosis induced by ferric chloride. Loss of TSC1 and hyper-activation of mammalian target of rapamycin complex 1 including mTOR and S6 proteins were observed by immunoblot analysis of carotid artery tissue lysates of TSC1SM22+/- mice compared with that of WT. Conclusion: These findings suggest that regulation of TSC1 and mTOR might be useful for therapeutic intervention in vascular injury and thrombosis.

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Kcne2 Gene Deletion Combines With A Western Diet To Cause Early-onset Diabetes Mellitus And Atherosclerosis In Mice

Soo-Min Lee, Ritu Kant, Geoffrey W Abbott, Univ of California Irvine, Irvine, CA

The KCNE2 single transmembrane domain protein regulates multiple types of cardiac voltage-gated potassium (Kv) channel *in vivo* and its disruption causes the ventricular arrhythmia, Long QT Syndrome. Interestingly, a SNP near the human KCNE2 locus was also previously linked to early-onset myocardial infarction (MI). Because KCNE2 is also expressed outside the heart, we are interested in understanding extracardiac defects caused by *Kcne2* deletion in mice, and also the potential contribution of these extracardiac defects to cardiac dysfunction. Thus, recently, we found that *Kcne2* deletion creates a multifactorial substrate for sudden cardiac death (SCD) that includes diabetes mellitus and dyslipidemia. Because atherosclerosis is the most common cause of MI, here, using *Kcne2*$^{-/-}$ mice, we investigated atherosclerosis and other potential predisposing factors in MI and SCD, and quantified the impact of a Western diet (high fat/high cholesterol) on these processes. Previously, we discovered impaired glucose tolerance in adult *Kcne2*$^{-/-}$ mice using a standard glucose tolerance assay in which plasma glucose is quantified 0-2 hours following glucose injection. Here, we found that in *Kcne2*$^{-/-}$ mice as young as 5 weeks, just 2 weeks on a Western diet induced impaired glucose tolerance, whereas 5-week-old wild-type mice regardless of diet, or *Kcne2*$^{-/-}$ mice on a control diet, had normal glucose tolerance. This indicates a strong interaction between diet and *Kcne2* deletion in creating early-onset diabetes mellitus.

Atherosclerosis was also investigated, using Sudan IV staining of plaques in the aorta of wild-type and *Kcne2*$^{-/-}$ mice fed on either standard (control) or Western diet. Strikingly, *Kcne2*$^{-/-}$ mice as young as 4.5 months exhibited aortic plaques, and this was accelerated by a Western diet. In contrast, wild-type littermates did not exhibit plaques at this age, regardless of diet. Ongoing studies to determine potential metabolic defects in the liver and pancreas are aimed at understanding the molecular mechanisms underlying these findings. Our data provide further evidence of the unexpected complexity of monogenic cardiovascular syndromes caused by disruption of genes associated with cardiac arrhythmias.

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Single Nucleotide Polymorphism of the B-Type Natriuretic Peptide Helps Predict the Presence of Significant Coronary Artery Disease

Terry Y Li, M. Yat Tse, Nicole M. Ventura, Marie-France Hetu, Amer M. Johri, Stephen C. Pang, Queen's Univ, Kingston, ON, Canada

Early detection and diagnosis of coronary artery disease (CAD) is crucial in reducing the morbidity and mortality. The clinical standard for detecting CAD is by angiography which is associated with rare but important clinical risks. Recent studies have shown that up to 40% of patients referred for angiography do not have significant disease. This discrepancy between referral and diagnosis may be improved by the utilization of genetic screening. A single nucleotide polymorphisms (SNP) of the B-type natriuretic peptide gene (NPPB), rs198389, was previously found to be associated with several cardiovascular diseases. Our objective was to determine whether detection of genetic variation could contribute to better selection of patients referred for angiography. We hypothesized that an SNP analysis of the NPPB gene may help differentiate patients with significant disease from those with non-significant CAD. Ninety-three patients referred for coronary angiography at the Kingston General Hospital Cardiac Catheterization Lab were consented for genetic screening. Blood samples were collected during angiography procedure. Genomic DNA was isolated from leukocytes, and screened for SNPs using a real-time PCR-based TaqMan SNP Assay. We found that more males were referred for coronary angiography than females: 69% versus 31%. Two out of ten males (20%) were found to have no or minimal CAD. In contrast, 48% of females were found to have non-significant CAD. Older age (≥ 69 years) was deemed to be a significant predictor of CAD in the total recruited population (odds ratio of 3.4), but no age difference was found between healthy and diseased females. Additionally, a mutation of the B-type natriuretic peptide gene (NPPB) was found to be a significant predictor of CAD in the younger population (< 69 years), with an odds ratio of 5.9. We found a significant difference between patterns of CAD development in males and females, suggesting that different diagnostic criteria should be used depending upon gender. Moreover, younger individuals with two copies of the major allele for the NPPB SNP were more likely to develop CAD, making this SNP a potential factor in the prediction of CAD in younger population.

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Genetic Basis of Isoproterenol-Induced Cardiac Fibrosis

Christoph D Rau, Rozeta Avetisyan, Dorit Stein, Milagros Romay, Jessica Wang, Yibin Wang, Aldons J Lusis, UCLA, Los Angeles, CA

Background: Cardiac fibrosis is a common pathology in the diseased heart, which can cause a loss of elasticity and contractile dysfunction. Cardiac fibrosis is a complex process driven by many pathological triggers which involve numerous genes, pathways and cell types. Despite its importance, the genetic basis for the development of cardiac fibrosis has not been systematically explored. Methods and Results: We have developed a resource, the Hybrid Mouse Diversity Panel (HMDP) for high resolution GWAS and systems genetics study of quantitative traits in mice. Eight week old female mice from 80 unique inbred strains of the HMDP were given 30 ug/g/day of isoproterenol (ISO) for three weeks and cardiac

fibrosis was assessed by Masson Trichrome staining which revealed a wide spectrum in the degree of fibrosis among the HMDP strains both before and after treatment. Using the Efficient Mixed Model Algorithm, we identified 13 significant or suggestive loci contributing to cardiac fibrosis, many containing numerous gene candidates. Within one of these loci, Abcc6, an orphan ABC transporter linked to the human disease pseudoxanthoma elasticum, was identified as a possible candidate for ISO-induced cardiac fibrosis. A splice-site mutation present in 19 strains of the HMDP was significantly linked to a higher degree of ISO-induced cardiac fibrosis(P=1E-4) but was not linked to increased fibrosis in untreated animals(P=0.25). Targeted genetic knockout of Abcc6 promoted ISO-induced cardiac fibrosis while reintroducing the wildtype Abcc6 allele to an genetic strain homozygous for the Abcc6 splice site mutation significantly alleviated ISO-induced cardiac fibrosis. Conclusion: A GWAS performed on levels of cardiac fibrosis observed in ISO treated animals using HMDP mice as model system uncovered significant genetic contributions to stress-induced cardiac fibrosis. Abcc6 is a novel gene contributing to ISO-induced cardiac fibrosis in the heart.

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β-adrenergic Stimulation Exacerbates Preclinical Arrhythmogenic Right Ventricular Cardiomyopathy Due To Desmoplakin Mutation

Subat Turdi, Nan Gong, Jeanne James, Patrick Winters, Lori Stanton, Enkhsaikhan Purevjav, Jeffrey A Towbin, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH

Background: Arrhythmogenic right ventricular cardiomyopathy (ARVC) is associated with ventricular arrhythmias and sudden cardiac death. We previously demonstrated that cardiac-specific overexpression of human mutant desmoplakin (DSPR2834H) leads to ARVC in mice at 6 months of age. However, the potential role and mechanism(s) of DSP in preclinical ARVC under cardiac stress remains unclear. Objectives: This study is aimed to elucidate the impact of DSP in the development of ARVC under adrenergic stimulation. Methods: Three-month-old non-transgenic (NTg), wild-type DSP (Tg-DSPWT) and Tg-DSPR2834H mice without obvious signs of ARVC, including right and left ventricular dysfunction, arrhythmias, were infused with either vehicle or isoproterenol (30mg/kg/d) for 2 weeks using mini-osmotic pumps. During isoproterenol infusion, electrocardiography (ECG) was monitored daily on conscious mice. Echocardiography and cardiac MRI were performed before and after 2-week of isoproterenol infusion. Myocardial tissues from both RV and LV were subjected to cellular, biochemical and histopathological analysis. Results: Isoproterenol resulted in cardiac hypertrophy to a similar degree amongst all genotypes; however, mortality occurred only in Tg-DSPR2834H mice. Vehicle-treated mice from all genotypes showed largely normal ECGs, whereas isoproterenol led to various types of arrhythmia in Tg-DSPR2834H mice including ventricular tachycardia and QT prolongation. Echocardiography analysis revealed LV dysfunction (decreased factional shortening) in isoproterenol-treated Tg-DSPR2834H mice compared to other treatment groups. Interstitial fibrosis and lipid infiltration was prominent in the Tg-DSPR2834H myocardium. MRI analysis is being performed to understand the RV and LV geometry and function. Conclusion: Our preliminary data confirms the essential role of desmoplakin in response to adrenergic stimulation. Studies investigating the electrophysiological, geometrical and cellular mechanisms of the pro-arrhythmic nature of DSPR2834H dysfunction are ongoing which may ultimately provide critical experimental data on prevention of asymptomatic preclinical ARVC in humans.

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The Transcriptional Cofactor Eyes Absent 4 Is A Critical Regulator In Cardiac Physiology

Tatjana Williams, Univ Hosp of Wuerzburg, Molecular Cardiology, Wuerzburg, Germany; Moritz Hundertmark, Peter Nordbeck, Univ Hosp of Wuerzburg, Wuerzburg, Germany; Sabine Voll, Univ Wuerzburg, Dept of Experimental Physics V (Biophysics), Wurzburg, Germany, Wuerzburg, Germany; Ines Elsner, Univ Hosp of Wuerzburg, Molecular Cardiology, Wuerzburg, Germany; Jost Schoenberger, Univ Hosp of Wuerzburg,, Wuerzburg, Germany; Oliver Ritter, Univ Hosp of Wurzburg, Comprehensive Heart Failure Ctr, Wuerzburg, Germany

Introduction:
Eyes absent 4 (Eya4) is a transcription cofactor involved in a number of cellular and developmental processes. We have previously shown that a truncating mutation in Eya4, E193, causes hearing impairment followed by terminal heart failure suggesting Eya4 is a regulator in cardiac physiology.
Methods and Results:
Transgene- or adenovirus mediated overexpression of Eya4 or E193 altered the expression of p27kip1, a critical mediator of cardiac hypertrophy in adult cardiomyocytes. Luciferase reporter and EMSA assays revealed that Eya4 directly binds to and suppresses p27kip1 promoter activity, while E193 exerts an opposing effect, respectively. Activity and phosphorylation of downstream molecules were significantly altered in Eya4 and E193 transgenic (TG) mice in a contradictory manner. Cardiac phenotypes evolved in both TG models already under basal conditions. Eya4 TG hearts developed hypertrophy as judged by increases in heart weight and cross-sectional cell surfaces and re-activation of fetal genes as well as fibrosis. E193 TG animals showed onset of DCM along with wall thinning, ventricular dilation, fibrosis and slightly compromised cardiac function. These two distinct cardiac phenotypes were even more aggravated upon pressure overload or Angiotensin II infusion. Finally, we just recently identified a new mutation in Eya4, E215, which also causes hearing impairment and heart failure.
Conclusion:
Our data indicate that Eya4, in a physical complex with Six1, plays a critical role in regulating normal cardiac function via p27/CK2-α/HDAC2 and allude that mutations within the Exa4/Six1 transcriptional complex interfere with this newly established signalling pathway, finally leading to age-related onset of cardiomyopathy.
Clinical perspective:
Gaining a better understanding of this disease mechanism could help identify new treatment options for heart failure patients.

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Matrix Metalloproteinase-9 Mediates Post-hypoxic Vascular Pruning Of Cerebral Blood Vessels By Degrading Laminin And Claudin-5

Amin Boroujerdi, Jennifer V Welser-Alves, Richard Milner, The Scripps Res Inst, La Jolla, CA

Objective: Vascular remodeling involves a highly coordinated break-down and build-up of the vascular basal lamina and inter-endothelial tight junction proteins. The goal of this study was to examine the role of matrix metalloproteinase-9 (MMP-9) in remodeling of cerebral blood vessels, both in hypoxia-induced angiogenesis and in the vascular pruning that accompanies the switch from hypoxia back to normoxia. Approach and Results: In a chronic mild hypoxia model of cerebrovascular remodeling, gel zymography revealed that MMP-9 levels were increased, both in the hypoxic angiogenic response and in the post-hypoxic pruning response. Compared to wild-type mice, MMP-9 KO mice showed no alteration in hypoxic-induced angiogenesis, but did show marked delay in post-hypoxic vascular pruning. In wild-type mice, vascular pruning was associated with fragmentation of vascular laminin and the tight junction protein claudin-5, while this process was markedly attenuated in MMP-9 KO mice. In vitro experiments showed that hypoxia stimulated MMP-9 expression in brain endothelial cells (BECs) but not pericytes. While immunofluorescent and flow cytometry analyses showed that hypoxia led to reduced expression of laminin and claudin-5 in wild-type BECs, this decrease was absent in MMP-9 KO BECs. Conclusions: These results show that while MMP-9 is not essential for hypoxic-induced cerebral angiogenesis, it plays an important role in post-hypoxic vascular pruning by degrading laminin and claudin-5. Our data support the concept that MMP-9 inhibition might provide

therapeutic benefit in the treatment of ischemic stroke, by preventing post-hypoxic vascular pruning, thereby optimizing vascular density and integrity.

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Growth Induction to Reverse Congenital Aortic Developmental Hypoplasia

Daniel F Labuz, James M Berry, Lee A Pyles, John E Foker, Univ of Minnesota Medical Sch, Minneapolis, MN

Objectives: Congenital heart disease may include hypoplastic heart valves, ventricles or great arteries. Infants with coarctation of the aorta (CoA) often have a hypoplastic transverse aortic arch (TAA) which can greatly complicate surgical repairs. Although these defects are often considered to be genetic in origin, our hypothesis was that they are problems of development which are potentially reversible. We tested the corollary hypothesis that hypoplastic TAAs retain normal developmental potential and the increased aortic flow after CoA repair provides the biomechanical signal for catch-up growth.
Methods: Infants (N = 19) with TAA hypoplasia who underwent surgical CoA repair were studied for TAA growth by echocardiography done prior to and at intervals up to 13 years later. The TAA diameters were indexed using nomograms and calculated as standard deviations from expected size (SDE). Normal range is ± 2 SDE and < -2 indicates hypoplasia.
Results: 1) TAA growth was rapid and significant within 3 months. 2) The initial average TAA SDE = -3.7 (range: -7.6 to -2.1) (0/19 normal); 3 months = -1.5 (-4.1 to 0.9) (12/17); 1 year = -1.0 (-4.2 to 1.3) (15/19); 5 to 13 years = -0.6 (-2.1 to 0.9) (16/17 normal).
Conclusions: 1) Hypoplastic aortic arches grew rapidly to normal size with increased flow following CoA repair. 2) The results suggest the cause of hypoplasia was underdevelopment from low flow and, when increased, flow provided the biomechanical signal to induce catch up growth. 3) Infants were a relevant model for demonstrating the aortic growth signal. 4) Growth induction by increased blood flow could be used to reverse aortic underdevelopment in other selected patients.

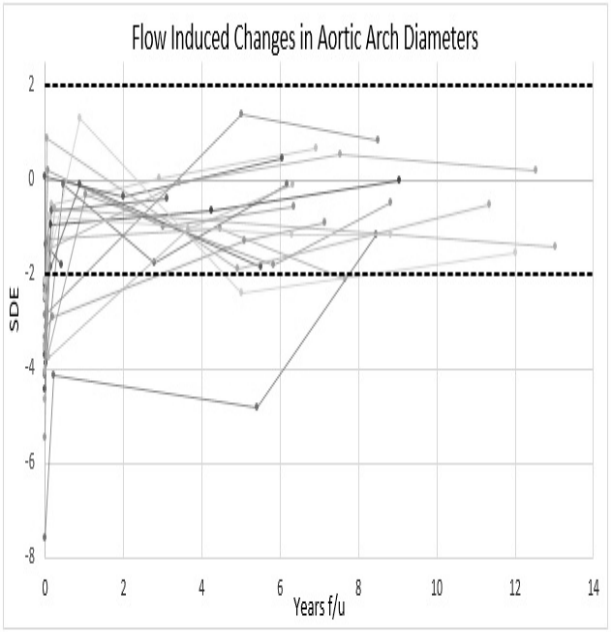

Figure 1: Changes in TAA diameters following CoA repair. Year= 0 signifies pre-op diameters; SDE -2 to 2 are considered the normal range. Each colored line corresponds to an individual patient.

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Contribution of Vascular Endothelial Growth Factor Signaling to Fate Specification in Cardiomyocytes

Taylor Y Lu, Courtney K Domigan, Vaspour Antanesian, Yasuhiro Nakashima, Atsushi Nakano, Luisa Iruela-Arispe, Univ of California, Los Angeles, Los Angeles, CA

Vascular endothelial growth factor (VEGF) is one of the pivotal proangiogenic growth factors that has long contributed to our knowledge of blood vessel and circulatory maintenance as well as angiogenesis in both pathology and pathophysiology. However, the non-canonical functions of VEGF in cardiac morphogenesis have not been well characterized. Here, we

examined how VEGF regulates cardiomyocyte cell fate.
Using chimeric embryos harboring both wild type and VEGF-null embryonic stem cells, we observed that derivatives of VEGF null cells were preferentially recruited to the atrium of the heart in comparison to the ventricles. To further provide physiologic context of this finding, we used reporter-LacZ staining and RT-PCR and found that endogenous VEGF was indeed expressed at much lower levels in the atrium but highly expressed in the ventricle early in cardiac morphogenesis. These data lead to our hypothesis that cell-autonomous expression of VEGF is a determinant of atrial vs. ventricular cardiomyocyte cell fate. To test this hypothesis, we used a VEGF knock-in mouse model of Sm22Cre x Rosa 26 VEGF. VEGF overexpression in cardiomyocytes (and smooth muscle) at E8.5 resulted in lethality by P1 and thickened atrial and ventricular walls in mutant embryos as characterized by histology (H&E, IF). We further explored the molecular changes underlying this phenotype via microarray and RT-PCR and find disruptions in molecular markers necessary for wall development, specifically: Notch-1, BMP10, Nrg-1. Taken together, our data indicates that aberrant embryonic VEGF signaling disrupts several critical signaling pathways and that overexpression leads to disruption of cardiomyocyte proliferation and cardiac morphogenesis. These findings add to the foundation of better understanding heart development, laying the groundwork for future therapy of congenital and acquired cardiac disease.

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Elucidating the role of GRK5 in Physiological Hypertrophy

Christopher J Traynham, Alessandro Cannavo, Jonathan Hullmann, Jessica L Gold, Walter J Koch, Temple Univ, Philadelphia, PA

Cardiac function is dynamically regulated by various G protein-coupled receptors (GPCRs). GPCR kinases (GRKs) are important in cardiac GPCR regulation through phosphorylation and desensitization of these receptors. GRK2 and GRK5 are the predominant GRKs in the heart, and the most widely characterized as they are both up-regulated in the failing heart. Prior studies from our Lab have determined that GRK5 plays a crucial role in pathological cardiac hypertrophy. Another type of hypertrophy termed, “physiological hypertrophy” occurs with exercise training and is defined as an enlargement in cardiac myocyte size leading to favorable cardiac adaptations. At present, it is unclear if GRK5 is a regulator of physiological hypertrophy in addition to its role in maladaptive hypertrophy. We hypothesize that GRK5 will not regulate physiological hypertrophy such that mice with cardiac-specific overexpression of GRK5 (TgGRK5) will yield a similar post-exercise cardiac physiological hypertrophic response as that of control wild-type (WT) mice. To test this hypothesis, TgGRK5 and WT mice were exposed to a 21 day high-intensity swimming exercise protocol. For each line, sham mice, which did not swim served as appropriate controls. At the conclusion of this protocol, mice were sacrificed and heart weight (HW), body weight (BW), and tibia length (TL) measured. TgGRK5 and WT mice both exhibited a characteristic 10-15% increase in HW/BW and HW/TL ratios, which are standard measures of cardiac hypertrophy. In addition, hearts were sectioned and H&E stained to evaluate myocyte size. Both TgGRK5 and WT mice exhibited a significant increase in myocyte size. Cardiac function was evaluated via echocardiography both prior to and after exercise training, and no changes were observed between TgGRK5 and WT mice after training. These data were re-affirmed in H9C2 cells and neonatal rat ventricular myocytes overexpressing either GFP or GRK5, which exhibited similar increases in cell size and AKT phosphorylation after IGF-1 treatment, a physiological hypertrophy stimulus. Taken together, these data suggest that physiological hypertrophy is similar in both control and TgGRK5 mice, confirming that GRK5 is solely a regulator of pathological cardiac hypertrophy.

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Hippo Signaling Regulates Epicardial Derived Cell Fate through Controlling Mechanical Property

Yang Xiao, Min Zhang, Texas A&M Health Science Ctr, Houston, TX; Yuka Morikawa, Texas Heart Inst, Houston, TX; James F Martin, Baylor Coll of Med, Houston, TX

Epicardium is the outmost layer of heart and regulates heart development by contributing to the heart tissue and secreting signaling molecules. Epicardial-derived cells, EPDC, is a group of multi-potent cells mainly derive into cardiac fibroblasts and smooth muscle cells. Hippo signaling pathway controls organ growth through regulating cell proliferation. Yap, the effector of Hippo pathway has been shown to regulate mechanosensing and cell fate. However, the function of Hippo signaling in EPDC development has not been studied yet. To study the role of Hippo signaling in development of epicardium and EPDC, we used epicardial specific line, $Wt1^{CreERT2}$, to conditionally delete the components of the pathway, Lats1/2. Cre activity was induced at embryonic day (E)11.5 and Lats1/2 CKO hearts exhibited disorganized coronary vasculature. We used genetic lineage tracing approach to track EPDC and revealed increased endothelial cells deriving from EPDC, suggesting that EPDC changed cell fate. We observed that shape of the epicardial cells became rounded with decreased F-actin in Lats1/2 CKO, which suggested that cell tension was reduced. Since mechanical properties of cellular microenvironment are known to regulate cell shape and influence cell fate determination, we hypothesize that Hippo pathway regulates mechanical property to control cell fate in EPDC. Transcription analysis showed that signals regulating cytoskeleton organization were disrupted, which suggests Hippo regulates cytoskeleton gene expression and affects mechanical force that cell sensed autonomously. Proliferation assay using EdU incorporation showed increased proliferation in EPDC of Lats1/2 CKO was observed, which suggests the changes of external tension cell sensed. To recapitulate the changes of external tension, we plated EPDC onto different stiffness substrate. EPDC of Lats1/2 CKO differentiated into endothelial cells on the soft substrate. On the other hand, Lats1/2 CKO did not derive endothelial cells on the hard substrate and wild type EPDC did not differentiate into endothelial cells on neither soft nor hard substrate. Taken together, our results suggested that Hippo pathway affected EPDC fate determination through controlling internal and external mechanical cues.

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T Cell Mediated Immune Responses Regulate Cardiac Remodeling and Survival in Pressure Overload Induced Heart Failure

Pilar Alcaide, Tania Nevers, Ane Salvador, Anna Grodecki-Pena, Andrew Knapp, Mark J Aronovitz, Francisco Velazquez, Tanya Kershaw, Navin K Kapur, Robert Blanton, Richard Karas, Tufts Medical Ctr, Boston, MA

Background: Clinical data support that inflammation and the improper regulation of the immune response are intimately associated with Heart Failure (HF), however, the type of immune response involved and whether it regulates cardiac remodeling remains largely unexplored. We **hypothesize** that T cell mediated immune responses and their recruitment into the heart influence cardiac remodeling and contribute to the pathogenesis of pressure overload induced HF.

Methods and results: Using quantitative flow cytometry we found that T cells infiltrated the heart as Wild-type mice (WT) developed systolic dysfunction and LV hypertrophy in response to transverse aortic constriction (TAC) ($p<0.01$ TAC *vs* Sham). Real time imaging demonstrated that T cells from TAC mice adhered to activated heart endothelial cells in higher numbers than T cells from Sham mice under physiological flow conditions in vitro ($P<0.05$) indicating a systemic T cell activation to pressure overload induced by TAC. Similarly, circulating T cells from patients with HF adhered more to activated human umbilical vein endothelial cells (HUVEC) than T cells from healthy volunteers. Based on these findings, we performed similar TAC studies in T cell deficient mice ($TCR\alpha^{-/-}$). In contrast with WT TAC mice, $TCR\alpha^{-/-}$ had preserved LV systolic and diastolic function ($p<0.01$) determined by echocardiography and hemodynamic studies, reduced LV fibrosis ($p<0.001$) and TGFβ1,

collagen Iα and αSMA gene expression ($p<0.05$), and reduced LV hypertrophy and gene expression of ANP and BNP ($p<0.05$), but unaltered expression of SerCA. Remarkably, $TCR\alpha^{-/-}$ had improved survival after 4 weeks of TAC [100%(16/16) $TCR\alpha^{-/-}$ *vs* 73.7%(14/19) WT, p=0.023]. Ongoing studies will determine the mechanisms regulating T cell recruitment into the heart, the type of T cell response involved and its contribution to pathological remodeling of the heart.
Conclusion: Our studies demonstrate that T cell immune responses and their recruitment into the LV contribute to the pathogenesis of pressure overload induced HF by mechanisms involving T cell regulation of cardiac hypertrophy and fibrosis, and open a window to develop novel therapeutic strategies to improve the structural, functional and molecular deficits of the failing heart.

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Lumican-null Mice Are Susceptible to Aging and Isoproterenol-induced Myocardial Fibrosis

Pao-Hsien Chu, Chang Gung Memorial Hosp, Taipei, Taiwan

Aims: With aging and stresses, the myocardium undergoes structural remodeling and often leading to fibrosis.
Main methods: To examine whether lumican, one of the class II small leucine-rich proteoglycans, has a role in cardiac remodeling and fibrosis, we analyzed the basic cardiac phenotypes of lumican-null (Lum-/-) mice in both youth and elder, and then used the isoproterenol-induced cardiac fibrosis model to study the roles of extra-cellular matrix and apoptosis in cardiac remodeling.
Key findings: Higher mortality resulted from significantly impaired systolic function, and an increase of atrial natriuretic peptide secreted by the ventricles in response to excessive stretching of myocytes of Lum-/- mice in comparison to wild type littermates. In addition, Lum-/- mice exhibited higher level of TGF-β, collagen I/III, and membrane-type matrix metalloproteinase-1 (MT1-MMP, or MMP-14) during cardiac remodeling.
Significance: Our data implicates that the lumican protein plays an important role in the pathogenesis of cardiac fibrosis.

P. Chu: None.

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Role of Cardiomyocyte-Localized Nuclear Factor (NF)-κB in Diet-Induced Diabetic Cardiomyopathy

Mehak Goel, Mohamed A Ismahil, Tariq Hamid, Sumanth D Prabhu, Div of Cardiovascular Disease, Dept of Med, Univ of Alabama at Birmingham, and Birmingham VAMC, Birmingham, AL

Role of Inflammatory signaling in the development of obesity- and type 2 diabetes (T2D)-associated cardiomyopathy remains unclear. We hypothesized that cardiomyocyte-specific inflammatory activation, independent of systemic inflammation, is sufficient for the genesis of diabetic cardiomyopathy. Wild-type (WT) and transgenic (Tg) C57BL/6 mice with myocyte-restricted overexpression of phosphorylation-resistant IκBα (IκBαS32A,S36A) were fed a high fat (HFD, 45% kcal fat) or control diet (CD, 10% kcal fat) for 28 weeks to induce obesity and T2D. These Tg mice exhibit attenuated NF-kB activation, a central regulator of inflammation. After 28 weeks of feeding, WT HFD mice exhibited increased body weight, hyperglycemia, hyperinsulinemia, insulin resistance, and glucose intolerance. In contrast, although HFD-fed Tg mice exhibited significantly ($p < 0.05$) increased body weight, the mice still maintained normal fasting blood glucose, insulin levels, and insulin sensitivity compared to CD Tg mice, indicating absence of overt T2D. Echocardiography revealed that both HFD-fed WT and Tg mice developed concentric left ventricular (LV) hypertrophy with increased septal and posterior wall thickness, decreased LV end-diastolic diameter and had unchanged

LV ejection fraction suggesting development of LV hypertrophy in both strains regardless of the severity of the metabolic disorder. Analysis of mononuclear phagocyte cell profiles revealed similar trends in WT and IκB Tg mice. HFD fed mice had higher levels of pro-inflammatory (CD11b+Ly6chi) and anti-inflammatory (CD11b+Ly6clo) monocytes in the blood, heart, spleen and adipose tissue compared to CD fed mice. Analysis of antigen presenting cells indicated that HFD fed mice in both genotypes had significantly increased levels of both classical (CD11c+CD45R–) and plasmacytoid (CD11cintCD45R+) dendritic cells in blood, heart, spleen and adipose tissues compared to respective CD fed mice. Thus, we conclude that cardiomyocyte inflammation is central to the development of metabolic abnormalities related to obesity, whereas neither cardiomyocyte-specific nor systemic insulin resistance is required for the development of the hypertrophic cardiomyopathic phenotype.

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Genetic Ablation Of S-nitrosoglutathione Reductase In Mice Enhances Proliferative Expansion Of Adult Heart Progenitors And Myocytes Post Myocardial Infarction

Konstantinos E Hatzistergos, Ellena C Paulino, Raul A Dulce, Lauro M Takeuchi, Shathiyah Kulandavelu, Wayne Balkan, Rosemeire M Kanashiro-Takeuchi, Joshua M Hare, Univ of Miami, Miami, FL

Objectives. The molecular pathways underlying the proliferative activity of adult cardiac myocytes and stem/progenitors in response to heart damage remain elusive. Since perturbing nitroso-redox balance in mice by genetic deletion of GSNOR ($GSNOR^{-/-}$) confers resilience to experimental myocardial infarction (MI), we investigated whether GSNOR knockout influences the proliferative activity of the post-MI heart.
Hypothesis: Knockout of GSNOR in mice enhances the proliferative expansion of CMs and $cKit^{+}$ cardiac stem cells (CSCs) in response to MI.
Methods: Wild-type (WT) and $GSNOR^{-/-}$ mice (n=5/ group) underwent experimental MI. To assess proliferative activity, animals received intraperitoneal injections of 5-bromodeoxyuridine (BrdU) at selected time-points during the first 2 weeks post-MI. Immunnohistochemical evaluation was performed 1 month post-MI. **Results:** Confocal immunofluorescence revealed that $GSNOR^{-/-}$ hearts exhibited higher rates of BrdU incorporation in CSCs after MI (10.9%±4.99% of WT CSCs compared to 15.9%±3% of $GSNOR^{-/-}$ CSCs, p=0.02). Similarly, there were ~3-fold more $BrdU^{+}$/Tropomyosin^{+} cardiomyocytes in the infarct zone of $GSNOR^{-/-}$ mice compared to WT (p<0.05). Immunohistochemical evaluation of cardiac troponin-T^{+} cardiomyocytes co-expressing the mitotic marker ser-10 phosphorylated histone H3 (H3P) showed further that cardiomyocyte mitosis was 2.4-fold greater in $GSNOR^{-/-}$ compared to WT mice (p<0.05), whereas the presence of aurora-b kinase in the cleavage furrow of $GSNOR^{-/-}$ cardiomyocytes substantiated their competence for mitosis after MI. The rate of cardiomyocyte apoptosis after MI, was not different between $GSNOR^{-/-}$ and WT mice, as shown by activated cleaved caspase-3 immunofluorescence. **Conclusions**: Collectively, our findings suggest that protein S-nitrosothiol turnover by GSNOR regulates proliferation of cardiomyocytes and CSCs in the adult heart in response to damage. These findings have therapeutic implications for the treatment of heart disease since they reveal novel pathways by which nitroso-redox balance influences cardiac repair.

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Saxagliptin and Tadalafil Differentially Alter Global and Cellular Cardiac Function in a Translational Miniature Swine Model of Left Ventricle Hypertrophy

Jessica A Hiemstra, Anne K Gibson, Jan R Ivey, Melissa S Cobb, Christopher P Baines, Maike Krenz, Timothy L Domeier, Craig A Emter, Univ of Missouri-Columbia, Columbia, MO

Left ventricular (LV) hypertrophy is a common characteristic of heart failure with preserved ejection fraction (HFpEF). Our lab recently characterized a mini-swine model of LV hypertrophy induced by aortic banding (AB) that displays clinical features associated with HFpEF including LV hypertrophy, diastolic dysfunction, and depressed contractile reserve. Disrupted cGMP signaling, a result of impaired production or enhanced catabolism, may play a role in development of HFpEF. We hypothesized preservation of cGMP signaling would attenuate pathological remodeling and improve cardiac function. The purpose of this study was to promote cGMP signaling via two mechanisms: 1) the DPP4 inhibitor saxagliptin; and 2) the PDE5 inhibitor tadalafil. We assessed whole heart and individual cardiomyocyte function 6 months post-AB in: control non-banded (CON; n=6), AB-control (AB; n=7), AB saxagliptin-treated (AB-SAX; n=7), and AB tadalafil-treated (AB-TAD; n=8) swine. Heart weight:body weight ratio increased to a similar extent in all AB groups. However, changes in cardiomyocyte morphology were variable. Cardiomyocyte length was increased only in the AB-TAD group, while cell width increased in both AB and AB-TAD animals. Cardiomyocyte length:width ratio decreased in the AB and AB-TAD groups, commensurate with decreased LV end diastolic (ED) and end systolic (ES) volumes. These changes were prevented in AB-SAX animals, as LV volumes and cell morphology were similar to CON. Pressure-volume analysis showed resting LV wall stiffness (ED pressure volume relationship [EDPVR] slope) was increased similarly in all AB groups. Increased resting LV contractility (ESPVR and preload recruitable stroke work) was observed in AB and AB-TAD animals. Interestingly, cardiomyocyte shortening was reduced in the AB-TAD group contrasting findings observed at the whole heart level. Saxaglitpin attenuated hypercontractile LV function at rest and preserved normal cardiomyocyte shortening. In conclusion, LV and cardiomyocyte function was distinctly altered in response to separate methods of pharmacological cGMP regulation. Our data suggest different pharmacological approaches to augment cGMP signaling promote distinct LV functional adaptations to developing HF.

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Promotion Of Nitroso-redox Balance By Beta 3 Adrenoceptor Agonism: Therapeutic Implications For Cardiovascular Complications Of Diabetes

Keyvan Karimi Galougahi, Chia-chi Liu, Alvaro Garcia, Natasha A Fry, Clare L Hawkins, Helge H Rasmussen, Gemma A Figtree, Kolling Inst for Medical Res, Sydney, Australia

Rationale: Disrupted balance between NO and O2.- is central in pathobiology of diabetes-induced cardiomyopathy and vascular dysfunction. We examined if stimulation of β3 adrenergic receptors (β3 ARs), coupled to endothelial nitric oxide synthase (eNOS) activation, would re-establish NO/O2.- balance, relieve oxidative inhibition of key caveolar proteins and protect against diabetes-induced cardiovascular dysfunction. Methods/Results: A hyperglycemic, hyperinsulinemic state was established in male White New Zealand rabbits by infusion of the insulin receptor antagonist S961 (12 μg/kg/h). Diabetes induced NADPH oxidase-dependent glutathionylation (GSS-) of the caveolar proteins Na+-K+ pump's β1 subunit and eNOS in cardiac myocytes and aorta, an oxidative modification that inhibits the pump and uncouples eNOS. Consistent with this, diabetes was associated with reduced electrogenic Na+-K+ pump current in voltage-clamped cardiac myocytes and impaired endothelium-dependent vasorelaxation. Selective β3 AR agonist CL316243 (CL, 40 μg/kg/h) restored NO levels analysed by spin-trapping of NO-Fe(DETC)2 complexes; decreased diabetes-induced elevation in O2.- measured by HPLC analysis of dihydroethidium oxidation products, improved endothelium-dependent vasorelaxation, and restored the Na+-K+ pump function in cardiac myocytes. These effects were mediated by CL abolishing diabetes-induced increase in eNOS-GSS and β1-GSS through a decrease in forward reaction

rate for glutathionylation by suppressing diabetes-induced NADPH oxidase activation, which was further amplified by promotion of de-glutathionylation via enhancement in association of glutaredoxine-1, the enzyme catalysing de-glutathionylation, with eNOS and Na+-K+ pump. Conclusion: β3 AR activation re-established nitroso-redox balance and relieved oxidative inhibition of key caveolar proteins in diabetes. β3 AR agonists are promising in treatment of diabetes-induced cardiovascular complications.

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Exercise Augments Cardiac Akt Activity And Reverses Aging-related Systolic Dysfunction.

Haruya Kawase, Yasuko K. Bando, Morihiko Aoyama, Akio Monji, Xieng Wu Cheng, Masafumi Kuzuya, Toyoaki Murohara, NAGOYA UNIV, Nagoya Aichi, Japan

[Purpose] Aging is one of the primary factors causing left-ventricular (LV) remodeling and susceptibility to heart failure (HF). Clinical evidences demonstrate a sustained exercise (EX) ameliorates HF; however, the molecular mechanism underlying the aging-related LV remodeling remains uncertain and few data have demonstrated whether EX may be beneficial for the aging-related LV remodeling and contractility. Because preclinical studies indicate the pivotal role of protein kinase Akt in aging, we thus hypothesized whether EX may exert benefits on HF induced by aging in which Akt may play an essential role. [Methods]
Male aged (40 w/o) and young (14 w/o) C57BL6 mice were subjected to the EX (45-min running (10~20m/s, 5-degree) on treadmill every second day for 15 weeks; agedC57EX and agedC57CON). To elucidate the role of Akt in aging heart, age- and gender-matched Akt knockout mice were also enrolled. [Results] Aging impairs both systolic and diastolic function without any changes in cardiac geometry. The systolic dysfunction of agedC57 reversed by EX with concomitant Akt activation; however, its diastolic dysfunction remained unaffected. EX enhanced cardiac Akt activity independently of aging. Aged AktKO exhibited systolic dysfunction to the more severe extent, which was reversed by EX. [Conclusions]
Our study demonstrates that #1 Akt is essential for adaptive cardiac contractility both to EX and aging. #2 Aging promotes diastolic dysfunction independently of Akt axis.

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Synergistic Salutary Effects of HMG-CoA Reductase Inhibitor and Angiotensin II Receptor Blocker on Load-induced Heart Failure

Yasuhiro Maejima, Mitsuaki Isobe, Tokyo Medical and Dental Univ, Tokyo, Japan

We have shown previously that combined HMG-CoA reductase inhibitor (statin) and angiotensin II receptor blocker (ARB) therapy significantly improves both symptoms and left ventricular (LV) function over time in patients with heart failure (HF) by a clinical study [*HF-COSTAR* Trial]. We elucidated the mechanisms of combination therapy with the ARB (losartan, LOS) and long-acting and statin (simvastatin, SIM) for the treatment of load-induced heart failure. Salt-loaded Dahl salt-sensitive (DS) rats were treated with vehicle, LOS (5mg/kg/day), SIM (2mg/kg/day) and LOS + SIM for 16 weeks. LOS and SIM in combination improved LV dysfunction (ΔLV fractional shortening; LOS = 60%, SIM = 42%, LOS + SIM = 24%, $p<0.05$), limited LV hypertrophy (ΔLV septal thickness; LOS = -21%, SIM = -18%, LOS + SIM = -13%, $p<0.05$) and reduced cardiac fibrosis (ΔLV collagen density; LOS = -26%, SIM = -16%, LOS + SIM = -28%, $p<0.05$) more than LOS or SIM alone. Both Rho and matrix metalloprotease-9 (MMP-9) activity in LV tissue were increased in untreated DS rats, and LOS and SIM in combination decreased these changes more than did LOS and SIM monotherapies. We confirmed that the plasma level of Exp-3174 (E3174), a LOS metabolite and a potent inverse

agonist of angiotensin II receptor type 1, was higher in rats treated with LOS and SIM in combination than in those treated with LOS alone (E3174/LOS ratio; LOS = 2.6 ± 0.3 vs. LOS + SIM = 3.2 ± 0.2, $p<0.05$). Next, to mimic the response of volume-overload heart failure *in vitro*, cultured neonatal rat cardiomyocytes (CMs) were cyclically stretched. Stretch-induced increased CM hypertrophy was suppressed by pretreatment with both SIM and E3174 more than by pretreatment with LOS, E3174, SIM, or LOS and SIM in combination. Mechanical stretch also induced activation of extracellular signal regulated kinase (ERK) and the stretch-induced ERK activation of CMs was also significantly suppressed by SIM + E3174. In conclusion, LOS and SIM had beneficial myocardial effects in rats with salt-sensitive hypertension, partly through promoting the accumulation of plasma E3174. SIM enhanced the myocardial protective effects of LOS through suppression of Rho and MMP-9 activity. Thus, a combination of ARB with statin has a promising potential as a therapeutic strategy for HF.

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Myozap Deficiency Promotes Adverse Remodeling And Cardiomyopathy In Response To Biomechanical Stress

Ashraf Y Rangrez, Derk Frank, Reza Poyanmehr, Alexander Bernt, Norbert Frey, Univ Medical Ctr Campus Kiel, Kiel, Germany

Background: Myozap is a new addition to the list of intercalated disc (ID) proteins, which we previously identified using a bioinformatics screen. In vitro characterization of Myozap revealed that it activates Rho-dependent SRF signaling. Moreover, cardiac restricted overexpression of Myozap in mice resulted in protein aggregate-associated cardiomyopathy, whereas, knockdown of its ortholog in Zebrafish led to severe contractile dysfunction and cardiomyopathy. The objective of the current study was to elucidate the cardiac consequences of targeted deletion of Myozap in mice.
Methods and Results: We generated a Myozap null mutant (MZP-ko) by global deletion of Myozap in mice. Unchallenged MZP-ko mice did not exhibit a baseline cardiac phenotype. However, upon biomechanical stress due to TAC (transverse aortic constriction), deficiency for Myozap led to accelerated cardiac hypertrophy (significant increases in the heart to body weight, left ventricular to body weight ratios) and fibrosis, accompanied by “super”-induction of the hypertrophic gene program (ANF/BNP). Moreover, MZP-ko mice revealed a severe reduction of fractional shortening and signs heart failure (increased lung/body weights) as well as a markedly increased mortality in response to TAC). Additional molecular data exhibited a significant decrease in the levels of native and phosphorylated Connexin 43 after transverse aortic constriction (TAC) in MZP-ko mice compared to wildtype animals. Finally, we observed a downregulation of dysbindin, a novel interaction partner of Myozap and known inducer of ERK1/2 signaling in TAC operated MZP-ko mice. Consistently, activation of ERK1/2 in response to TAC was blunted compared to wild-type littermates.
Conclusions: We here show that myozap deficiency in vivo leads to a maladaptative response to increased biomechanical stress associated with cardiomyopathy, heart failure and cardiac death. Mechanistically, this phenotype can at least in part be explained by the interruption of the interaction between myozap and dysbindin with subsequent loss of ERK1/2 activation. Taken into a larger perspective, our data imply an essential role of the ID and its associated proteins in cardiac remodeling.

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Mimetic peptide overcomes dysregulated L-Type Calcium Channel density and recovers myocardial function

Francesca Rusconi, Inst of Genetic and Biomedical Res (IRGB)-CNR, UOS of Milan, Milan, Italy; Michele Miragoli, Humanitas Clinical and Res Ctr, Rozzano, Milan, Italy; Elisa Di Pasquale, Inst of Genetic and Biomedical Res (IRGB)-CNR, UOS of Milan, Milan, Italy; Marcella Rocchetti, Dept of Biotechnologies and Biosciences, Univ of Milan-Bicocca, Milan, Italy; Paola Ceriotti, Humanitas Clinical and Res Ctr,

ABSTRACTS

Rozzano, Milan, Italy; Pierluigi Carullo, Inst of Genetic and Biomedical Res (IRGB)-CNR, UOS of Milan, Milan, Italy; Silvia Caprari, Natl Inst of Nuclear Physics, Rome Tre Section, Rome, Italy; Giacomo Viggiani, Humanitas Clinical and Res Ctr, Rozzano, Milan, Italy; Magali Cazade, Jean Chemin, Dept de Physiologie, Inst de Génomique Fonctionnelle, Ctr Natl de la Recherche Scientifique Unité Mixte de Recherche 5203, INSERM U661, Univs de Montpellier, Montpellier, France; Marie-Louise Bang, Inst of Genetic and Biomedical Res (IRGB)-CNR, UOS of Milan, Milan, Italy; Fabio Polticelli, Dept of Sciences, Univ of Roma Tre, Rome, Italy; Antonio Zaza, Dept of Biotechnologies and Biosciences, Univ of Milan-Bicocca, Milan, Italy; Gianluigi Condorelli, Humanitas Clinical and Res Ctr, Rozzano, Milan, Italy; Daniele Catalucci, Inst of Genetic and Biomedical Res (IRGB) -, UOS Milan, Milan, Italy

Voltage dependent L-Type calcium-channels (LTCCs) are located on the cardiomyocyte membrane and regulate cardiac contraction and rhythmicity. In human pathologies, such as heart failure (HF), decreased inward calcium current (I_{Ca}) is frequently observed. Here, we generated a mimetic peptide (MP) that targets LTCCs and restores impaired intracellular calcium homeostasis through a novel mechanism. Effective delivery of MP, fused with a cell penetrating peptide, was found to correct Ca2+ alterations in a mouse model of HF, in human cardiomyocytes derived from induced pluripotent stem-cells. These data provide a proof-of-concept supporting a therapeutic role for MP to treat human diseases related to LTCC abnormalities.
Category: heart failure biology

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Branched-Chain Amino Acid Catabolic Reprogramming in Heart Failure

Haipeng Sun, UCLA, Los Angeles, CA; Kristine Olson, Penn State Univ, Hershey, PA; Chen Gao, UCLA, Los Angeles, CA; Domenick A Prosdocimo, Case Western Reserve Univ, Cleveland, OH; Meiyi Zhou, Shanghai Jiao Tong Univ Sch of Med, Shanghai, China; Darwin Jeyaraj, Case Western Reserve Univ, Cleveland, OH; Ji-Youn Youn, Shuxun Ren, Hua Cai, Peipei Ping, UCLA, Los Angeles, CA; Paul Christian Schulze, Columbia Univ Medical Ctr, New York, NY; Christopher Lynch, Penn State Univ Coll of Med, Hershey, CA; Mukesh K Jain, Case Western Reserve Univ, Cleveland, OH; Yibin Wang, UCLA, Los Angeles, CA

Abnormal branched-chain amino acids (BCAA) catabolism has been strongly linked with cardiovascular and metabolic diseases in recent studies. In heart, metabolic reprogramming associated with the onset of heart failure has been established based on fetal-like changes in fatty acid and glucose metabolism, while little is known about the changes in amino acid homeostasis. In this report, we demonstrated a signature of suppressed BCAA catabolism in failing rodent and human hearts. The branched-chain α-keto acids (BCKA), intermediate of BCAA catabolism, accumulates, closely coordinated with reduced expression of key enzymes of BCAA catabolism in pathologically stressed myocardium. The down-regulation of these genes mimics a similar expression pattern observed in neonatal heart, indicating a fetal-like genetic reprogramming. Using both *in vitro* and *in vivo* models, we identified KLF15 as a key transcriptional regulator of the BCAA catabolic circuitry in heart. Genetic inactivation of BCAA catabolic pathway resulted in elevated cardiac BCKA levels and promoted cardiac dysfunction in response to mechanical overload, associated with increased oxidative stress and impaired mitochondrial respiration. Taken together, our data established for the first time that BCAA catabolic reprogramming is an integral component of metabolic remodeling that contributes to heart failure progression.

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Exposure To Air Pollution In Utero Causes Persistent Cardiovascular Dysfunction

Loren Wold, Matthew Gorr, Clayton Eichenseer, Dane Youtz, The Ohio State Univ, Columbus, OH; Markus Velten, Rheinische Friedrich-Wilhelms Univ, Bonn, Germany

Exposure to air pollutants during pregnancy can have devastating effects on offspring, inducing intrauterine growth retardation and maturation deficits. These developmental insufficiencies can affect the formation and function of the adult heart. We hypothesized that exposure to air pollution *in utero* would induce abnormal cardiac function at adulthood. FVB mice were exposed (6h/day, 7d/wk) to environmentally relevant concentrations of ambient particulate matter (PM2.5) or filtered air (FA) beginning when animals were paired for breeding. After birth, both groups remained in FA. Cardiac echocardiography was performed at 10 weeks of age. Birth weight was reduced in pups exposed to PM2.5 during intrauterine development compared to FA exposed pups, and litter size did not differ significantly between groups. Echocardiography revealed reduced left ventricular fractional shortening with greater left ventricular end systolic diameter in PM2.5 exposed mice at 10 weeks of age. These results were similar to data from mice exposed perinatally (until weaning). This study supports the hypothesis that exposure to air pollution *in utero* can lead to heart dysfunction at adulthood.

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Tripartite Motif E3 Ubiquitin Ligase Family Proteins Regulate Intracellular Signaling During the Progression of Heart Failure

Jenna Alloush, Eric X Beck, Sayak Bhattacharya, Zhaobin Xu, Liubov Guschina, Anjella Manoharan, Noah Weisleder, The Ohio State Univ Wexner Medical Ctr, Columbus, OH

Heart failure is a major complication of cardiovascular disease that frequently involves initial cardiac hypertrophy that provides transient compensation for decreased heart function. Eventually, decompensation leads to compromised cardiac structure and progression into heart failure. Investigation of the downstream effector pathways for these growth factors has identified molecules involved in the progression of cardiac hypertrophy and heart failure, including phosphoinositide 3-kinase (PI3K) and Akt (Protein Kinase B). MG53, a tripartite motif (TRIM) protein family member designated as TRIM72, is highly expressed in skeletal and cardiac muscle and is known to have cardioprotective effects through modulation of PI3K signaling mechanisms. It is essential for the activation of PI3K-mediated intracellular signaling in cardiomyocytes and TRIM72 overexpression is sufficient to induce PI3K signaling. As TRIM72 regulates PI3K signaling it may play a role in regulation of heart failure, which is supported by our findings that TRIM72 levels increase in the failing mouse heart. Our recent studies also show that TRIM72 can form heterodimers with other members of the TRIM family proteins that contains approximately 70 different members in the human genome. Many TRIM family proteins are known to act as E3 ubiquitin ligases that target the ubiquitin proteasome to particular proteins. Through this activity, TRIM72 homodimers and heterodimers can resolve specific substrates that can modulate aspects of the PI3K/Akt signaling cascade. Our recent studies have resolved multiple binding partners for TRIM72 in the TRIM family that are co-regulated during heart failure. Resolving the target substrates of the heterodimers formed by these various family members and determining their role in regulating PI3K/Akt signaling mechanisms during cardiac hypertrophy will be further defined as our studies continue.

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Identification of Novel Protein Kinase G I Alpha Antiremodeling Substrates in the Myocardium

Robert M Blanton Jr., Robert A Baumgartner, Robrecht Thoonen, Tufts Medical Ctr, Boston, MA; Shewit Giovanni, Tufts Univ Sch of Med, Boston, MA; Suresh Govindan, Dept of Cell and Molecular Physiology, Loyola Univ, Chicago, IL; Mark Aronovitz, Tufts Medical Ctr, Boston, MA; Sakthivel Sadayappan, Dept of Cell and Molecular Physiology, Loyola Univ, Chicago, IL; David A Kass, Johns Hopkins Medical Insts, Baltimore, MD; Richard H Karas, Tufts Medical Ctr, Boston, MA

Protein kinase G I α (PKGIα) inhibits cardiac remodeling, and this effect requires the PKGIα leucine zipper (LZ) binding domain. However, PKGIα LZ-dependent cardiac substrates remain poorly understood. Clinical trials of PKGI activating drugs have been limited to date by hypotension arising from vascular PKGI activation. Therefore, we explored downstream PKGIα substrates in the heart which may inhibit remodeling, yet circumvent the hypotensive effects of systemic PKGI activation. A screen for PKGIα LZ-interacting proteins identified: 1)cardiac myosin binding protein-C (cMyBP-C) and 2) mixed lineage kinase 3 (MLK3). cMyBP-C is a cardiac myocyte protein known to inhibit remodeling when phosphorylated. Co-precipitations with cGMP-conjugated beads confirmed the PKGIα-cMyBP-C interaction. Purified PKGIα phosphorylated cMyBP-C in vitro at Ser-273, Ser-282, and Ser-302. cGMP induced cMyBP-C phosphorylation at these sites in COS cells transfected with WT PKGIα, but not in cells transfected with either LZ mutant PKGIα or kinase-inactive PKGIα. In hearts of 9 month old PKGIα Leucine Zipper mutant mice, which have LV hypertrophy (LVH) and diastolic dysfunction, we observed decreased phosphorylated cMyBP-C as well as decreased total cMyBP-C, compared with WT littermate hearts.

We next tested the effect of MLK3, which interacts with PKGIα in the heart, on remodeling in vivo. We performed 7 day Transaortic Constriction (TAC) on MLK3 KO mice and WT littermates (n=5 shams, 8 TAC per genotype). MLK3 KO TAC mice had increased LVH (LV mass/tibia length 71.1 ± 2.7 g/cm KO TAC vs 62.1 ± 2.7 WT TAC; $p<0.05$). Further, MLK3 KO mice developed overt CHF compared with WT littermates (LV end diastolic pressure 14.8 ± 1.9 mmHg KO TAC vs 7.7 ± 2.1 WT TAC, $p <0.05$), as well as accelerated decrements in LV preload recruitable stroke work (36.6 ± 11.9 mmHg/ul KO TAC vs 94.6 ± 12.9 WT TAC, $p<0.05$) and min dP/dt (-6292 ± 519 mmHg/s KO TAC vs -8157 ± 554 WT TAC , $p <0.05$). We observed no differences in LV structure or function between sham genotypes.

These studies reveal 2 novel PKGIα anti-remodeling substrates, and they support that exploring PKGIα substrates in the heart may identify novel therapeutic targets to inhibit cardiac remodeling but avoid excessive PKGI induced vasodilation.

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αB-crystallin Interacts And Prevents Stress-activated Proteolysis Of Focal Adhesion Kinase In Cardiomyocytes

Aline M dos Santos, Michelle Bueno Pereira, Danieli C Gonçalves, Alisson C. Cardoso, Silvio R. Consonni, Fabio C. Gozzo, Paulo S. Oliveira, Alana R. Figueiredo, Brazilian Natl Lab for Biosciences, Ctr for Res in Energy and Materials, Campinas, Brazil; André A. de Thomaz, Carlos L. Cesar, Gleb Wataghin Physics Inst, Univ of Campinas, Campinas, SP, 13083-859, Brazil, Campinas, Brazil; Carlos H. Ramos, Chemistry Inst, Univ of Campinas, Campinas, SP, 13083-970, Brazil, Campinas, Brazil; Kleber G. Franchini, Brazilian Natl Lab for Biosciences, Ctr for Res in Energy and Materials, Campinas, Brazil

Focal adhesion kinase (FAK) contributes to cellular homeostasis under stress conditions. Here, we show that αB-crystallin confers protection to FAK against calpain-mediated proteolysis under mechanical stress in cardiomyocytes. Biochemical assays, chemical cross-linking coupled to mass spectrometry experiments, mutational analyses and Förster resonance energy transfer (FRET) were combined to investigate the basis of FAK and αB-crystallin interaction. A hydrophobic patch mapped between helices 1 and 4 of the FAK FAT domain was found to bind to the β4-β8 groove of αB-crystallin. Such an interaction requires FAK tyrosine 925 and is enhanced

following its phosphorylation by Src, which occurs upon FAK stimulation by mechanical stress. αB-crystallin silencing results in calpain-dependent FAK depletion and in increased apoptosis of cardiomyocytes. The overexpression of a myc-FAK construct or treatment with a calpain inhibitor (E64) restored the survival of cardiomyocytes depleted of αB-crystallin. The association between FAK and αB-crystallin was also demonstrated to occur in response to pressure overload in rat left ventricle. The myocardial depletion of αB-crystallin by siRNA results in enhanced apoptosis of cardiomyocytes and myocardial fibrosis in overloaded hearts. These alterations were markedly attenuated in the overloaded left ventricles of transgenic mice with cardiac specific FAK expression. These findings define a novel mechanism by which αB-crystallin controls FAK function, with impact on cardiomyocyte survival and cardiac remodelling in response to stress.

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Regulation Of Acetylation Of Histone Deacetylase 2 By P300/CBP-associated Factor/histone Deacetylase 5 In The Development Of Cardiac Hypertrophy

Hyun Kook, Gwang Hyeon Eom, Yoon Seok Nam, Nakwon Choe, Hyun- Ki Min, Chonnam Natl Univ Medical Sch, Gwangju, Korea, Republic of

Rationale: Histone deacetylases (HDACs) are closely involved in cardiac reprogramming. Although the functional roles of the class I and class IIa HDACs are well established, the significance of interclass crosstalk in the development of cardiac hypertrophy remains unclear.
Objective: Recently, we suggested that casein kinase-2α1-dependent phosphorylation of HDAC2 leads to enzymatic activation, which in turn induces cardiac hypertrophy. Here we report an alternate posttranslational activation mechanism of HDAC2 that involves acetylation of HDAC2 mediated by p300/CBP-associated factor (pCAF)/HDAC5.
Methods and Results: Hdac2 was acetylated in response to hypertrophic stresses in both cardiomyocytes and a mouse model. The acetylation was reduced by a histone acetyltransferase inhibitor but was increased by a nonspecific HDAC inhibitor. The enzymatic activity of Hdac2 was positively correlated with its acetylation status. pCAF bound to Hdac2 and induced acetylation. The HDAC2 K75 residue was responsible for hypertrophic stress-induced acetylation. The acetylationresistant Hdac2 K75R showed a significant decrease in phosphorylation on S394, which led to the loss of intrinsic activity. Hdac5, one of class IIa HDACs, directly deacetylated Hdac2. Acetylation of Hdac2 was increased in Hdac5 null mice. When an acetylation-mimicking mutant of Hdac2 was infected into cardiomyocytes, the anti-hypertrophic effect of either nuclear tethering of Hdac5 with leptomycin B or Hdac5 overexpression was reduced.
Conclusions: Taken together, our results suggest a novel mechanism by which the balance of HDAC2 acetylation is regulated by pCAF and HDAC5 in the development of cardiac hypertrophy.

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βAR Resensitization At The Plasma Membrane Provides Beneficial Cardiac Remodeling

Elizabeth E Martelli, Neelakantan T Vasudevan, John George, Maradumane L Mohan, Manveen K Gupta, Sathyamangla V Naga Prasad, Cleveland Clinic Founation, Cleveland, OH

βAR downregulation and desensitization are hallmarks of heart failure. Agonist occupied βARs undergo desensitization through phosphorylation by G-protein coupled receptor kinases leading to βAR internalization. Phosphorylated βAR becomes resensitized following dephosphorylation by PP2A in the endosomes. In contrast to this paradigm our recent studies have shown that resensitization of βARs can occur at the plasma membrane

through the inhibition of PP2A activity by I2PP2A. We generated a stable HEK cell line expressing short hairpin RNA targeting I2PP2A (shRNA-I2PP2A). Confocal microscopy studies show cells expressing shRNA-I2PP2A resulted in significant loss of receptor phosphorylation despite the presence of the agonist. Radioligand binding and confocal imaging also showed marked inhibition of receptor internalization upon depletion of I2PP2A with significant PP2A activation. We also observed preservation of βAR function despite the presence of agonist measured by cAMP generation and adenylyl cyclase activity. To further dissect the interaction of I2PP2A-PP2A we generated mutants of PP2A from amino acids 263-309. Over expression of these PP2A mutant peptides significantly reversed receptor phosphorylation upon isoproterenol (ISO) stimulation compared to full length PP2A. Also, expression of PP2A mutant showed marked increase in adenylyl cyclase activity in response ISO stimulation suggesting that this mutant I2PP2A competes out inhibitory I2PP2A interaction with PP2A. To test whether alteration in βAR resensitization contributes to cardiac dysfunction with stress we generated transgenic (Tg) mice with cardiac specific over expression of wt I2PP2A and mutant I2PP2A (phopspho- and dephospho-). ISO treatment of wt I2PP2A Tg and littermate controls showed that wt I2PP2A Tg mice had significant βAR dysfunction and cardiac hypertrophy. Assessment of age dependent cardiac function of these mice showed that wt I2PP2A Tg and phospho-I2PP2A Tg mice have cardiac hypertrophic response followed by dilated cardiomyopathy; expression of dephospho-I2PP2A mutant reversed this phenotype. These studies suggest targeting I2PP2A alters receptor function and may have implications in cardiac remodeling and hypertrophy with cardiac stress.

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Disruption of P90 Ribosomal S6 Kinase Binding 3 to Muscle A-kinase Anchoring Protein In Vivo Via Adeno-associated Virus Expression of a Competing Peptide Attenuates Pressure Overload-induced Cardiac Hypertrophy

CATHERINE L PASSARIELLO, Michael D. Kritzer, Hrishikesh Thakur, Michael Sanders, Jinliang Li, Michael S. Kapiloff, UNIVERSITY OF MIAMI, MIAMI, FL

RATIONALE:
Cardiac myocyte hypertrophy is the main compensatory response to chronic stress in the heart. p90 ribosomal S6 kinase (RSK) family members are effectors for extracellular signal-regulated kinases that induce myocyte growth. RSK3 contains a unique N-terminal domain that mediates RSK3 binding to the muscle A-kinase anchoring protein (mAKAPβ) scaffold. We have previously published that disruption of RSK3-mAKAPβ complexes using a competing peptide inhibited the phenylephrine-induced hypertrophy of neonatal ventricular myocytes in vitro. In vivo, RSK3 gene deletion in mice attenuated the concentric cardiac hypertrophy induced by pressure overload. We hypothesize that RSK3 anchoring to mAKAPβ in myocytes is required for cardiac hypertrophy in vivo.
METHODS AND RESULTS:
Adeno-associated viruses (AAV) are gene therapy vectors in development for the treatment of human diseases owing to their nonpathogenic capability for transducing non-dividing cells and their long-term transgene expression. We have used a recombinant AAV2/9 vector to express a mAKAPβ RSK3-binding domain (RBD)-GFP fusion protein under the control of the cardiac myocyte-specific cardiac troponin T promoter. 3 day-old C57BL/6 mice were injected intraperitoneally with either AAV-RBD-GFP or AAV-GFP control virus. At 8 weeks of age mice were subjected to transverse aortic constriction to induce pressure overload (TAC) for two weeks. Cardiac hypertrophy was attenuated in mice injected with the AAV-RBD-GFP virus (biventricular weight indexed to tibial length (mg/mm): 7.7, 8.6, and 9.2 for AAV-RBD, AAV-GFP and non-injected TAC cohorts, respectively; $p<0.05$ vs. both controls). Echocardiography both corroborated the inhibition of hypertrophy and revealed no deleterious effect on cardiac function attributable to the AAV-RBD-GFP vector.
CONCLUSIONS:
Anchored RSK3 regulates pathologic myocyte growth. AAV can successfully deliver a competing peptide inhibiting pathological hypertrophy and should be investigated further as a prevention and/or treatment for heart failure.

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Ras GTPase-activating Protein SH3 Domain Binding Protein 1 (G3BP1) Regulates The Induction Of Stress Granules During Cardiac Hypertrophy

Danish Sayed, Rutgers New Jersey Medical Sch, Newark, NJ

Stress granules (SGs) are dynamic, microscopically visible, cytoplasmic bodies that play a major role in mRNA metabolism (e.g. sorting, storage, decay) and induced in cells during stress conditions like starvation, oxidative strain or growth. With substantial role in cancer and neurodegenerative diseases, these granules have never been studied during cardiac hypertrophy, or in the heart in general. Several studies have identified independent proteins, mostly mRNA binding proteins that are part of these granules, some of which are sufficient to nucleate the assembly in quiescent cells even without stress. One such mRNA binding protein is Ras GTPase-activating protein SH3 domain binding protein 1 (G3BP1), which increases during cardiac hypertrophy via posttranscriptional regulation. Thus, we hypothesized that G3BP1 might be involved in the induction of SGs during hypertrophy and hence in regulating mRNA processing and gene expression. Our aim was to investigate, 1) if these SGs appear in hypertrophied hearts and 2) if G3BP1 is necessary and sufficient to induce them during hypertrophic stimuli. In vivo staining of TIA-1/TIAR (SG marker) in mouse hearts subjected to sham or transaortic coarctation (TAC) surgeries showed accumulation of these granules with cardiac hypertrophy. Similar induction was seen in isolated, cultured, rat neonatal cardiac myocytes with hypertrophic stimulation (Endothelin1) or overexpression of G3BP1 alone (>60% of myocytes stained for SG). Conversely, switch to growth-inhibited conditions or knockdown of G3BP1 in hypertrophying myocytes was sufficient to prevent the assembly of these structures. Co-staining with other components of these granules like TIA-1/TIAR or proteins specific to P bodies, like decapping enzyme 1 validated these structures as SGs in cardiac myocytes. Interestingly, a long non-coding RNA, Gas5 (Growth Arrest Specific 5) that is validated binding partner of G3BP1 sequestered to perinuclear focal locations in myocytes stimulated with ET1, suggesting growth-induced recruitment to SGs. While we are still in process of examining G3BP1 targets that are recruited to SGs and their role in hypertrophy development, we have concluded that G3BP1 is required for the induction of SGs during cardiac hypertrophy

D. Sayed: None.

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Scleraxis is a Required Component of the Cardiac Extracellular Matrix Gene Expression Program

Michael P Czubryt, Rushita A Bagchi, Patricia L Roche, Ryan Wang, Shreyas Devalapurkar, UNIVERSITY OF MANITOBA, Winnipeg, MB, Canada; Ronen Schweitzer, Oregon Health and Science Univ, Portland, OR

The collagenous cardiac extracellular matrix (ECM) reinforces the myocardium and facilitates intercellular communication, but excessive ECM production in fibrosis results in dysfunction and heart failure. The transcription factor scleraxis directly governs expression of type I fibrillar collagen in ECM-rich tissues including tendons and the heart. We have also shown that scleraxis is required for collagen 1α2 gene expression induced by the potent pro-fibrotic TGFβ-Smad signaling pathway. We therefore examined the broader role of scleraxis in myocardial ECM production, including mechanisms regulating the expression of scleraxis itself. Scleraxis knockout in mice resulted in a dramatic ~50% loss of cardiac ECM components including fibrillar collagens, proteoglycans and matrix metalloproteinases, concomitant with a significant decrease in fibroblast number. Scleraxis knockdown in primary cardiac proto-myofibroblasts recapitulated these changes without increasing cell death, suggesting a reduction in fibroblast precursors in knockout mice in vivo. Conversely, over-expression of scleraxis had the opposite effect, up-regulating expression of ECM genes and numerous markers indicative of increased commitment to a myofibroblast cell fate. Scleraxis increased proto-myofibroblast contractility via direct transactivation of the α-

smooth muscle actin promoter. Similar to TGFβ, pro-fibrotic angiotensin II and Connective Tissue Growth Factor induced scleraxis expression, suggesting that scleraxis behaves as a common transcriptional effector for multiple fibrotic pathways. Fibrillar collagen gene expression induced by these factors was significantly attenuated by scleraxis knockdown, further implicating scleraxis in fibrotic ECM synthesis. Intriguingly, the histone deacetylase inhibitor trichostatin A, which has been reported to exert anti-fibrotic effects in the heart, significantly reduced scleraxis expression in cardiac myofibroblasts. These data collectively identify scleraxis as a central and requisite transcriptional regulator of fibroblast phenotype and the ECM gene expression program in the heart, and provide rationale for the investigation of anti-scleraxis strategies to attenuate fibrosis in patients.

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18-HEPE, An EPA Metabolite Released By Macrophages, Prevents Against Pressure Overload-induced Cardiac Remodeling

Jin Endo, Motoaki Sano, Keiichi Fukuda, Keio Univ Sch of Med, Tokyo, Japan; Makoto Arita, Graduate Sch of Pharmaceutical Sciences, the Univ of Tokyo, Tokyo, Japan

N-3 polyunsaturated fatty acids (PUFAs) have potential cardiovascular benefit, although the mechanisms underlying this effect remain poorly understood. The present study investigated the impact of the enrichment of n-3 PUFAs in the setting of pressure overload induced cardiac hypertrophy using fat-1 mouse, a genetic model with elevated n-3 PUFA levels in tissues, as well as the possible cellular mechanism involved in such a role.
The fat-1 mice subjected to pressure overload by transverse aortic constriction showed less infiltration of macrophages and decreased interstitial fibrosis, leading to the preserved cardiac function as compared to wild type (WT) mice. Bone marrow (BM) transplantation experiments revealed that fat-1 transgenic BM cells, but not fat-1 transgenic cardiac cells, contributed the anti-remodeling effect, which was comparable between fat-1 and WT mice transplanted with fat-1 transgenic BM, but was absent in fat-1 mice transplanted with WT BM. In the co-culture assay with cardiac fibroblasts, the fat-1 transgenic macrophages showed anti-inflammatory and anti-fibrotic activities. Lipidomic analysis revealed selective enrichment of eicosapentaenoic acid (EPA) in fat-1 transgenic bone marrow (BM) cells and an EPA-metabolite 18-hydroxyeicosapentaenoic acid (18-HEPE) in fat-1 transgenic macrophages. 18-HEPE-rich milieu in the fat-1 transgenic heart was generated by BM-derived cells, most likely macrophages. 18-HEPE inhibited macrophage-mediated proinflammatory activation of cardiac fibroblasts in culture, and in vivo administration of 18-HEPE significantly attenuated the inflammation and fibrosis of hypertrophied hearts, resulting in improvement of cardiac function.
We found that the elevated n-3 PUFA level in BM-derived macrophages is responsible for cardiac protection, and 18-HEPE exhibited cardioprotective potential when administered in vivo.

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Relaxin Receptor-Ligand Expression in a Fibrotic Environment

Jamie L Haney, Univ of Pittsburgh, Pittsburgh, PA; David S. Schwartzman, Univ of Pittsburgh Medical Ctr, Pittsburgh, PA; Sanjeev G. Shroff, Univ of Pittsburgh, Pittsburgh, PA

The renin-overproducing transgenic rat (mREN2) has recently been proposed as a model of left ventricular (LV) hypertension and fibrosis. Comparing mREN2 (n=7) to sex- and age-matched Wistar-Han control rats (WHAN, n=11), we have confirmed hypertension (mean blood pressure: 174±9 vs. 107±3 mmHg, P<0.001), hypertrophy (LV weight:body weight: 4.0±0.1 vs. 2.7±0.1 mg/g, P<0.001), and cardiac fibrosis. At the protein level, LV from mREN2 exhibited significantly higher percentage of interstitial collagen per total protein area versus WHAN (5.95±1.01% vs. 1.96±0.06%, P=0.02). At the level of LV function, mREN2 hearts demonstrated significant diastolic dysfunction (increased stiffness coefficient from stress-strain

relationship: 198±30 vs. 78±10, P=0.01, and reduced magnitude of dP/dtmin: 1254±125 vs. 2000±110 mmHg/s, P<0.001), and impaired relaxation (increased relaxation time constant: 0.048±0.001 vs. 0.043±0.001 s, P=0.002). The naturally occurring hormone relaxin is known to have antifibrotic properties in multiple organ systems and it affects both synthesis and degradation aspects of collagen homeostasis. It is known that relaxin (RLN1) and receptor (RXFP1) are expressed locally in LV tissue, yet the biological function of the endogenous relaxin message-receptor system is not fully understood. Interestingly, the relative mRNA expressions of relaxin and relaxin receptor were increased in this model of LV fibrosis (2-ΔΔCT by qRT-PCR for RLN1: 3.04±0.56 (mREN2) vs. 1.0±0.18 (WHAN), P=0.001; and RFXP1: 7.06±1.57 (mREN2) vs. 1.00±0.20 (WHAN), P=0.003). Thus, the unregulated endogenous relaxin-receptor system in the mREN2 fibrosis model seems to be an adaptive response which is trying to keep the fibrosis in check. However, this endogenous adaptive response is insufficient to completely block collagen accumulation and prevent LV diastolic dysfunction. We are currently conducting experiments to examine whether exogenous administration of relaxin can assist the endogenous adaptive response and reverse LV fibrosis and normalize diastolic function in this model.

J.L. Haney: None. **D.S. Schwartzman:** None. **S.G. Shroff:** None.

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RhoA Controls Myofibroblast Characteristics

Aline Jatho, Naim Kittana, Kerstin Schenk, Beate Ramba, Wolfram-Hubertus Zimmermann, Susanne Lutz, Univ Med of Göttingen, Göttingen, Germany

Purpose: RhoA has been shown to be beneficial in cardiac disease models when overexpressed in cardiomyocytes whereas its role in cardiac fibroblasts (CF) is still poorly understood. During cardiac remodeling CF undergo a transition towards a myofibroblastic phenotype showing an increased proliferation and migration rate. Both processes involve the remodeling of the cytoskeleton, a process known to be strongly regulated by RhoA.
Methods: To investigate the role of RhoA in neonatal rat cardiac fibroblasts we used a lentivirus-based knockdown (RhoA-KD-CF). In addition, wild type (wt) CF were treated with 10 μM Fasudil for ROCK inhibition or 5 μg/mL Tubastatin A (Tub A) for tubulin-specific deacetylase HDAC6 inhibition. Cytoskeletal proteins were analyzed by immunoblot and immunofluorescence. Adhesion velocity and migration was determined by microscopy and the serum-driven proliferation rate by nuclei counting.
Results: Compared to control cells (shControl) RhoA-KD-CF develop an epithelial-like morphology lacking stress fibers and higher order actin structures like geodesic domes. The orientation of focal adhesions sites along the cell stress axis was also impaired. This phenotype could be mimicked by the treatment of CF with Fasudil. Furthermore, in RhoA-KD-CF cytoskeletal proteins were found to be unchanged except for a decrease in the myofibroblast marker smooth muscle actin by 43% and an increase in acetylated tubulin by 57% without a change in the expression of HDAC6. In order to analyze the impact of both changes we investigated the migration and proliferation rate of CF. First, the reduction of RhoA accelerated the adhesion but decelerated migration (shControl 4043±316 nm/h versus shRhoA 3021±153 nm/h) and second, the serum-driven proliferation rate of RhoA-KD-CF was reduced by 50%. Interestingly, treatment of wt-CF with Fasudil significantly decreased migration velocity by 62% but had no effect on the proliferation rate, whereas Tub A only slightly decreased migration velocity but reduced proliferation rate by 60%.
Conclusion: RhoA influences the two main myofibroblast characteristics, migration and proliferation, by interfering with the actin and tubulin cytoskeleton via ROCK and HDAC6, respectively.

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DDAH1 Knockout Exacerbates Hypoxia-induced Pulmonary Arterial Hypertension In Mice

Dongmin Kwak, Univ of Minnesota, Minneapolis, MN; Dachun Xu, Sanghai Tenth People's Hosp of Tongi Univ, Sanghai, China; Dorothee Atzler, Univ Medical Ctr Hamburg-Eppendorf, Hamburg, Germany; Xinli Hu, Xin Xu, Huan Wang, John Fassett, Hailing Li, Shuo Yan, Univ of Minnesota, Minneapolis, MN; Haipeng Guo, Qilu Hosp of Shandong Univ, Jinan, China; Yingjie Chen, Univ of Minnesota, Minneapolis, MN

Pulmonary arterial hypertension (PAH) is a progressive disease with a very poor prognosis. Recent studies have demonstrated that PAH is associated with diminished nitric oxide bioavailability, increased levels of endogenous nitric oxide synthase (NOS) inhibitor ADMA, and decreased lung dimethylarginine dimethylaminohydrolase (DDAH) activity. We have demonstrated that DDAH1 is essential for degradation of endogenous NOS inhibitor ADMA, and is important for optimal vascular endothelial NO production. However, it is not clear whether decreased DDAH activity and ADMA accumulation exacerbates development or progression of PAH and right ventricular hypertrophy. In addition, the impact of cardiomyocyte specific DDAH1 dysfunction on right ventricular hypertrophy is unknown. Using global DDAH1 gene deficient mice, we demonstrate that chronic ADMA accumulation by global DDAH1 gene deletion did not cause spontaneous PAH under control conditions, but significantly exacerbated chronic hypoxia-induced PAH, as indicated by significantly increased right ventricular (RV) pressure, more RV hypertrophy, and enhanced pulmonary vascular remodeling in DDAH1 deficient mice as compared to wild type mice. Chronic hypoxia resulted in reduced lung DDAH activity and increased circulating ADMA content in wild type mice. Cardiomyocyte specific DDAH1 gene deletion did not exacerbate hypoxia-induced increases in RV pressure or lung vascular remodeling, but significantly exacerbated hypoxia-induced RV hypertrophy in comparison to wild type littermates, indicating DDAH1 distributed in cardiomyocytes protect the heart against RV hypertrophy independent to pressure overload. Collectively, our data indicate that lung DDAH1 plays a critical role in protection against hypoxia-induced PAH and right ventricular hypertrophy.

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Determining the Potential Role of cTnT Isoform Switching In the Development of Early Childhood Tropomyosin-Linked DCM

Melissa Lynn, Univ of Arizona, Tucson, AZ; Lauren Tal-Grinspan, Albert Einstein Coll of Med, Bronx, NY; J.P. Jin, Wayne State Univ, Detroit, MI; Jil Tardiff, Univ of Arizona, Tucson, AZ

An oft-noted component of sarcomeric HCM and DCM is the observation that patients within families, carrying the same primary mutation, often exhibit significant phenotypic variability. This lack of a distinct link between genotype and phenotype has greatly complicated clinical management. In a recent study of two large unrelated multigenerational families carrying 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 congestive heart failure, while adults developed a mild to moderate DCM in mid-life. Of note, children who survived the initial presentation often recovered significant systolic function in adolescence and young adulthood. Therefore, to better understand the mechanism of this “bimodal” phenotype, we began to investigate the potential modulating role of isoform switching by other sarcomeric components. We hypothesize 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). Initial biophysical studies (circular dichroism and regulated *in vitro* motility, R-IVM) show that while D230N does not alter Tm’s thermal stability it does have a profound impact on myofilament activation. Both maximal velocity of filament sliding and calcium sensitivity were decreased. Furthermore, an additive decrease was observed in these parameters for R-IVM solutions containing $cTnT_1$(fetal)+D230N Tm filaments as compared to $cTnT_3$(adult)+D230N. Preliminary *in vivo* studies utilizing our novel double transgenic Tm-D230N x $cTnT_1$ mice show profound changes in wall thickness and

chamber dilation, as compared to age-matched non-transgenic mice and D230N Tm mice. Further studies aim to model the "bimodal" clinical phenotype seen in families with D230N Tm and assess the potential for disease reversibility using a cardiac specific inducible $cTnT_1$ transgenic mouse model. Our goal is to use a translational approach to better understand the mechanism by which primary mutations lead to distinct clinical phenotypes in order to improve clinical management.

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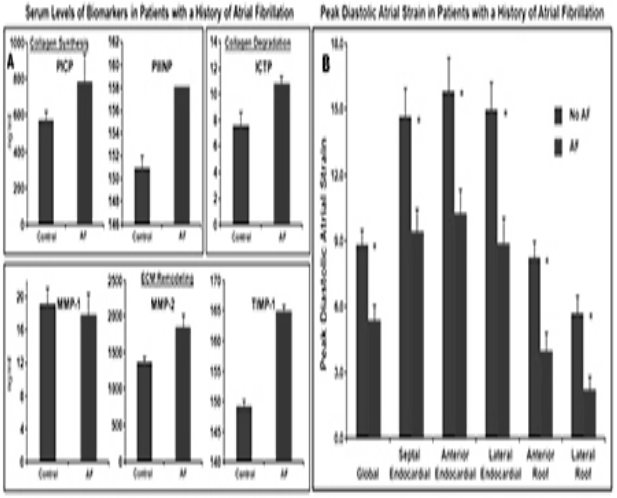
Noninvasive Approach Assessing Atrial Mechanics and Serum Biomarkers of Collagen Turnover Provides a Surrogate for Fibrosis and Atrial Fibrillation

Mahek Mirza, Farhan Rizvi, Melissa Albrecht, Anton Strunets, Larisa Emelyanova, Ekhson Holmuhamedov, Ctr for Integrative Res in Cardiovascular Aging, Aurora Univ of Wisconsin Medical Group, Milwaukee, WI; Paul H. Werner, David C. Kress, Aurora Medical Group-Cardiovascular and Thoracic Surgery, Aurora St. Luke's Medical Ctr, Milwaukee, WI; Bijoy K. Khandheria, Aurora Cardiovascular Services, Aurora Sinai/Aurora St. Luke's Medical Ctrs, Univ of Wisconsin Sch of Med and Public Health, Milwaukee, WI; Jasbir Sra, Aurora Cardiovascular Services, Aurora Sinai/Aurora St. Luke's Medical Ctrs, Univ of Wisconsin Sch of Med and Public Health, Milwaukee, WI, United States, Milwaukee, WI; Arshad Jahangir, Ctr for Integrative Res in Cardiovascular Aging, Aurora Univ of Wisconsin Medical Group, Milwaukee, WI

Introduction: Atrial fibrosis alters myocardial electrophysiological properties, increasing susceptibility to postoperative atrial fibrillation (PoAF); however, its estimation is problematic. We hypothesized that a noninvasive approach using history of AF (HxAF), LA mechanics and serum biomarkers of collagen turnover provides a surrogate for the extent of interstitial atrial fibrosis to identify patients at risk for PoAF. Methods: In patients undergoing cardiac surgery from April-Dec 2013, concentrations of biomarkers reflecting collagen synthesis/degradation and extracellular matrix remodeling were determined in serum from preoperative blood using an enzyme-linked immunosorbent assay, and echocardiographic evaluation was performed using M-mode, 2D, Doppler and 3D speckle tracking. Results: Of 66 patients (68 ±11 y, 67% men), 15 had HxAF and 11 of 51 with no HxAF (22%) developed new onset PoAF. In patients with HxAF, biomarkers for collagen turnover were elevated (Fig A) and correlated with a reduction in LA ejection fraction and global and regional relaxation of the LA wall (p=0.01, Fig B). In patients with no HxAF, procollagen type III (PIIINP) was significantly different in those who developed PoAF (p=0.01) and correlated with reduction in contractility in the posterior LA roof (p=<0.001) with a prolonged time to peak end-diastolic volume (p=0.03). LA size or ventricular structure and function were not different between groups. Conclusion: Surrogate serum and imaging biomarkers correlate with the substrate abnormality that promotes AF. These results need to be validated in larger cohorts to assess the power of these parameters in predicting new onset PoAF.



Propeptides of procollagen types I (PICP) and III (PIIINP), carboxy-terminal telopeptide (ICTP), matrix metalloproteinases (MMP1 and 2) and tissue inhibitor of MMPs (TIMP1), extracellular matrix (ECM).

M. Mirza: None. **F. Rizvi:** None. **M. Albrecht:** None. **A. Strunets:** None. **L. Emelyanova:** None. **E. Holmuhamedov:** None. **P. Werner:** None. **D. Kress:** None. **B. Khandheria:** None. **J. Sra:** None. **A. Jahangir:** None.

ABSTRACTS

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Specific MicroRNAs Regulate Cardiac Fibroblast-to-Myofibroblast Transition And Fibrosis.

Varun Nagpal, Rahul Rai, Aaron Place, Sheila Murphy, Douglas E Vaughan, Northwestern Univ, Chicago, IL

Cardiac fibrosis is the pathological consequence of fibroblast-to-myofibroblast transition (FMT) within the human myocardium, resulting in heart dysfunction. Transforming growth factor-β (TGF-β) plays a pivotal role in the induction of both FMT and cardiac fibrosis. However, the molecular basis of TGF-β-induced FMT in cardiac fibrosis is not clear. In this study, we propose a novel miRNA-mediated approach to attenuate cardiac fibrosis by blocking TGF-β-induced FMT. We observed that the canonical TGF-β/SMAD pathway, and not the MEK pathway, plays a pivotal role in the induction of FMT in primary cultures of human cardiac fibroblasts. Importantly, we have demonstrated that the specific miRNA is significantly upregulated during cardiac FMT. In addition, we observed significant upregulation of the same miRNA in fibrotic human myocardium and two murine models of cardiac fibrosis (transverse aortic constriction and Angiotensin II). Furthermore, overexpression of the miRNA using mimics augmented TGF-β-induced FMT. Downregulation of the miRNA using an antagomiR approach attenuated TGF-β-induced FMT. Notably, in silico analysis and qRT-PCR analysis revealed that this miRNA directly targets apelin, an anti-fibrotic mediator. Next, efficient delivery of cy3-tagged antagomiRs in the heart, liver and spleen was confirmed by confocal microscopy. In vivo silencing of miRNAs in the heart was achieved by systemic delivery of locked nucleic acid (LNA), both in the presence and absence of Angiotensin II. We conclude that TGF-β-induced specific miRNA is both sufficient and necessary for the induction of cardiac FMT and is a novel repressor of apelin. Our data suggests that the inhibition of miRNAs necessary for FMT may serve as a novel therapeutic strategy to prevent human cardiac fibrosis.

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Novel Interaction of Spinophilin with Alpha1a-Adrenergic Receptor and its Genetic Variant in Cardiovascular Cells

Ekaterina Babaeva, Irina Gradinaru, Univ of Washington, Seattle, WA; Debra A Schwinn, Univ of Iowa, Seatlle, WA; **Anush Oganesian**, Univ of Washington, Seattle, WA

Activation of α_1-Adrenergic Receptors (α_1ARs), members of the G protein-coupled receptor (GPCR) superfamily, in response to stimulation of the sympathetic nervous system by catecholamines plays a major role in regulating cardiovascular (CV) function. Among three α_1AR subtypes (α_{1a},α_{1b},α_{1d}), α_{1a}ARs predominate in human resistant vessels and in heart. Recently, we discovered that naturally occurring human α_{1a}AR-G247R (247R) genetic variant, identified in the 3rd intracellular loop (3iL) of the receptor in highly hypertensive patient, triggers constitutive hyperproliferation in CV cells (cardiomyoblasts, smooth muscle cells (SMC) and fibroblasts), which may lead to myocardial fibrosis and remodeling. In fibroblasts and cardiomyoblasts 247R triggered hyperproliferation is due to constitutive active coupling to Gq-independent βarrestin1/MMP/EGFR/ERK dependent pathway, while in SMC it is Gq- and MMP/EGFR/ERK-dependent. Here we report that α_{1a}AR-WT (WT) and 247R differentially interact with ubiquitous multi-domain scaffold protein spinophilin (SPL) that binds to 3iL of several GPCRs competing with arrestin thereby prolonging their signaling. The role of SPL in CV regulation is poorly studied. We hypothesized that SPL mediates constitutive signaling of 247R and examined whether SPL directly interacts with α_{1a}AR-WT or 247R. Our preliminary results reveal a direct interaction of SPL with WT and 247R: the SPL-WT interaction appears to be stronger as determined by co-immunoprecipitation. Different domains of SPL differentially interact with WT or 247R. SPL 1-480aa fragment interacts stronger with WT indicating interaction with 3iL, while SPL 480-817 fragment interacts stronger with 247R. Our preliminary results also demonstrate that 247R expression in all three cell types elevates endogenous SPL protein levels. Importantly, inhibition of SPL expression with specific siRNA

reduces 247R-triggered hyperproliferation in SMC and cardiomyoblasts to near normal levels, while SPL knockdown has no effect in WT cells. Thus, we identified SPL as a novel protein involved in interacting and signaling of α_{1a}AR and its genetic variant in CV cells and that SPL could be considered as a potentially novel target in α_{1a}AR-mediated cardiovascular disorders.

E. Babaeva: None. **I. Gradinaru:** None. **D.A. Schwinn:** None. **A. Oganesian:** None.

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MED13 Dependent Signaling From The Heart Confers Leanness By Enhancing Metabolism In Adipose Tissue And Liver

Kedryn K Baskin, UTSW, Dallas, TX; Chad E Grueter, U of Iowa Carver Coll of Med, Iowa City, IA; Christine M Kusminski, William L Holland, Santhosh Satapati, Shawn C Burgess, Philipp E Scherer, UTSW, Dallas, TX; Christopher B Newgard, Duke Unversity, Durham, NC; Rhonda Bassel-Duby, Eric N Olson, UTSW, Dallas, TX

The heart requires a continuous supply of energy but has little capacity for energy storage and thus relies on exogenous metabolic sources. We previously demonstrated that cardiac MED13 modulates systemic energy homeostasis in mice. In the present study, we sought to define the extra-cardiac tissue(s) that respond to cardiac MED13 signaling. Cardiac over-expression of MED13 in mice (MED13cTg) confers a lean phenotype that is associated with increased lipid uptake, beta-oxidation and mitochondrial content in white adipose tissue (WAT) and liver. Cardiac expression of MED13 decreases metabolic gene expression and metabolite levels in heart and liver but enhances them in WAT. Although exhibiting increased energy expenditure in the fed state, MED13cTg mice are metabolically flexible and adapt to fasting. These findings demonstrate that MED13 acts within the heart to promote systemic energy expenditure in extra-cardiac energy depots and point to an unexplored metabolic communication system between the heart and other tissues.

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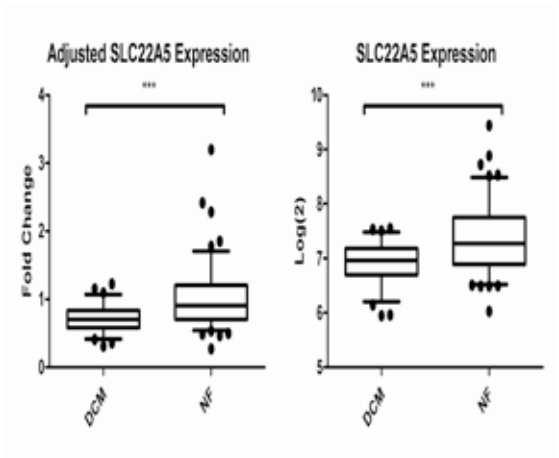
Dysregulation of the Small Organic Carnitine Transporter and Carnitine in Heart Failure

Kenneth C Bedi Jr., Nathaniel W Snyder, Jeff Brandimarto, Clementina Mesaros, Ian A Blair, Kenneth B Margulies, J. Eduardo Rame, Univ of Pennsylvania, Philadelphia, PA

Introduction: Advanced heart failure (HF) is characterized by metabolic abnormalities. We have recently shown myocardial lipid species to be significantly decreased in non-diabetic HF. The finding of deficient carnitine species has directed us to examine a key gene SLC22A5 the high-affinity carnitine transporter. Known mutations of this gene lead to primary carnitine deficiency and cardiomyopathy. **Methods:** Left ventricular samples procured at the time of orthotopic heart transplantation (OHT) from non-diabetic idiopathic dilated cardiomyopathy (DCM) n=16, and brain-dead organ donors without a history of diabetes or HF (NF) n=18 formed the study sample. All hearts received in situ cold cardioplegia. Lipids including carnitines were quantitated with liquid chromatography and high-resolution mass spectrometry (LC-HRMS) following the Folch extraction method. mRNA expression data of target genes including SLC22A5 from a separate study of n=117 DCM patients and n=67 NF patients $p \leq 0.05$ were analyzed. **Results:** Representative trends for all carnitine species assayed were shown to be significantly reduced in DCM. The figure below represents the mRNA expression between DCM and NF patients. There was a statistically significant reduction of SLC22A5 expression in the DCM population (Figure: ***$p \leq 0.0001$, dots 95th percentiles). **Conclusions:** A statistically significant deficiency of carnitine species and a reduction in the expression of the carnitine transporter SLC22A5 was demonstrated in non-diabetic HF. Future studies will attempt to resolve the mechanisms behind these observations.



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Differences Between Mammalian and *Drosophila* Mitochondrial Calcium Handling Help Identify Mechanisms Regulating the Permeability Transition.

Dipayan Chaudhuri, David E Clapham, Boston Children's Hosp, Boston, MA

Studies of cardiomyocyte death during calcium overload induced by ischemia-reperfusion injury or heart failure have implicated the mitochondrial permeability transition as a key pathway. During the permeability transition, an opening of a channel in the inner membrane leads to mitochondrial depolarization and swelling. Despite extensive studies in mammalian systems, the machinery responsible for this phenomenon remains only partially identified. If present in non-mammalian species, the components of the permeability transition may be further elucidated, given potential advantages within these systems for high-throughput screens. However, the existence of a permeability transition remains controversial in non-mammalian organisms. In *Drosophila*, prior studies have documented calcium-induced mitochondrial depolarization, but no obvious swelling. Here we show that *Drosophila* S2R+ cells do possess the machinery for permeability transition, but that the threshold for a calcium trigger is significantly higher than in mammalian systems. Using a calcein-loading method, we show that *Drosophila* permeability transition can be triggered by calcium overload, using ionomycin, and by cysteine oxidation, using phenylarsine oxide. As in mammalian systems, blockade of mitochondrial cyclophilin or the ATP/ADP transporter appears to inhibit the *Drosophila* permeability transition. Finally, we examine three alternative hypotheses that may explain these differences in permeability transition. First, we test if perturbing the pathways for calcium influx into S2R+ mitochondria can trigger this phenomenon. Second, we test if the discrepancy in the calcium threshold is due to structural differences in the key regulators, particularly the mitochondrial cyclophilin. Third, we compare *Drosophila* and human genomes to see if any novel molecules may be responsible for setting the lower threshold for calcium-induced permeability transition in mammalian cells. Since the *Drosophila* cells possess such significant resistance to permeability transition, the results of our investigations suggest potential new strategies for the development of therapeutics inhibiting mitochondrial permeability transition in cardiac calcium-induced injury.

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HAX-1: A Novel Regulator of Energetics and Oxidative Stress in the Heart

Chi K Lam, Wen Zhao, Wenfeng Cai, Guansheng Liu, Phil Bidwell, George Gardner, George Adly, Evangelia G Kranias, Univ of Cincinnati, Cincinnati, OH

Sarcoplasmic reticulum (SR) calcium handling is central not only in the control of heart function during excitation-contraction coupling but also in mitochondrial energetics and apoptosis. Recent studies have identified the anti-apoptotic protein, HS-1 associated protein X-1 (HAX-1) as a novel regulator of SR calcium cycling. Although HAX-1 has been shown to localize to mitochondria in various tissues, we found out that it also localizes to SR through its interaction with phospholamban (PLN) in cardiac muscle. Acute or chronic overexpression of HAX-1 in

cardiomyocytes promoted PLN inhibition on the calcium ATPase (SERCA) and decreased cardiomyocyte calcium kinetics and contractile parameters. Accordingly, ablation of HAX-1 significantly enhanced SERCA activity and calcium kinetics. Furthermore, the HAX-1/PLN interaction appeared to also regulate cardiomyocyte survival. Indeed, overexpression of HAX-1 and the associated depressed SR Ca-load attenuated endoplasmic reticulum stress induced apoptosis, as evidenced by reduction of both caspase-12 activation and pro-apoptotic transcription factor C/EBP homologous protein induction during ischemia/reperfusion injury. In addition, the depressed SR Ca-cycling by HAX-1 overexpression was associated with reduced mitochondrial Ca-load as reflected by: a) hyper-phosphorylation of pyruvate dehydrogenase (PDH) and decreases in its activity, to diminish ATP production consistent with the attenuated energetic demand in these hearts; and b) reduced levels of reactive oxygen species, indicating protection from oxidative damage and preserved mitochondrial integrity. These findings suggest that HAX-1 is a key regulator of Ca-cycling, apoptosis and energetics in the heart. Thus, decreases in HAX-1 levels, observed during ischemia/reperfusion injury, may contribute to the deteriorated function and progression to heart failure development.

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Mitochondrial Sirt3 Is Upregulated By Glucagon-like Peptide-1 Receptor Activation And Contributes To Reversal Of Cardiac Mitochondrial Remodeling Induced By Type 2 Diabetes.

Akio Monji, Yasuko K. Bando, Haruya Kawase, Morihiko Aoyama, Toyoaki Murohara, NAGOYA UNIV, Nagoya Aichi, Japan

[RATIONALE] Sirtuin 3 (SIRT3) is a mitochondrial protein deacetylase that maintains basal ATP yield and its expression level is increased by fasting, exercise, and some NAD+ intermediates. We recently reported that the glucagon-like peptide-1 (GLP-1) receptor agonist exendin-4 (Ex-4) ameliorated cardiac mitochondrial remodeling in diabetic cardiomyopathy via increase in cardiac cyclic AMP (AJP2013). Because changes in cyclic AMP level is regulated by adenylyl cyclase which is one of the downstream target of Ex-4, we hypothesized that SIRT3 may involve in the Ex-4-mediated myocardial reverse remodeling of mitochondria in diabetic mice. [METHODS] Type 2 diabetic Mice (16-week old male) were allocated into experimental groups as follows: Ex-4 (24 nmole/kg/day, subcutaneously administrated by osmotic pump for 40 days, DIO/Ex-4) and vehicle control (DIO/CON). Heart samples and cultured rat neonatal cardiomyocytes were subjected to mitochondrial fractionation using density gradient.
[RESULTS] Cardiac cyclic AMP concentration and phosphorylation of CREB were elevated in DIO-ex4, suggesting successful administration of Ex-4. Electron microscopic analysis revealed that Ex-4 reversed destroyed cristae structure and defragmented mitochondria of DIO/CON heart. The ratio of expression levels of Mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) was consistently suppressed by Ex-4 treatment, suggesting normalization of mitochondrial morphology. Of note, DIO-CON exhibited marked decrease in cardiac SIRT3 level compared to lean/nondiabetic counterpart, which was reversed by exendin-4 treatment. Pharmacological production of intracellular cAMP levels (Isoprotelenol (10 microM) and 8-bromo-cyclic AMP (1 mM)) increased SIRT3 mRNA in cultured primary cardiomyocytes. The AMP-activated protein kinase (AMPK) inhibitition blocked the increase in SIRT3 mRNA, indicating that the SIRT3 expression was regulated by AMPK-dependent manner. [CONCLUSIONS] Our results indicate that Ex-4 reversed abnormal suppression of SIRT3 in mitochondria via activating the PKA/AMPK-dependent pathway.

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Accumulation of Mitochondrial DNA Mutations Impairs Survival, Proliferation, and Differentiation of Cardiac Progenitor Cells

Amabel M Orogo, Dieter A Kubli, Anne N Murphy, Åsa B Gustafsson, Univ of California San Diego, La Jolla, CA

Activation and participation of cardiac progenitor cells (CPCs) in regeneration are critical for effective repair in the wake of pathologic injury. Stem cell activation and commitment involve increased energy demand and mitochondrial biogenesis. To date, little attention has been paid to the importance of mitochondria in CPC survival, proliferation and differentiation. CPC function is reduced with age but the underlying mechanism is still unclear. Mitochondrial DNA (mtDNA) is more susceptible to oxidative attacks than nuclear DNA due to its proximity to the mitochondrial respiratory chain and lack of protective histone-like proteins. With age, mtDNA accumulates mutations that can impair mitochondrial respiration and increase ROS production. In this study, we examined the effects of accumulating mtDNA mutations on CPC proliferation and survival. We have found that incubation of uncommitted c-kit+ CPCs in differentiation medium increased mitochondrial mass and expansion of the mitochondrial network, which correlated with increased cell size and expression of cardiac lineage commitment markers. Differentiation activated mitochondrial biogenesis, increased mtDNA copy number, and enhanced oxidative capacity and cellular ATP levels in CPCs. To investigate the effect of mtDNA mutations and aging on CPC survival and function, we utilized a mouse model in which a mutation in the mtDNA polymerase γ ($POLG^{m/m}$) leads to accumulation of mtDNA mutations, mitochondrial dysfunction, and accelerated aging. Isolated CPCs from hearts of 2-month old $POLG^{m/m}$ mice had reduced proliferation and were more susceptible to oxidative stress and chemotherapeutic agents compared to WT CPCs. The majority of $POLG^{m/m}$ CPCs contained fragmented mitochondria as shown by immunostaining. Incubation in differentiation medium resulted in fewer GATA-4 positive $POLG^{m/m}$ CPCs compared to WT CPCs. The reduced differentiation in these $POLG^{m/m}$ CPCs correlated with reduced PGC-1α expression and OXPHOS protein levels, suggesting that mitochondrial biogenesis is impaired. 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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Unc-51-like Kinase 1 (ULK1) Plays A Crucial Role In Mitophagy In Cardiomyocytes

Toshiro Saito, Junichi Sadoshima, Rutgers New Jersey Medical Sch, Newark, NJ

The mitochondrion is an essential organelle that supplies ATP in cardiomyocytes (CMs). However, damaged mitochondria are harmful via the production of reactive oxygen species and induction of apoptosis in pathological conditions. Therefore, quality of mitochondria should be controlled tightly through various mitochondrial quality control mechanisms. Mitochondrial autophagy (mitophagy) is considered an integral part of this mechanism, and recent investigations uncovered the role of PINK1 and Parkin in mitophagy. However, these observations were made under artificial conditions, such as over-expression of Parkin or treatment with CCCP, and thus the precise mechanism has not been fully elucidated in more pathophysiologically relevant conditions. Recent evidence suggests that mitophagy can take place independently of ATG7, a molecule essential for the conventional form of autophagy, and that this form of autophagy is ULK1-dependent. We investigated the role of ULK1 and ATG7 in mediating mitophagy using mitochondria-targeted Keima (Mito-Keima) in cultured rat neonatal CMs. Keima has a bimodal excitation spectrum peaking at 440 and 560 nm, corresponding to the neutral and acidic pH, respectively. In CMs transfected with Mito-Keima, the fluorescent dots with a high 560nm/440nm ratio represent the mitochondria incorporated into autolysosomes which indicate mitophagy. Here we report that ULK1 plays a more predominant role in glucose deprivation (GD) -induced mitophagy than ATG7. Control CMs exhibited 8.7±1.0 % of the area of high-

ratio dots per cells after GD. Knockdown of ULK1 significantly reduced the area to 2.3±0.9 % in CMs after GD ($p<0.01$, vs sh-Control). The reduction was significantly greater in CMs with knockdown of ULK1 than that of ATG7 (7.0±1.6 %, $p<0.05$, sh-ULK1 vs sh-ATG7). In addition, knockdown of Beclin1 and Drp1 also significantly decreased the area of high-ratio dots (about 1.0 % and 0.5 %, respectively). Overexpression of ULK1 was sufficient to induce mitophagy without starvation, whereas that of ATG7 was not. These results suggest that ULK1, Beclin1 and Drp1 play an essential role in mediating GD-induced mitophagy in CMs.

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Mitochondrial-targeted Grk2 Increases Superoxide Formation And Impairs Cardiomyocyte Respiratory Reserve Capacity

Priscila Y Sato, J Kurt Chuprun, Jessica Ibetti, John W Elrod, Walter J Koch, Temple Univ Medical Sch, Philadelphia, PA

β-adrenergic receptors (βARs) are powerful regulators of cardiovascular function and are impaired in heart failure (HF). Signal transduction of βARs is canonically shut down by phosphorylation via G protein-coupled receptor kinase 2 (GRK2) and the subsequent binding of β-arrestins. This process of receptor desensitization is enhanced in HF via the up-regulation of GRK2 and contributes to disease progression. We have recently reported non-canonical actions of GRK2, which contribute to the development of HF independent of βAR desensitization. We have previously shown that GRK2 can act as a pro-death kinase in cardiomyocytes bytranslocating to mitochondria and activating mitochondria permeability transition. This study was designed to gain more understanding of the mitochondrial function of GRK2. We isolated adult cardiomyocytes from cardiac-specific transgenic mice overexpressing GRK2 at levels found in human HF (TgGRK2), and examined superoxide production using the redox sensitive reporter MitoSox Red. Confocal imaging revealed a 4.6 fold increase in superoxide levels in cardiomyocytes overexpressing Grk2 as compared to non-transgenic (NLC) cardiomyocytes (corrected total cell fluorescence 11.59±1.06, TgGRK2 (n= 3 hearts, 88 cells) vs 2.54±0.02 NLC (n=3 hearts, 52 cells), ($p<0.001$). This indicates that the chronic elevation of GRK2 induces mitochondrial oxidative stress priming the myocyte for enhanced injury. To further explore the mitochondrial actions of GRK2 and consequences of redox stress we examined oxidative phosphorylation by performing oxygen consumption measurements in neonatal rat ventricular myocytes overexpressing GRK2 or GFP-expressing control myocytes. Seahorse analysis showed that cells overexpressing GRK2 have a significant decrease in spare respiratory capacity indicating that cells with elevated GRK2 levels have an impaired capacity to generated ATP during times of stress. Further studies with mutants that limit GRK2 kinase activity or mitochondrial localization demonstrate that mitochondrial GRK2 may be a significant contributor to cellular dysfunction as seen in heart failure.

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Different Reaction To Lactate Between The Atrial And The Ventricular Muscle In Excitation-contraction Coupling

Daisuke Shimura, Waseda Univ, Tokyo, Japan; Yoichiro Kusakari, Jikei Univ, Tokyo, Japan; Nobuhito Goda, Waseda Univ, Tokyo, Japan; Susumu Minamisawa, Jikei Univ, Tokyo, Japan

[Background]The effect of a metabolic change on excitation-contraction (EC) coupling is poorly understood in the atrial myocardium, while the previous studies have mainly focused on EC coupling in the ventricular myocardioum. Although the myocardium mostly uses fatty acid as an energy source, we have reported that metabolic substrate, includes lactate, can be used for energy production and that the metabolomic profile is different between the atria and the ventricles in mouse heart. In addition, it is still under discussion whether lactate can be an energy source for muscles. In the present study, we aimed to investigate the effect of

lactate exposure on EC coupling in the atrial and the ventricular myocardium. [Methods]We micro-injected aequorin (a Ca2+-sensitive photoprotein) into superficial cells of the left atrium and/or the left ventricular papillary muscle isolated from mice (C57/BL6, 12 - 17 weeks of age), and simultaneously measured intracellular Ca2+ concentration and tension (1 Hz at 36). We added lactate (~10 mM) into HEPES-Tyrode solution (pH was adjusted at 7.4) and observed the changes in the peak tension and the peak Ca2+ concentration. [Results and Conclusion]Lactate at a concentration of 10 mM significantly decreased the peak tension (61.7±6.0%; n = 3; P < 0.05.) and the peak Ca2+ concentration (78.8±4.8%; n = 3; P < 0.05.) in the atrial myocardium. Although we observed similar effect of lactate on the ventricular papillary muscle, it was modest compared with the atrial myocardium (72.8±5.6%, 91.6±13.5%, peak tension and Ca2+, respectively; n = 3; no significance). Our results suggest that the atrium has different characteristic of EC coupling from the ventricles in response to an increase in lactate, of which condition is often observed in myocardial ischemia. Moreover, lactate did not seem to contribute to make energy in terms of the tension in the heart. Simultaneous measurement of tension and intracellular Ca2+ concentration can be useful to analyze the atrial physiological property.

D. Shimura: None. **Y. Kusakari:** None. **N. Goda:** None. **S. Minamisawa:** None.

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Progressive Mitochondrial Protein Acetylation and Microsteatosis is Associated with Diastolic Dysfunction in a Hypertrophic Cardiomyopathy Model

Amanda R Stram, Gregory R Wagner, P Melanie Pride, R Mark Payne, Indiana Univ, Indianapolis, IN

We tested the hypothesis that increased mitochondrial protein acetylation is associated with impaired fatty acid metabolism and diastolic dysfunction in Friedreich's Ataxia (FRDA). FRDA results from deficiency of the mitochondrial protein, frataxin (FXN), and causes hypertrophic cardiomyopathy. FRDA hearts show decreased ATP production. We previously showed that FXN loss results in loss of activity of the NAD+-dependent mitochondrial deacetylase, sirtuin 3 (SIRT3), and cardiac mitochondrial protein hyperacetylation in a mouse model of FRDA. Long-chain and medium chain acyl CoA dehydrogenases (LCAD, MCAD) are targets of SIRT3, suggesting that abnormal acetylation may alter fatty acid metabolism. A cardiac specific mouse model with conditional deletion of FXN in heart and skeletal muscle (FXN MCK-Cre-/-) was compared to healthy controls (FXNfl/fl). Mice underwent echocardiogram and left heart catheterization in vivo at age 30 and 65 days. Heart lysate was examined for overall lysine acetylation at ages 30, 45 and 70 days, and acetylated LCAD and MCAD. Myocardial cells were stained with oil red to detect lipids. Hearts of FXN MCK-Cre-/- animals showed mitochondrial hyperacetylation, increased LCAD and MCAD acetylation, and microsteatosis compared to controls. Diastolic dysfunction was evident in FXN MCK-Cre-/- at day 65 by increased mitral valve E/A ratios and IVRT ($p<0.04$ and $p<0.03$, respectively, n=7), prolonged tau, and decreased -dP/dt ($p<0.02$ and $p<0.03$, respectively, n=6). Cardiac mechanics at day 65 showed systolic failure with reduced EF and FS ($p<0.002$ and $p<0.003$, respectively, n=7). Comparison of FXN deficient mice and controls at day 65 trended toward flattened ESPVR and left shifted EDPVR curves, but differences were not significant. Early diastolic dysfunction was evident at day 30 in FXN MCK-Cre-/- by high E/A ratio and EDP compared to day 65 controls ($p<0.03$, n=7, and $p<0.03$, n=6, respectively). We conclude that progressive mitochondrial protein acetylation and abnormal fatty acid metabolism is associated with early diastolic dysfunction and later systolic failure. This metabolic dysregulation and pathophysiology may share characteristics with other metabolic heart disorders, such as diabetes and metabolic syndrome.

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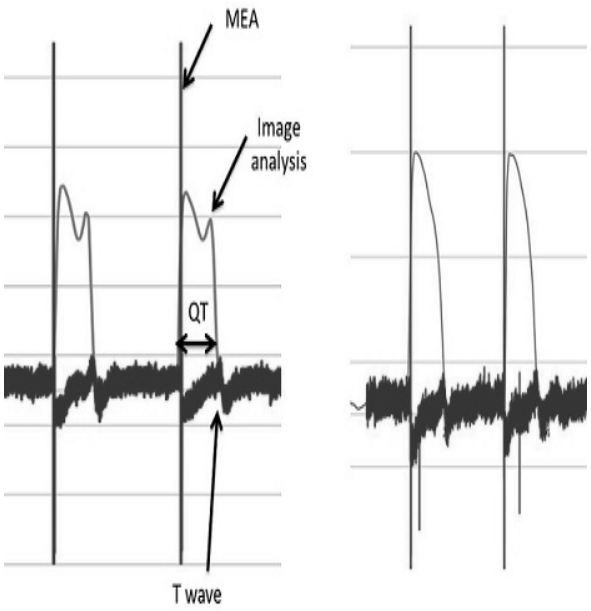
Automated and Non-invasive Estimation of QT Interval from Microscopy Images of Stem Cell-Derived Cardiomyocytes

Mahnaz Maddah, Kevin Loewke, Cellogy Inc., Menlo Park, CA

A promising application of induced pluripotent stem cells (iPSCs) is the generation of patient-specific cardiomyocytes (CMs), which can be used for drug development and safety testing related to cardiovascular health. iPSC-derived CMs can be used for preclinical testing of new drugs that may cause drug-induced arrhythmia or long QT syndrome, as well as post-market safety testing of existing drugs. The measurement of QT interval for iPSC-derived CMs is commonly analyzed using electrophysiological potentials captured by a micro-electrode array (MEA). While such systems are the current standard for characterization, they can be expensive and low-throughput, require high cell plating density, and due to the direct contact between cells and electrodes, may cause undesirable cellular response.

Here, we present a new method to non-invasively measure the QT-interval in iPSC-derived CMs using video microscopy and computer vision analysis. Our algorithms can reliably and automatically extract beating signal characteristics such as frequency, irregularity, and duration through image analysis of cardiomyocyte motion. Through a correlative study with MEA, we demonstrate that a non-invasive measurement of QT interval can be derived from the duration of visible cellular motion that occurs during contraction and relaxation. We also show that our system can accurately characterize the cellular response from the addition of compounds known to modulate beating frequency and irregularity. Our measurement technique is robust, automated, and requires no physical or chemical contact with the cells, making it ideal for cardiovascular drug development and cardiotoxicity testing.



M. Maddah: None. **K. Loewke:** None.

212

Prophylactic Anti-inflammatory Treatment Attenuates The Neuroinflammatory And Behavioral Effects Of Silent Cerebral Infarction

Christina L Nemeth, Gretchen N Neigh, Emory Univ, Atlanta, GA

Silent brain infarction is a frequent complication of cardiac surgery and is associated with mood changes and cognitive disruption. Microsphere embolism (ME) rodent models recapitulate both the diffuse ischemic infarcts and the delayed subtle behavioral disturbances characteristic to silent infarction (SI). Previously, we have shown that ME leads to increased hippocampal inflammation, weakening of the blood brain barrier, and the infiltration of peripherally circulating inflammatory cells in rats. Given long-term increases in inflammatory activity following SI, the current study tests the efficacy of anti-inflammatory versus anti-depressant treatment strategies to reduce the inflammatory and behavioral sequelae of injury. Adult rats were administered either chronic meloxicam (preferential COX-2 inhibitor) or fluoxetine (SSRI) beginning five days prior to ME surgeries. After a two week recovery, animals

were tested for anxiety-like behaviors in the open field paradigm and the hippocampus was examined for gene expression of inflammatory cytokines. Meloxicam treated animals showed a decrease in hippocampal gene expression of inflammatory markers (SPP1; p = 0.0272) and greater than a 3-fold change improvement in open field central tendency (p = 0.0003). No differences in inflammatory gene expression were observed in fluoxetine treated animals (SPP1; p = 0.3288); however, fluoxetine treatment resulted in a 2-fold change improvement in open field central tendency (p = 0.0138) suggesting that while both treatment strategies attenuate SI induced behavioral disruption, only meloxicam acts via inflammatory mechanisms. Given the long term negative consequences of increased central and peripheral inflammatory activity, the data suggest that anti-inflammatory therapeutic strategies may benefit patients at risk for SI as well as cardiac surgery candidates.

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Dystrophic iPSC-derived Cardiomyocytes Have Mislocalization of eNOS and Increased Susceptibility to Cell Death which is Reversed by the Nitrate-like Properties of Nicorandil.

Jennifer L. Strande, Melanie Reiter, Courtney Gastonguay, M. Zeeshan Afzal, Medical Coll of Wisconsin, Milwaukee, WI

Background: Cardiomyopathy is a leading cause of death in Duchenne and Becker muscular dystrophy (D/BMD) patients and specific therapies are lacking. Dystrophin deficiency is associated with mislocalization of nitric oxide synthase (NOS) whose dysregulation contributes to the pathogenesis of dystrophic cardiomyopathy. We have modeled dystrophic cardiomyopathy using patient-specific induced pluripotent stem cells (iPSCs) and show that DMD- and BMD- iPSC derived cardiomyocytes have mislocalization of endothelial (eNOS) but not neuronal NOS (nNOS). In this study, we determined whether nicorandil, a drug with nitrate-like properties, protects against stress-induced dystrophic cardiomyocyte injury. **Methods/Results:** DMD-iPSC (exon 3-6 deletion), BMD-iPSC (exon 45-53 deletion) and non-dystrophic (N)-iPSC lines were differentiated into cardiomyocytes (iCMs) and matured for 35-38 days. Immunofluorescence revealed eNOS co-localized with dystrophin along the sarcolemma in N-iCMs but not in DMD- or BMD-iCMs. eNOS also co-localized with caveolin-1 in all groups. nNOS did not co-localize with dystrophin but with the ryanodine receptor 2 in all groups. Both DMD- and BMD-iCMs have increased cell death as determined by TUNEL + staining when subject to 2 hours of metabolic stress and 4 hours of recovery (DMD: 66±3%, BMD: 29±4% vs. 1.5±0.04%; n=3-6). This was associated with dissipation of the mitochondrial membrane potential as monitored by laser-scanning confocal microscopy (DMD: complete loss, BMD: 441±168 vs. N: 891±183 AU; n=3-5). Nicorandil pretreatment was able to restore mitochondrial membrane potential and decrease cell death. The protective effects of nicorandil were abolished by ODQ, a selective inhibitor of NO-sensitive guanylyl cyclase. **Conclusion**: Nicorandil protects against stress-induced cell death and preserves mitochondrial function in dystrophic cardiomyocytes through its nitrite-like properties. This suggests a potential therapeutic role of nicorandil in the treatment of dystrophic cardiomyopathy.

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214

Reduction of Scar Tissue after GHRH-A Treatment in a Swine Model of Sub-acute Ischemic Cardiomyopathy

Luiza Lima Bagno, Rosemeire Kanashiro Takeuchi, Bo Wang, Viky Suncion, Vasileios Karantalis, Courtney Premer, Samuel Golpanian, Wayne Balkan, José E. Rodriguez, Univ of Miami, MIAMI, FL; David Valdes, Univ of Miami, Miami, FL; Marcos Rosado, Ariel Wolf, Norman L. Block, Univ of Miami, MIAMI, FL; Peter Goldstein, Biscayne Pharmaceuticals, MIAMI, FL; Azorides Morales, Andrew V. Schally, Joshua M. Hare, Univ of Miami, MIAMI, FL

Background: Growth hormone-releasing hormone receptor agonists (GHRH-A) stimulate cardiac repair following myocardial infarction (MI) through the activation of the GHRH signaling pathway within the heart. We tested the hypothesis that the administration of GHRH-A prevents ventricular remodeling in a swine sub-acute MI model. Methods: Twelve female Yorkshire swine (25-30 Kg) underwent transient occlusion of the LAD coronary artery (MI). Two-weeks post-MI, swine were randomized to receive injections of either 30 μg/Kg GHRH-A (MR-409) (GHRH-A group; n=6) or vehicle (placebo group; n=6). Cardiac MRI, pressure volume loops and measures of endothelial function were obtained at multiple time points. Infarct-, border- and remote- (non-infarcted) zones were assessed by immunohistochemistry for the growth hormone-releasing hormone receptor (GHRHR). Results: Four-weeks of GHRH-A treatment resulted in reduced scar mass (GHRH-A group: –21.9±6.42%; p=0.02; placebo group: 10.9±5.88%; p=0.25; Two-way ANOVA; p=0.003), and reduced scar size (percent of left ventricle mass) (GHRH-A group: –38.38±4.63; p=0.0002; placebo group: –14.56± 6.92; p=0.16; Two-way ANOVA; p=0.02). Moreover, peripheral endothelial function was significantly increased compared to baseline values in the GHRH-A group (paired t-test; p=0.006) but not in the placebo group (p=0.99). Unlike in rats, this reduced infarct size in swine was not accompanied by improved cardiac function as measured by serial hemodynamic pressure-volume analysis. GHRH receptors were abundant in cardiac tissue, with a greater density in the border zone of the GHRH-A group compared to the placebo group. These data support the concept of direct post-infarction activation of cardiac signal transduction, and of enhancing this activation with systemic treatment by GHRH. Conclusions: Daily subcutaneous administration of GHRH-A is feasible and safe in female swine. Furthermore, GHRH-A therapy significantly reduced infarct size and increased endothelial function, suggesting that a local activation of the GHRH pathway leads to the regenerative process and preservation of peripheral endothelial function.

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The Redox Activation of DUSP4 by N-Acetyl Cysteine Protects Endothelial Cells and the Myocardium against Oxidative Damage

Chun-An Chen, Alma Barajas-Espinosa, Ariel Basye, Haixu Yan, Erin Jesse, David Quan, Ohio State Univ, Columbus, OH

Background: Redox imbalance is the primary cause for endothelial dysfunction (ED), obstructed blood flow, and subsequent heart attack and failure. Under oxidant stress, many critical proteins regulating endothelial function undergo oxidative modifications that lead to ED. Cellular levels of glutathione (GSH), the primary reducing source, can significantly regulate cell function via reversible protein thiol modification. N-Acetyl cysteine (NAC), a precursor for GSH biosynthesis, is beneficial for many vascular diseases; however, the detailed mechanism of these benefits is still not clear. Methods: We employed EPR spin-trapping, HPLC, fluorescent microscopy, immunoblotting, and qPCR of both in vitro and ex vivo experiments using either cultured cells or the Langendorff heart preparation. Results: NAC treatment increases NO generation from endothelial cells, as well as the enzyme and cofactor responsible for its production, ie eNOS and BH4. Interestingly, NAC treatment also increased the expression of DUSP4, an inducible nuclear dual-specificity phosphatase implicated in cardiovascular function.

We hereby establish that DUSP4 redox modulates two important kinases (p38 and ERK1/2) of MAPK signaling pathways and provides protection against Cd2+-induced ROS damage as well as hypoxia-reoxygenation insult to endothelial cells. Furthermore, a four week oral NAC pre-treatment promotes DUSP4 both protein and mRNA expression in the rat myocardium and renders the heart less susceptible to ex vivo ischemia-reperfusion injury. Protein expression profiles in the myocardium closely mimic those observed with cultured endothelial cells. The infarct size of NAC-treated hearts is significantly reduced. The

myocardial rate pressure product is much improved above vehicle treated rats. Conclusion: NAC serves as a direct antioxidant and as a regulator of transcription and translation of DUSP4, modulating the activity of its downstream effectors: ERK1/2 and p38, and thus protects against ROS-induced damage both in vitro and ex vivo. As such, NAC-derived drugs can provide a novel therapy to oxidant-induced diseases via the specific up-regulation of protective proteins such as DUSP4 and eNOS.

C. Chen: None. **A. Barajas-Espinosa:** None. **A. Basye:** None. **H. Yan:** None. **E. Jesse:** None. **D. Quan:** None.

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Traditional Chinese Medicine Tongxinluo Modulates The Secretion Of Cytokines In Vitro By Cardiac Microvascular Endothelial Cells In Ischemia/Reperfusion Injury.

Hehe Cui, Xiangdong Li, Fuwai Hosp, Beijing, China; Zhigang Wang, Peking Union Medical Coll, Beijing, China; Na Li, Kang Qi, Qing Li, Chen Jin, Yuejin Yang, Fuwai Hosp, Beijing, China

Cardiac microvascular endothelial cells (CMECs) regulate the function of cardiomyocytes and blood cells in myocardial ischemia/ reperfusion injury (MIRI) via paracrine methods. Tongxinluo (TXL) is a Traditional Chinese Medicine compound to treat angina pectoris in China, which is constituted of ginseng and other 11 natural products. It is proved to improve the endothelial function and be protective against MIRI. Thus, we aimed to find alterations in paracrine function of CMECs under the hypoxia/reoxygenation (H/R) situation and its modulation by TXL. CMECs were exposed to different concentrations of TXL for 30 min and then subjected to H/R for 12 h/2 h. Apoptotic rates were measured to determine the optimal concentration. Protein antibody arrays were used to find the alterations of cytokines in conditioned medium (CM) secreted by CMECs. Gene Ontology project was adopted to describe the functions of changed cytokines. TXL inhibited apoptosis of CMECs dose-dependently under H/R and reached its peak effect at 800 μg/mL. Thirty-three types of cytokines were significantly changed by H/R (19 factors decreased and 14 increased), and TXL at 800μg/ml changed 121 types of cytokines compared to the H/R group (93 factors decreased and 28 increased). The cytokines with significant alterations were involved in cell differentiation and proliferation, positive chemotaxis, and endothelial cell migration. Among these cytokines, bFGF, PDGF and IL-7 were attenuated by H/R and further decreased by TXL, while IL-21, MCP-2 and GROa were increased by H/R but decreased by TXL. The study elucidated the paracrine function of CMECs in MIRI and partly illuminated the protective mechanisms of TXL.

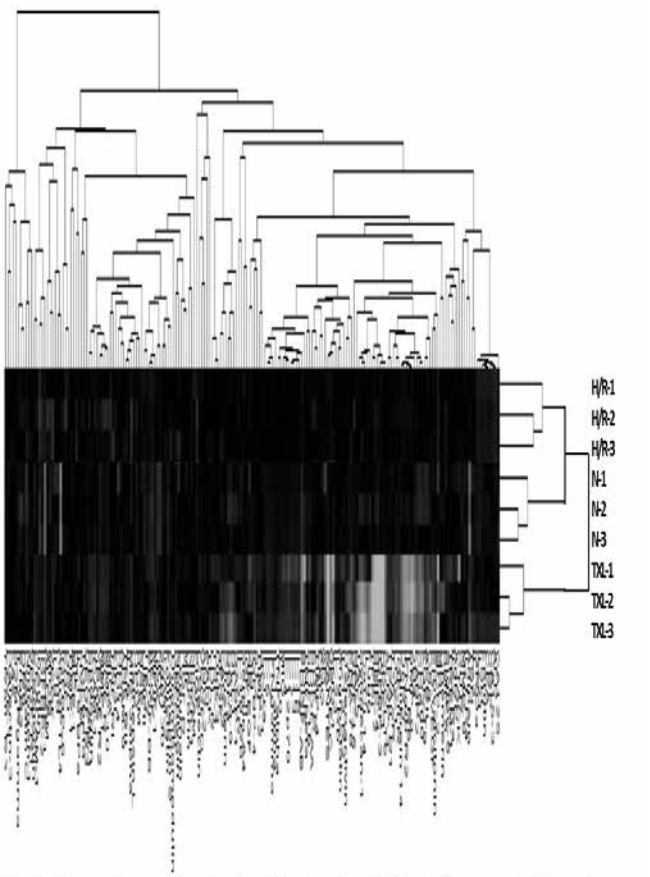

Fig. Protein antibody arrays of cytokines in conditioned medium. H/R: hypoxia/reoxygenation ; N: Normal; TXL: Tongxinluo; Three samples were analyzed in one group.

H. Cui: None. **X. Li:** None. **Z. Wang:** None. **N. Li:** None. **K. Qi:** None. **Q. Li:** None. **C. Jin:** None. **Y. Yang:** None.

ABSTRACTS

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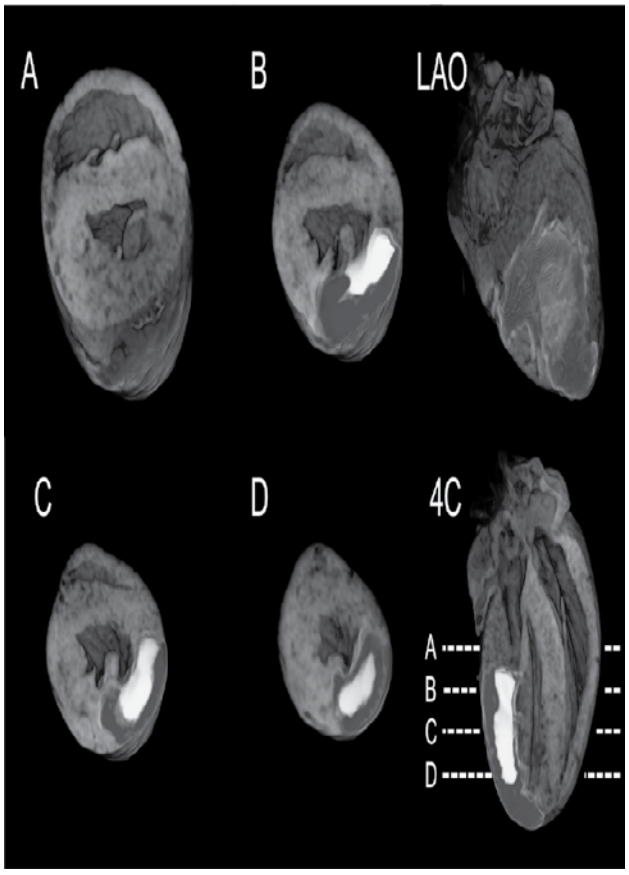
Use of Magnetic Resonance Imaging Method For Quantification Of Myocardial Salvage In A Rat Model Of Ischemia-reperfusion Injury.

Stuart M Grieve, Jawad Mazhar, Fraser Callaghan, Cindy Y Kok, Ravinay Bhindi, Gemma A Figtree, Univ of Sydney, Camperdown, Australia

Introduction: Quantification of myocardial "area-at-risk" (AAR) and infarct (MI) zone is critical for assessing novel therapies targeting myocardial ischemia-reperfusion (IR) injury. The current standard method involves perfusion with Evan's Blue (EB), staining with TTC and manual slicing and analysis. We have developed an MRI method for quantifying MI and AAR in whole hearts which provides superior 3D resolution to the standard approach.
Methods: Rats were given an IR injury, recovered for 24 hours then infused with Gd-DTPA via the tail vein. The coronary artery was then religated, and a solution containing both iron oxide microparticles and EB was infused. For comparison, hearts were then harvested and transversally sectioned for TTC staining. Some hearts were kept intact for MRI only analysis. Ex-vivo MRI T2* and T1 images were acquired on a 9.4T magnet. The AAR (red) was quantified by comparing the T2* signal loss in perfused regions (blue) and high T1-signal in infarcted zones (white) from Gd-DTPA retention.
Results: MRI and EB/TTC measures on the same slice for both AAR and MI were highly correlated ($r=0.92-0.94$;$p<0.05$). 3D MRI acquisition and analysis of whole hearts reduced intra-observer variability, and automated segmentation and analysis further reduced inter-observer variation.
Conclusion: This novel MRI technique allows precise assessment of infarct and AAR zones and is highly suited to automation of both analysis and acquisition. This method could remove the need for tissue slicing, and via a centralised MRI facility, could permit 3D digital analysis of hearts at high anatomical resolution, accessible for all laboratories already performing IR experiments.



S.M. Grieve: None. **J. Mazhar:** None. **F. Callaghan:** None. **C.Y. Kok:** None. **R. Bhindi:** None. **G.A. Figtree:** None.

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The Role of Novel Tripartite Motif Proteins in Sarcolemmal Membrane Repair

Liubov V. Gushchina, Jenna Alloush, Sayak Bhattacharya, Zhaobin Xu, Eric X. Beck, Noah L. Weisleder, The Ohio State Univ, Columbus, OH

Tripartite motif (TRIM) proteins are a superfamily of coiled-coil-containing RING E3 ligases that function in many cellular processes, particularly in membrane repair pathways. Mitsugumin 53 (MG53) also known as TRIM72, is primary expressed in skeletal muscle and heart. Our experimental data confirm that during membrane damage, MG53 translocates to the injury site and acts as a molecular glue to reseal the damage area. The role of MG53 in membrane repair has been demonstrated in both in vitro studies using molecular approaches and in vivo

using rodent wild type and knockout models. Thus, our data indicate that recombinant human MG53 protein can be directly applied as a therapeutic agent to increase the membrane repair capacity of many cell types, including cardiomyocytes during acute injury or in chronic disease progression. However, the precise mechanism and potential partners by which MG53 executes its membrane repair function are not completely understood. On the basis of the global TRIM family protein alignment, we hypothesize that there are other TRIM proteins that, alone or together with MG53, may facilitate repair by targeting the site of an injury. Moreover, data from our lab demonstrated that MG53 and these TRIM proteins can form homo- and hetero-oligomeric assemblies due to the presence of the coiled-coil region in these proteins and, further, that this may be necessary for the active membrane resealing process. Using E. coli protein expression methodology we can generate and isolate new TRIM recombinant proteins and test if these protein complexes are effective when applied externally to cardiac and non-cardiac cells. These novel proteins will also be tested for their pharmacokinetic properties to determine their efficacy in both acute and chronic applications. Our studies should increase our knowledge of the mechanisms controlling cardiac membrane repair and also provide novel therapeutic targets.

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Cardiac Autophagy in Response to Fasting is Impaired in Mice with Metabolic Syndrome

Bruce R Ito, Nandini Ravindran, San Diego State Univ, San Diego, CA; Allen M Andres, Roberta A Gottlieb, Cedars-Sinai Heart Inst, Los Angeles, CA; Robert M Mentzer Jr., Wayne State Univ, Detroit, MI

Introduction: Macroautophagy (autophagy) is highly responsive to nutrient/energy status and cellular stress. It plays an adaptive role in limiting ischemia/reperfusion (I/R) injury in the heart. Nutrient depletion is a potent inducer of autophagy and is mediated by reciprocal regulation of AMPK and mTOR pathways which in turn regulate autophagy initiation via ULK1/Atg1. Autophagy is reduced in the setting of obesity, dyslipidemia, and insulin resistance associated with metabolic syndrome (MetS). The purpose of this study was determine whether impaired autophagy in MetS is due to altered upstream signaling or a disruption in the autophagic pathway. Methods:, Mice were ad-lib fed a chow (Lean) or a high fat diet (HFD) (D12492, 60% fat) for 18 weeks. In both groups, half of the mice were freely fed with the other half fasted for 24 hours prior to cardiac harvest. Protein levels were determined with Western blotting and gene expression with quantitative PCR. Results: Consistent with previous data, HFD animals exhibited obesity and insulin resistance as reflected by increased body weight, and elevated serum insulin/HOMA compared to Lean animals. Fasting resulted in a marked reduction in serum insulin in both Lean (p=0.013) and HFD (p =0.002) animals. This was accompanied by marked increases in cardiac AMPK activation as reflected by increased phosphorylation in both Lean (p =0.002) and HFD (p=0.002) groups with parallel reciprocal suppression of mTOR activity as reflected by decreased pS6K and pS6. As expected, the Lean group exhibited a fasting-induced increase in LC3-II (p=0.020) indicating an increase in autophagy. This did not occur in the HFD group despite a similar induction in LC3B and p62 gene expression. This lack of autophagy stimulation with fasting in HFD animals was not explained by differences in autophagy initiation as the phosphorylation status of ULK1 decreased similarly with fasting in both groups. Conclusions: These data indicate that the impairment of cardiac autophagy in the presence of MetS is not attributable to abnormal upstream nutrient sensing pathways. The findings suggest a MetS-associated defect in the autophagy/lysosomal fusion pathway. This could explain the increased vulnerability of the heart to I/R injury in the setting of MetS.

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Transcriptional Analysis Of Caveolin And Cavin In The Male And Female Ageing Mouse Heart Following Ischemic Stress

Can J Kiessling, Bond Univ, Gold Cost, Australia; Melissa Reichelt, Univ of Melbourne,

ABSTRACTS

Melbourne, Australia; John Headrick, Griffith Univ, Gold Cost, Australia; Kevin Ashton, Bond Univ, Gold Cost, Australia

Cardioprotection against infarction and dysfunction in the myocardium involves G-protein-coupled receptor signalling orchestrated by specialised membrane microdomains termed caveolae. The caveolin protein family consist of three subtypes: caveolin-1, -2 and -3 (Cav1-3) and are responsible for the formation of caveolae and hypothesized to orchestrate cardioprotective signalling. Caveolin-3 deficiency and overexpression has been shown to attenuate and restore cardioprotection, respectively. Recently, a family of four related proteins known as cavins (Cavin1-4) have been implicated as regulators of caveolae formation and function. The roles and expression distribution of the cavin family is currently unknown in cardiac tissue. In this study hearts were isolated from 8, 16, 32 and 48 week male and female mice and subjected to normoxic perfusion (80 min) or ischemic stress (20 min global ischemia, 60 min reperfusion). RT-qPCR was used to assess differential gene expression of caveolin and cavin subtypes across these ages in both sexes. Decreased post-ischemic pressure development and increased LDH release were observed in 32 and 48 week old relative to 8 week old male hearts hearts, indicative of age-related loss of ischemic tolerance. Females showed greater tolerance to ischemia at 32 and 48 week old hearts when compared to male counterparts. In normoxic male 48 week old hearts, Cav1,-2,-3 and Cavin1 were significantly repressed, whilst post-ischemic male 48 week old hearts demonstrated significant repression of Cav3 and Cavin1 only. Normoxic female hearts showed no significant changes in caveolin and cavin transcript expression over the aging time course. However, post-ischemic female 48 week old hearts showing significant down-regulation of Cav3 only. Taken together, alterations in caveolin and cavin expression may contribute to the age-related loss of ischemic tolerance and G-protein-coupled receptor-mediated protection in aging male and female mice hearts.

C.J. Kiessling: None. **M. Reichelt:** None. **J. Headrick:** None. **K. Ashton:** None.

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Prognostic Role of elevated Myeloperoxidase in Patients with Acute Coronary Syndrome: A Systemic Review and Meta-Analysis.

Andrew R Kolodziej, Charles L. Campbell, Richard Charnigo, Univ of Kentucky, Lexington, KY; Raphael Twerenbold, Christian Mueller, Univ Hosp Basel, Basel, Switzerland; Khaled M Ziada, Ahmed Abdel-Latif, Univ of Kentucky, Lexington, KY

BACKGROUND: It has been shown that Myeloperoxidase (MPO) is intimately involved in pathogenesis of atherosclerosis and Acute Coronary Syndrome (ACS). Small studies have shown that high levels of MPO are a poor prognostic factor in patients presenting with ACS. However, due to the small nature of these studies, the relationship between MPO and outcomes has not been confirmed. Here we aimed to examine the prognostic value of MPO in patients with ACS. METHODS: We performed a meta-analysis to compare the long-term prognosis of ACS patients with high MPO and low MPO levels. The literature was retrieved by formal searches of electronic databases (PubMed, EMBASE, Medline, OVID, and web of knowledge) from inception to November 2013. A total of 16 trials were included in this meta-analysis involving 10572 patients. Data were analyzed using random-effects model and study quality was assessed using appropriate scales. RESULTS: High MPO group was associated with overall worse outcomes than low MPO group in terms of recurrent myocardial infarction (9% [211 of 2336] vs. 7.7% [240 of 3101], odds ratio [OR] 1.4, 95% confidence interval [CI]: 0.92-2.15, p 0.11); all cause mortality (8% [236 of 2920] vs. 5% [209 of 4263], OR 1.83, 95% CI: 1.31-2.54, p <0.0004) and Major Adverse Cardiovascular Events (MACE) (24% [334 of 1400] vs. 14% [133 of 949], OR 2.04, 95% CI: 1.46-2.85, p< 0.0001) (Figure). CONCLUSIONS: In this meta-analysis examining the long-term outcomes in ACS patients, high MPO levels were associated with worse outcomes. These observations support prospective trials tailoring more aggressive therapy to patients with suspected worse prognosis.

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ABSTRACTS

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Intralipid-induced Cardioprotection In Late Pregnancy Is Fully Abolished By Inhibition Of Stat3, But Not Pi3k Signaling Pathway

Jingyuan li, Mansoureh Eghbali, UCLA, Los Angeles, CA

We have recently shown that the heart of late pregnant (LP) rodent is more prone to ischemia/reperfusion (I/R) injury compared to non-pregnant. Here we explored the therapeutic potential of Intralipid (ITLD) in the protection of LP hearts against I/R injury, and investigate the involvement of the signal transducer and activator of transcription-3 (STAT3) and phosphoinositide 3-kinase (PI3K) signaling pathways in ITLD-induced cardioprotetion. Isolated LP mouse hearts were subjected to 20 min ischemia followed by 40 min reperfusion with 1) Krebs Henseleit buffer (CTRL group), 2) 1% intralipid (ITLD group) or 3) ITLD+STAT3 inhibitor Stattic (20 μM, Stattic group), and 4) ITLD+PI3K inhibitor LY294002 (45 μM). The heart function and the infarct size were measured. The Intralipid-induced cardioprotection was fully abolished by Stattic, as RPP was significantly lower in the presence of Stattic (RPP=8881±1331 mmHg*beats/min vs. 1186±563 mmHg*beats/min in ITLD+Stattic, p<0.01) at the end of reperfusion. In fact, all of the hemodynamic indexes in ITLD+Stattic were not significantly different from CTRL. The infarct size was also significantly larger in ITLD+Stattic group when compared to Intralipid alone (47.9±2.5% in ITLD+Stattic vs. 21.7±2.6 % in ITLD, p<0.01). The Intralipid-induced cardioprotection was only partially abolished by LY294002, as at the end of 40 min reperfusion the RPP was significantly lower compared to the group treated with Intralipid alone, but still significantly higher than ITLD+Stattic ((RPP=8881±1331 mmHg*beats/min in ITLD vs. 5212±1955 mmHg*beats/min in ITLD+LY, p<0.05; RPP=5212±1955 mmHg*beats/min in ITLD+LY vs.1186±563 mmHg*beats/min in ITLD+Stattic, p<0.05). The infarct size was also larger when compared to Intralipid alone (32.8±3.1% in ITLD+LY vs. 21.7±2.6% in ITLD, p<0.05), but lower than ITLD+Stattic group (32.8±3.1% in ITLD+LY vs 47.9±2.5%, p<0.05). In conclusion, Intralipid protects the late pregnant heart against I/R injury via the STAT3 rather than the PI3K signaling pathway.

J. li: None. **M. Eghbali:** None.

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The Role Of Angiogenesis In The Myocardial Ischemia/reperfusion Injury In Pregnancy

Jingyuan li, UCLA, Los Angeles, CA; Zoltan Pierre Arany, Beth Israel Deaconess Medical Ctr, Boston, MA; Mansoureh Eghbali, UCLA, Los Angeles, CA

Angiogenesis plays an important role in the pathogenesis of cardiovascular disease. Pro-angiogenic and anti-angiogenic treatments have provided new insights into the impact of angiogenesis-based approaches on coronary artery disease. We have recently reported that the hearts of late pregnant (LP) mice are more prone to ischemia/reperfusion (I/R) injury compared to non pregnant(NP) mice. Provided the significant change of angiogenesis status in pregnancy, here we explored whether stimulating the angiogenesis with VEGF is able to protect the heart against I/R injury in late pregnancy, and whether anti-antigenic treatment with soluble endoglin(sENG), an anti-angiogenic factor, aggravates cardiac I/R injury in NP. Pregnant mice at day 12 either received daily injection of VEGF (100 ug/kg daily subcutaneous injection) or PBS(LP CTRL) for 7 days, and at day 19 the LP mice hearts were subjected to 20 min ischemia followed by 40 min reperfusion in Langendorff. NP mice either received a single adenovirus sENG(2×10 8particles via tail vein injection) or vehicle(NP CTRL), and 10 days later NP mice were subjected to 20 min ischemia followed by 40 min reperfusion in Langendorff. The heart function was recorded throughout the experiments, and the infarct size was measured by TTC staining at the end of experiments. Exogenous VEGF treatment significantly improved the cardiac function of LP mice after ischemia. The rate pressure product (RPP) at the end of reperfusion was improved from 1617±287 mmHg*beats/min (n=6) in LP CTRL to 11287±1783 mmHg*beats/min (n=3) in the VEGF group(p<0.01). The infarct size was also significantly reduced by VEGF treatment to 25.0±4.3% (n=3) from 57.4±5.2%(n=6) in CTRL (p<0.01). While sENG aggravated the cardiac

I/R injury in NP, as the RPP at the end of reperfusion in the sENG group (4523±1281 mmHg*beats/min, n=4) was significantly lower compared with NP CTRL group(12818±1213 mmHg*beats/min, n=6)($p<0.01$). Furthermore, the infarct size in the sENG group was markedly higher compared with NP CTRL group (34.0±3.3% (n=4) vs. 16.3±1.4%(n=6) in NP CTRL, $p<0.05$). In conclusion, anti-angiogenic treatment aggravates the cardiac I/R injury in NP, while angiogenic therapy protects the heart against I/R injury in LP.

J. li: None. **Z. Arany:** None. **M. Eghbali:** None.

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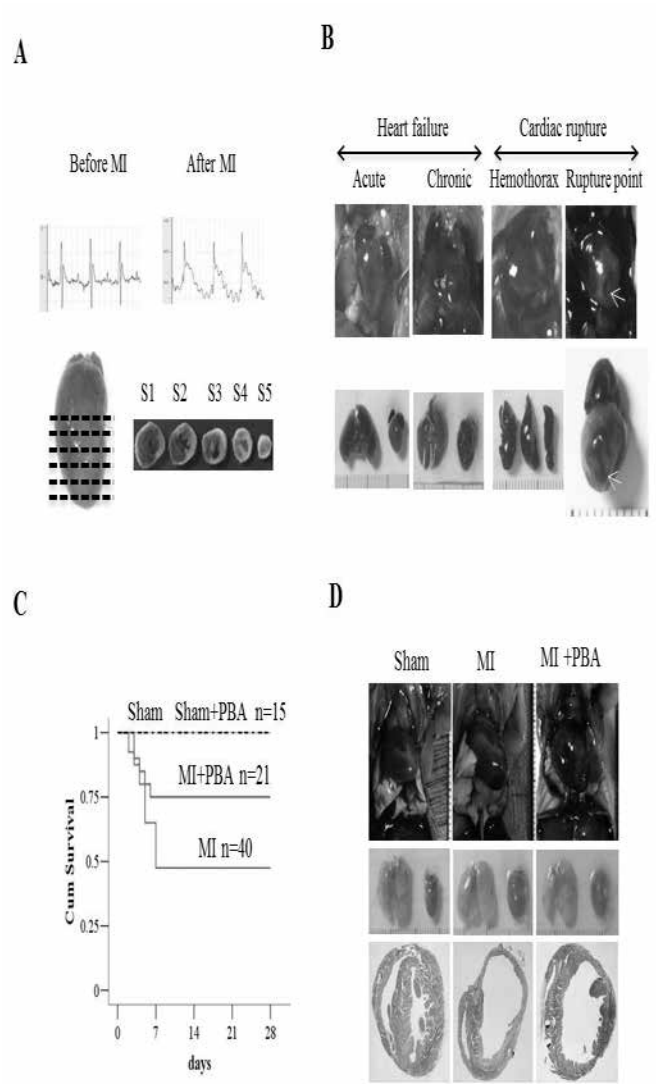
4-phenylbutyric Protects The Heart From Heart Failure, Cardiac Rupture And Remodeling By Alleviating Cardiac Apoptosis And Fibrosis In A Mouse Model Of Myocardial Infaction

Tao Luo, Dept of pathophysiology, Sch of Med, Jinan Univ, Guangzhou, China; Baihe Chen, Dept of Cardiology, Nanfang Hosp, Southern Medical Univ, Guangzhou, China; Xianbao Wang, Dept of Cardiology, Zhujiang Hosp, Southern Medical Univ, Guangzhou, China; Yanping Wang, Dept of Pathophysiology, Sch of Med, Jinan Univ, Guangzhou, China; Masafumi Kitakaze, Cardiovascular Div of Internal Med, Natl Cerebral and Cardiovascular Ctr, Suita, Japan; Yulin Liao, Dept of Cardiology, Sch of Med, Southern Medical Univeristy, Guangzhou, China; Liang Yan, Dept of Pathophysiology, Sch of Med, Jinan Univ, Guangzhou, China

Evidences showed that endoplasmic reticulum (ER) stress has involved in cardiac hypertrophy and inhibition of ER stress exerts an effective cardioprotective effect. But little is known about the effect of ER stress inhibition on myocardial infarction (MI). The aim of this study is to investigate whether direct inhibition of ER stress by 4-phenylbutyric acid (4-PBA), an ER stress chemical chaperone, could attenuate MI induced by left coronary artery (LCA) ligation in male C57BL/6 mice. Either NaCl or 4-PBA (20 mg/kg/d) alone was intra-peritoneal injected to the mice immediately after MI for 4 weeks. Heart failure, cardiac rupture and remodeling occurred after MI. There was 52.5% *vs* 24% death rate in control group *vs* 4-PBA group during the early phase after MI (2-7 days). The survivals underwent cardiac remodeling with impaired systolic and diastolic functions at the end. The ventricular aneurysm and fibrosis were much smaller in 4-PBA group and the heart functions were improved. Western blot showed that the ER stress markers, Bax, Caspase 3, TGFβ1 and Smad 2/3 in the heart tissues of the control group were significantly increased after MI, in which Bax and Caspase 3 were increased markedly in the early phase of MI, while TGFβ1 and Smad 2/3 were increased obviously in the late phase. 4-PBA decreased those protein levels in the early and late phases of MI respectively. These findings indicate that inhibition of ER stress using 4-PBA could be an effective therapeutic agent protects the heart from post-infarcted heart failure, cardiac rupture and remodeling.



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Chronic Alcohol Consumption Alters the Epigenetic Fingerprint of Cardiac Cell Types

Alexander R Mackie, Erin E Vaughan, Mohsin Khan, Suresh K Verma, Srikanth Garikipati, Prasanna Krisnamurthy, Veronica Ramirez, Tatiana Abramova, Kenneth Ellingson, Sol Misener, Aiko Ito, Raj Kishore, Northwestern Univ, Chicago, IL

The cardiac influence of chronic ethanol (EtOH) consumption in humans is dictated by the frequency of ethanol ingestion. Whereas moderate consumption (i.e. 1-2 alcoholic drinks/day) imparts a cardiac benefit to patients by reducing adverse cardiovascular event (ACE) occurrences, consumption below or beyond these moderate levels significantly increases the incidence of ACEs. Despite these observations, little is known regarding the functional impact of chronic EtOH consumption on post-myocardial infarct repair or the cellular mechanisms involved in this process. Thus, we investigated the post-AMI functional consequences of chronic ethanol consumption in mice. Mice received chronic ethanol via the Lieber-DeCarli paradigm (i.e. 0%, 1% (moderate) or 5% (high) ethanol v/v) in an isocaloric fashion for 8 weeks. After 8 weeks, mice underwent a 60-minute ischemic/reperfusion injury and the subsequent assessment of their cardiac function for 4 weeks post-AMI. As early as two weeks post-AMI, mice fed the 1% EtOH displayed modest yet significant improvements in ejection fraction and systolic ventricular volumes as compared to control mice. Conversely, the 5% EtOH diet group displayed diminished ejection fraction and increases in both systolic chamber volume and infarct size. To explore the cellular basis of these observed functional changes, primary cardiac cell types (fibroblasts and endothelial cells) treated chronically (5 days) with ethanol in vitro (i.e. 0%, 0.1% (moderate) or 0.5% (high) ethanol v/v) displayed robust changes in their epigenetic histone-modification profiles indicating a high likelihood for changes in cell specific gene expression. In addition, conditioned media from ethanol treated primary cardiac fibroblasts (PCFBs) altered the tube forming capacity of endothelial cells in a matrigel tube-formation assay in a dose pattern akin to what was observed in vivo. This data suggests that chronic ethanol directly invokes epigenetic changes in PCFBs that can modify their contribution to cardiac repair processes following AMI. Lastly, we are actively investigating whether ethanol-induced changes to endothelial cell epigenetic patterns alter the cells responses in the face of an ischemic insult.

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ABSTRACTS

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GGF2 Requires Activation Of PI3 Kinase Pathway To Mediate Cytoprotective Effects In The Mouse Atrial-derived HI-1 Cardiomyocyte-like Cell Line

Maya Srinivas, Jing Cao, Roni Zilinski, Anthony O Caggiano, Donald Button, Acorda Therapeutics, Ardsley, NY

Glial growth factor (GGF2) is a Neuregulin-1 (NRG-1), which binds ErbB receptors and activates downstream cell signaling processes. NRG-1 ligands and their receptors are required for cardiac development and adult cardiac function. GGF2 has been shown to improve left ventricular function in several models of heart failure though the mechanisms by which GGF2 is efficacious are poorly understood. GGF2 is currently in clinical trials for treatment of congestive heart failure. Many studies have focused on GGF2 regulation of PI3 kinase (PI3K) as measured by formation of pAKT *in vivo* and in numerous cell lines, including HL-1 cells, neonatal rat ventricular myocytes (NRVMs) and human iPSC-derived cardiomyocytes. NRG-1s also activate the MAP kinase (MAPK) pathway as measured by pERK1/2 formation. To better understand GGF2 action in the stressed heart, we have developed *in vitro* systems to study GGF2 protection of cardiomyocytes from doxorubicin induced toxicity. After serum starvation, cells were pre-incubated with GGF2 (0.15 pM to 9.5 nM) for 1 hr followed by exposure to 1 μM doxorubicin for 18 hrs. After doxorubicin treatment MTT labeling index of cultures was used as an endpoint measurement of cell metabolic status. Doxorubicin decreases MTT labeling index by 90% while GGF2 prevented this toxicity by almost 50% compared to untreated cells, with an EC_{50} of 83.3±19.4 pM . This effect appears to be dependent upon AKT signaling and not MAPK as assessed using inhibitors of both pathways. Inhibition of MAPK increases pAKT formation without changing extent of GGF2 protection. Blocking ErbB4 receptor, highly expressed in cardiomyocytes, causes a decrease in pAKT formation, suggesting that this receptor is important for mediating GGF2 action in these cells. Our results demonstrate that, in HL-1 cells, GGF2 mediates cytoprotective responses which require PI3K activation. Similar studies are being pursued in doxorubicin-treated NRVMs and human iPS derived cardiomyocytes. Initial results indicate GGF2 also mediates cytoprotective effects in these cell types. Future efforts will be aimed at elucidating potential roles for PI3K-dependent GGF2 regulation of mitochondrial function, Ca^{2+} signaling or REDOX regulation in the observed cytoprotective actions.

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Activation of Angiotensin II Type 2 Receptor Suppresses TNFα-induced ICAM-1 via NF-κB: Possible Role of ACE2

Liping Zhu, Oscar A. Carretero, Jiang Xu, Pamela Harding, Xiao-Ping Yang, Henry Ford Health System, Detroit, MI

Activation of the renin-angiotensin system (RAS) is a major factor contributing to the pathophysiology of cardiovascular disease (CVD). Blockade of the RAS with angiotensin receptor blockers (ARBs) has been a standard treatment for CVD. Activation of angiotensin II type 2 receptor (AT2) and angiotensin I-converting enzyme 2 (ACE2) contribute to the cardioprotective effects of ARBs. Both AT2 and ACE2 counteract the vasoconstrictor and pro-inflammatory effects of angiotensin II. However, the possible interaction between AT2 and ACE2 has never been established. Tumor necrosis factor (TNFα) is a cytokine involved in angiotensin II signaling and promotes the inflammatory response via NF-κB. We hypothesized that activation of AT2 increases ACE2, thereby preventing TNFα-stimulated intercellular adhesion molecule-1 (ICAM-1) expression via inhibition of NF-κB. Human coronary artery endothelial cells were pretreated with AT2 antagonist PD123319 or ACE2 inhibitor DX-600, and then stimulated with TNFα in the presence or absence of AT2 agonist CGP42112A. ACE2 mRNA was measured by real-time RT-PCR. ACE2 activity was measured using a Fluorimetric Kit. ICAM-1 and phospho-inhibitory κB (p-IκB) were measured by Western Blot. Activation of AT2 with CGP42112A increased ACE2 mRNA by 1.82 ± 0.09 fold ($p<0.01$) and ACE2 activity from 0.61 ± 0.05 to 0.95 ± 0.03 (pg/μl/h/μg protein) ($p<0.01$). This effect was diminished by inhibition of AT2 or ACE2. ICAM-1 expression was almost

undetectable in untreated cells but greatly increased by TNFα. Activation of AT2 reduced TNFα-induced ICAM-1 expression by 47% (from control value of 1 to 0.53 units) ± 5% ($p<0.01$), which was diminished by AT2 antagonist or ACE2 inhibitor. We also found that TNFα increased p-IκB by 7.46 ± 0.51 fold ($p<0.01$) compared to untreated cells and this effect was diminished by AT2 activation. Furthermore, ACE2 inhibitor blunted the effects of AT2 on TNFα-induced p-IκB expression. Our findings suggest that stimulation of AT2 reduces TNFα-induced ICAM-1 expression, which is partly through ACE2-mediated inhibition of NF-κB. Perspective: understanding the mechanisms underlying AT2-mediated anti-inflammatory effects could lead to new therapeutic strategies such as specific activation of AT2 and/or ACE2.

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Cardioprotection Of Recombinant Human Mg53 Protein In A Porcine Model Of Ischemia And Reperfusion Injury

Hua Zhu, The Ohio State Univ, Columbus, OH; Jianxun Liu, Yongqiu Zheng, Lei Li, China Acad of Chinese Medical Sciences, Beijing, China; Tao Tan, Ki Ho Park, The Ohio State Univ, Columbus, OH; Jincai Hou, China Acad of Chinese Medical Sciences, Beijing, China; Zhengguo Liu, Jianjie Ma, The Ohio State Univ, Columbus, OH

Rationale: Ischemic heart disease is a leading cause of death in human population and protection of myocardial infarction (MI) associated with ischemia-reperfusion (I/R) injury remains a challenge. MG53 is an essential component of the cell membrane repair machinery that protects injury to the heart.
Objective: We investigated the therapeutic value for using the recombinant human MG53 (rhMG53) protein for treatment of MI.
Methods and Results: Using Langendorff perfusion of isolated mouse heart, we found that I/R caused injury to cardiomyocytes and release of endogenous MG53 into the extracellular solution. The exogenous rhMG53 protein applied to the perfusion solution could concentrate at injury sites on the cardiomyocytes to facilitate cardioprotection. With rodent models of I/R-induced MI, we established the *in vivo* dosing range for rhMG53 in cardioprotection. Using a porcine model of angioplasty-induced MI, the cardioprotective effect of rhMG53 was evaluated. Intravenous administration of rhMG53, either prior to or post ischemia, reduced the infarct size and troponin I release in the porcine model when examined at 24 hrs post reperfusion. Biochemical study showed that the systemically delivered rhMG53 localized to the acute infarct zone of the porcine heart. Echocardiogram and histological analyses revealed that the protective effects for rhMG53 observed following acute MI led to long-term improvement in cardiac structure and function in the porcine model when examined at 4 weeks post operation.
Conclusions: Cardioprotection of rhMG53 in porcine model supports the concept that rhMG53 protein could have therapeutic value for treatment of MI in human patients with ischemic heart disease.

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Hydrogen sulfide donor, the Organic Polysulfide Diallyl Trisulfide Augments Ischemia-induced Angiogenesis by Upregulation of VEGF-Akt-eNOS Pathway

Ryo Hayashida, Kazuhisa Kondo, Satoshi Shintani, Toyoaki Murohara, Nagoya Univ, Nagoya city, Japan

Introduction: Hydrogen sulfide (H2S) upregulates anti-oxidant, anti-apoptosis, anti-inflammatory, and cell survival. H2S plays an extremely important role in the homeostasis of the cardiovascular system and in the pathogenesis of cardiovascular disease. The diallyl trisulfide (DATS) is a polysulfide constituent found in garlic oil, and known as H2S donor that is able to release H2S continuously. Peripheral artery disease (PAD) causes considerable morbidity and mortality. The aim of this study was investigate whether the DATS would augment ischemia-induced angiogenesis. Methods: The unilateral permanent femoral artery ligation was performed in C57BL/6J mice

(8-10 weeks old, n=8-10/group) and eNOS KO mice (8-10 weeks old, n=5-8/group), and vehicle or DATS (500 μg/kg/day) was injected intra-peritonealy up to 1 week following the hind limb ischemia (HLI). We evaluated the blood flow recovery using the laser Doppler perfusion image and capillary density stained with CD31 at 3 weeks following HLI. We assessed VEGF level by qPCR analysis and activations of eNOS and Akt by western blot analysis in ischemic tissues. Moreover, we cultured HUVECs in a hypoxic chamber with serum starvation for 24 hour, and then evaluated apoptosis by tunnel staining and oxidative stress by DHE staining with or without DATS (50 μmol). We also evaluated activations of eNOS and Akt in cultured HUVECs with or without DATS.
Results: DATS treatment significantly increased blood flow (0.45 vs 0.78,$p<0.05$) and capillary density (1.57 vs 1.85,$p<0.05$) at 3 weeks following HLI compared to vehicle. DATS also increased mRNA level of VEGF and activation of Akt and eNOS at 3 days following HLI. On the other hand, DATS treatment failed to increase blood flow and capillary density at 3 weeks following HLI in eNOS KO mice. The DATS treatment inhibited apoptosis and oxidative stress in cultured HUVECs via activation of Akt and eNOS pathway in vitro.
Conclusions: Our results indicate that DATS treatment could augment ischemia-induced angiogenesis by upregulation of VEGF-Akt-eNOS pathway that leads to inhibitions of apoptosis and oxidative stress. These results suggest that administration of H2S releasing agents might be efficacious for the treatment of PAD.

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ABSTRACTS

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Regional Variation in Intrinsic Mechanics of Arterial Vascular Smooth Muscle Cells in Spontaneously Hypertensive Rats

Jia-Jye Lee, Icahn Sch of Med at Mount Sinai, New york, NY; Lo Lai, New Jersey Medical Sch, Rutgers, The State Univ of New Jersey, Newark, NJ; Hongyu Qiu, Loma Linda Univ, Loma Linda, CA; Kevin D. Costa, Icahn Sch of Med at Mount Sinai, New york, NY

Aims. An increased aortic stiffness is a fundamental manifestation of hypertension (HT). Our previous study showed that intrinsic mechanical properties of aortic vascular smooth muscle cells (VSMC) are an important contributor to the increased large artery stiffness in HT. However, whether VSMC mechanics of smaller arteries is also altered in HT remains unknown. The goal of this study is to test our hypothesis that the VSMC elastic and adhesive properties vary along the arterial tree reflecting the regional heterogeneities of physiological loading and geometric properties of the artery wall.
Methods and Results. Primary VSMCs were isolated from the thoracic aorta (TA) and renal artery (RA) of adult spontaneously hypertensive rats (SHR) (16 weeks old, male) and age-matched Wistar-Kyoto normotensive (WKY) rats. Atomic force microscopy (AFM) was used to measure mechanical properties of individual VSMC at 37°C. Local apparent elastic modulus (Eap) was determined using Hertz contact analysis for a cone to model the indentation force curve, and maximum adhesion force (Fad) was obtained from the retraction force curve; results were shown as mean (±SD) (n=10 cells per condition) and compared using two way ANOVA. Eap of VSMCs from the TA was significantly higher in SHR (7.0± 1.3 kPa) vs. WKY (5.3 ± 1.5 kPa; $p < 0.001$) and Fad was significantly larger in SHR (39.9 7.2 pN) vs. WKY (30.6 10.9 pN; $p < 0.01$). No difference was found between SHR and WKY in VSMCs from renal artery in terms of Eap (4.5 0.8 kPa vs. 4.8 0.9 kPa; $p = 0.59$) and Fad (23.1 5.2 pN vs. 28.0 7.0 pN; $p = 0.17$). Although no difference was observed in cell shape represented by the cellular length:width ratio ($p > 0.25$), stiffness and adhesion of VSMC from TA were significantly higher vs. RA in SHR ($p<0.0001$) but not in WKY ($P>0.35$), indicating that the altered mechanics of VSMC in hypertension is more prominent in the large conduction vessel compared to the small artery.
Conclusion. Intrinsic stiffness and adhesion of isolated VSMC are elevated preferentially in the thoracic aorta of SHR rats; the regional variations may associate mechanistically with increased aortic tissue stiffness in hypertension.

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Uncovering the Mechanism and Function of Newly Discovered KCNQ1-Transporter Complexes

Daniel L Neverisky, Geoffrey W Abbott, UC Irvine, Irvine, CA

The KCNQ1 voltage-gated potassium channel is essential for human ventricular repolarization, permitting potassium efflux from excited cardiomyocytes to end each action potential and repolarize the heart. In cardiomyocytes, KCNQ1 is modulated by interaction with beta-subunits from the KCNE gene family, each of which significantly alters KCNQ1 channel function. KCNQ1 mutations are the most common identified genetic basis for Long QT syndrome (LQTS) and are also associated with lone atrial fibrillation (AF). The sodium-dependent myo-inositol transporter 1 (SMIT1) mediates cellular uptake of myo-inositol, an essential osmolyte that also represents an important substrate for phosphatidylinositol signaling pathways that regulate a plethora of ion channels including those essential for human cardiac function. We recently discovered that KCNQ1 can form heteromeric, co-regulatory complexes with Na+-coupled solute transporters including SMIT1, SMIT2 and glucose transporter SGLT1. These findings represent the first reported example of formation of an ion channel-solute transporter complex. Having discovered KCNQ1-SMIT1 complexes in mouse choroid plexus epithelium, we are currently investigating whether these types of complexes occur in the heart, how their function is altered by the various cardiac-expressed KCNE regulatory subunits or by arrhythmia-associated mutations, and which parts of KCNQ1 coordinate complex formation. Here, we present evidence of KCNQ1-SMIT1 co-assembly in pig heart based on co-immunoprecipitation experiments. Using KCNQ1-KCNQ4 chimeras we also begin to define which specific regions of KCNQ1 are required for complex formation with SMIT1. Finally, we present data showing the effects of SMIT1 on complexes formed by KCNQ1 and KCNE1, 2 and 3. KCNQ1-transporter complexes provide a potential hub for electrochemical crosstalk in normal cardiac function and in arrhythmogenesis.

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Studies of Cardiac transcriptome and Dilated Cardiomyopathy-causative genes in zebrafish

Yu-Huan Shih, Yuji Zhang, Tim Olson, Xiaolei Xu, MAYO CLINIC, Rochester, MN

Background*:* Rapid advance of genome technologies accelerate the discovery of genetic basis of cardiomyopathy and heart failure and also enable system biology studies to pinpoint underlying mechanism. However, the limited throughput of mammalian models restricted the number of genes that can be studied in a particular lab. Adult zebrafish has been recently pursued as a new model with higher throughput. However, as a non-mammalian model, its conservation is not tested. **Objective:** To assess the conservation of zebrafish for genetic studies of human dilated cardiomyopathy (DCM) via transcriptome analysis of 51 known DCM-causative genes. **Methods and Results:** By conducting RNA-sequencing (RNA-seq) analysis of larva and adult zebrafish, we identified genes with high expression level in the heart and fetal gene program using differential expression between embryonic and adult stages. We then searched zebrafish orthologues for 51 reported human DCM-causative or associated genes and identified zebrafish orthologues for 49 of them. While 30 genes have a single orthologue, 14 genes have two homologues and the remaining 5 genes have more than three. We then applied the transcriptome data to prioritize these homologues for the 19 DCM causative genes with more than one homologue. Based on the cardiac abundance and cardiac enrichment hypothesis, we are able to recommend a single zebrafish homologue of high priority for 12 out of 19 DCM genes, 2 zebrafish homologues of high priority for *ACTC1.* Interestingly, our expressional data suggested zebrafish othologues for human *MYH6* and *MYH7*, respectively. Similar to that in mammals, these two zebrafish othologues are oppositely expressed during zebrafish embryonic and adult stage.
Conclusions: Orthologues for the majority of DCM causative genes can be found in Zebrafish, supporting its usage as a conserved vertebrate model for studying DCM. The definition of cardiac transcriptome in zebrafish will facilitate the future system biology studies. This

vertebrate model with higher throughput can be further leveraged to validate the novel variants identified from human patients, to understand underlying signaling pathways and to develop novel therapeutics.

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Engineering Growth Factors For Cardiomyocyte Survival and Regeneration Following Ischemic Injury

Timothy R. Stowe, Shawdee Eshghi, Laura D. Jennings-Antipov, Yan Zhang, Phung Gip, Matthew D. Onsum, Silver Creek Pharmaceuticals, San Francisco, CA

Background: Despite the benefits of reperfusion therapy after myocardial infarction (MI), one-year mortality and morbidity rates remain high. Silver Creek Pharmaceuticals is engineering a new class of protein therapeutics, termed Smart Growth Factors (SGFs), designed to act directly on cardiomyocytes (CMs) to promote survival and regeneration after ischemia/reperfusion (I/R) injury. Our approach is to maximize pro-survival growth factor signaling in damaged cells by creating targeted molecules with optimized pharmacokinetics and dynamics.
Methods and Results: Computer model simulations guided the design of a panel of SGFs comprising various growth factors, targeting arms, half-life modulators, and linkers. SGF constructs were cloned and subsequently produced using a HEK 293F expression system. This panel was screened in vitro for signaling pathway activation and target affinity. The top hits were assayed for pro-survival activity in a chemical injury model in human CMs, where a targeted SGF significantly reduced caspase activation ($p<0.0084$; SGF-treated 3.82±0.64-fold, IGF1-treated 16.25±3.6-fold) Based on these data and additional simulations, a class of SGFs that utilized annexin V to target IGF1 to apoptotic cells via phosphatidylserine binding was selected for screening in animal models. In vivo, these SGFs extended pAKT signaling in heart tissue compared to wild-type IGF1 and had increased accumulation in the infarcted region of mouse hearts after MI. Preliminary data suggests that a single low intravenous dose (16 pmol) of SGF delivered one hour after LAD ligation could significantly reduce scar size in animals assessed at 12 weeks post MI ($p<0.05$; SGF-treated 9.3±1.3%, n=10; vehicle 18.1±3.13%, n=8). Finally, SGF treatment at the time of reperfusion in a rat I/R model was able to significantly reduce infarct size ($p<0.05$; SGF-treated 27.0±1.8% n=7; vehicle 33.6±1.92% n=8).
Conclusions: This work demonstrates an enhanced ability of SGFs in preventing apoptosis in vitro and reducing infarct sizes in animal models of MI. By taking a systems biology approach to SGF engineering, Silver Creek is developing cardioprotective therapies with the hope of improving patient health and addressing unmet medical need.

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Molecular Signature Of Cardiomyogenic c-kit-positive Bone Marrow Cells

Junghyun Kim, Fumihiro Sanada, João D Pereira, Anna Czarna, Brigham and Women's Hosp, Boston, MA; Jared Churko, Joseph C Wu, Stanford Univ, Stanford, CA; Marcello Rota, Piero Anversa, Annarosa Leri, Brigham and Women's Hosp, Boston, MA

Conflicting results have been reported concerning the ability of c-kit-positive bone marrow cells (c-kit-BMCs) to transdifferentiate into cardiomyocytes in vivo. We have raised the possibility that c-kit-BMCs may constitute a functionally heterogeneous pool, containing cells with different cardiomyogenic potential. To test this hypothesis, clonal analysis of individual c-kit-BMCs was introduced to track in vivo the progeny derived from a single founder cell. By employing viral gene-tagging, only a limited fraction of clonal c-kit-BMC was found to be able to generate cardiomyocytes in the acutely infarcted heart. On this basis, c-kit-BMC-derived

clones were classified as myogenic or non-myogenic. The gene profile of these two categories of cells was studied by RNA-sequencing to determine whether myogenic c-kit-BMCs had a distinct molecular signature. Genes that showed a significant ($p<0.05$) expression difference of at least 2-fold among cell groups were included in the analysis; 1243 genes were upregulated and 493 genes were downregulated in myogenic c-kit-BMCs when compared with non-myogenic c-kit-BMCs. An additional comparison involved freshly isolated c-kit-BMCs. 2512 genes were upregulated in myogenic c-kit-BMCs and 1844 genes were downregulated in this cell group. These results were subjected to functional annotation using gene ontology for the identification of molecular pathways showing different state of activation or repression in myogenic BMCs. The affected transcripts in myogenic c-kit-BMCs involved mostly signal transduction and muscle development including cardiac muscle. Similarly, a set of genes implicated in the regulation of angiogenesis were more represented in myogenic c-kit-BMCs, possibly reflecting the proficiency of this cell class to form vessels in the microenvironment of the infarcted heart. Our findings document that myogenic clonal c-kit-BMCs have a distinct molecular signature that differs from that of non-moygenic and non-expanded c-kit-BMCs. On this basis, c-kit-BMCs with high cardiomyogenic ability can be prospectively isolated and employed clinically for the treatment of patients with cardiac diseases.

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Sirt6 Heterozygosity Exacerbates Atherosclerosis in Apolipoprotein E Deficient Mice

De-Pei Liu, Zhu-Qin Zhang, Si-Cong Ren, Zuo-Zhi Li, Ying Tan, Xiao-Qiang Tang, De-Long Hao, Zhi-Chen Guo, Xiang Zhao, Hou-Zao Chen, Chinese Acad of Medical Sciences & Peking Union Medical Coll, Beijing, China

Sirt6 is a member of the class III histone deacetylase family and is reported to promote longevity. Whether Sirt6 is involved in atherosclerosis, one aging associated disease and the major cause of cardiovascular diseases, is unknown. We investigated effects of Sirt6 on atherosclerosis development. We found that in human atherosclerotic plaques, Sirt6 expression was decreased. Sirt6+/-ApoE-/- mice exhibited increased atherosclerosis development and decreased plaque stability than ApoE-/- mice. We found that Sirt6 downregulation showed increased expression of NKG2D ligands (H60b in mice and MICA/B in human). Sirt6 bound to promoters of these genes and regulated the H3K9 acetylation levels. Thus, atherosclerosis development was promoted by Sirt6 heterozygosity and epigenetic modification of NKG2D ligand expression is involved in this process.

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Intronic 27-nt Mirna Suppresses The Expression Of Endothelial Nitric Oxide Synthase Predominantly Through Nuclear Transcription Factor Ap-1

Patrick Liu, Limei Yan, Meimei Li, Hong Hao, Zhenguo Liu, Hesheng Ou, The Ohio State Univ Medical Ctr, Columbus, OH

The 27-base pair (bp) repeats in intron 4 were considered as the source of small RNA that suppressed endothelial nitric oxide synthase (eNOS) expression in endothelial cells. The present study was to investigate the role of polymorphic intronic 27-nucleotide (nt) miRNA and nuclear transcription factors (TFs) in eNOS expression in endothelial cells. The 27-nt or 54-nt miRNA or their mutants (with 3 mutations at 13, 14, and 15^{th} nt for 27-nt mutant and at $13,14,15,40,41,42^{th}$ nt for 54-nt mutant) were stably transfected and expressed in human aortic endothelial cells (HAECs). Overexpression of 27-nt miRNA and its duplexes and mutants significantly decreased eNOS mRNA level and protein content. Interestingly, more reduction in eNOS protein was observed in the cells overexpressing the mutant miRNAs than the cells overexpressing the wild-type ones for both 27-nt and 54-nt miRNAs. Western blotting showed that transcription factor Ap-1 expression

was substantially decreased in the cells that overexpressed either 27-nt or 54-nt miRNAs (both wild-type and mutants). Augmented inhibition of Ap-1 expression was observed in the cells overexpressing the mutant 27-nt miRNA as compared with the wild-type. However, Sp-1 protein expression was significantly suppressed only in the cells overexpressing 54-nt miRNA. Silencing Ap-1 abolished the polymorphic effect of miRNAs both in length and mutation on eNOS expression; while Sp-1 silencing only reversed the polymorphic effect of mutant miRNAs. These results suggested that overexpression of intron 4-based 27-nt miRNAs suppressed eNOS gene expression polymorphically through interactions predominantly with nuclear transcription factors Ap-1 in HAECs.

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miR-222 is Necessary for Exercise-induced Cardiac Growth and Protects Against Pathological Cardiac Remodeling

Xiaojun Liu, Cardiovascular Div of the Beth Israel Deaconess Medical Ctr and Harvard Medical Sch, Boston, MA; Junjie Xiao, Regeneration Lab and Experimental Ctr of Life Sciences, Shanghai Univ, Shanghai, China; Han Zhu, Xin Wei, Colin Platt, Federico Damilano, Chunyang Xiao, Cardiovascular Div of the Beth Israel Deaconess Medical Ctr and Harvard Medical Sch, Boston, MA; Vassilios Bezzerides, Cardiovascular Div of the Beth Israel Deaconess Medical Ctr; Cardiovascular Div of Boston Children's Hosp and Harvard Medical Sch, Boston, MA; Anthony Rosenzweig, Cardiovascular Div of the Beth Israel Deaconess Medical Ctr and Harvard Medical Sch, Boston, MA

Exercise induces cardiac growth, protects against adverse remodeling, and may also induce a regenerative response. Since microRNAs (miRNA) play important roles in cardiovascular disease, we investigated their role in the cardiac exercise response. We used the TaqMan rodent miRNA array to profile cardiac miRNA expressed at three weeks in two exercise models (swimming, running) compared to sedentary controls. Sixteen concordantly regulated miRNAs were identified and validated in both models, and examined for functional effects in neonatal rat ventricular cardiomyocytes (CMs). miR-222 was upregulated ~two-fold in both models and increased CM size (22%, $p<0.01$) and proliferation markers (EdU and Ki67, $p<0.01$). Bioinformatic and expression analyses identified four potential miR-222 targets (p27, Hipk1, Hipk2, and Hmbox-1) in CMs. These were confirmed as direct targets by luciferase assays, site-directed mutagenesis, and immunoblotting. siRNA knockdown (KD) of p27 or Hipk1 induced neonatal CM proliferation, while siRNA KD of Hmbox-1 increased CM size.
To examine miR-222's role in vivo, LNA-antimiR-222 was injected via tail vein or subcutaneously and shown to reduce cardiac miR-222 levels to 1.5% ($p<0.01$). Untreated animals subjected to three weeks of swimming had the expected increase in heart size (15% in HW/TL, $p<0.05$), CM size (26%, $p<0.05$), and markers of CM proliferation (Ki67 and pHH3, $p<0.05$). Increases in heart and CM size were unaffected by control LNA-antimiR but completely blocked by LNA-antimiR-222, while CM proliferation markers decreased (60%, $p<0.05$). To see if miR-222 is sufficient to mediate the benefits of exercise, we made transgenic mice with cardiac-specific, regulated expression of miR-222 (Tg-miR-222). Tg-miR-222 have normal heart size and function at baseline. After ischemia-reperfusion injury (IRI), Tg-miR-222 had similar initial 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 increased CM proliferation markers (63%, $p<0.05$).
Conclusion: Cardiac miR-222 is upregulated by exercise, necessary for exercise-induced cardiac growth, and protects against pathological cardiac remodeling after IRI.

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ABSTRACTS

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Selective Class I and II Histone Deacetylation Inhibitors Alter Stability of HDAC-Corepressor Complexes

Hsiao C Wang, Lillianne G. Harris, James C. Chou, Santhosh Mani, Donald Menick, Medical Univ of South Carolina, Charleston, SC

Introduction Alterations in expression and activity of different genes have been implicated in the pathogenesis of heart failure. Our lab has shown that HDAC-repressor complexes play a critical role in the upregulation Sodium Calcium Exchanger (*Ncx1)* and HDAC inhibition causes changes that attenuated cardiac remodeling during cardiac hypertrophy and heart failure. Thus, treatment with HDAC inhibitors has been proposed as a potential strategy for treatment of cardiac hypertrophy and heart failure. HDAC inhibitors repress deacetylase activity but we propose that they also affect HDAC confirmation and interaction with other protein factors. **We hypothesize that HDAC inhibitors affect the stability of the co-repressor complex with specific transcription factors and that this effect is dependent on the transcription factor.**

Results: Inhibition of HDACs in adult cardiomyocytes results in the greater stabilization of HDACs with co-repressor molecules that were recruited to the NCX1 promoter through Nkx2.5 transcription factor. HDAC class I specific inhibitor, MS 275 demonstrated stronger association between HDACs and co-repressors while other Class I inhibitors, PD106 and BML 210 failed on showing this phenomenal. The results suggested that class I HDACs inhibitors may affect formations of HDAC-complex via alternated active site interactions other than chelating with zinc binding domain. These results compliment ChIP experiments which also demonstrate the different recruitments of Sin3a at the proximal promoter of NCX1. *In vivo* analysis on HDAC5 knockout mice reveal that the Sin3a-HDAC1/2 repressor complex is not recruited to the *Ncx1* promoter in the absence of HDAC5, indicating not only Class I HDAC but also Class II HDACs play an important role on HDAC-complex formation.

Conclusions: This work gives insight into part of the molecular mechanism of how HDAC inhibitors can affect the stability of the HDAC co-repressor complex in cardiac hypertrophy and heart failure. In addition, we demonstrated the Class IIa HDACs are required for the recruitment of the Sin3a/HDAC1/2 co-repressor complex to specific transcription factors on the target promoter.

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Vascular Differentiation of C-kit+ Cardiac Progenitor Cells in Bioactive PEG Hydrogels

Srishti Bhutani, Kristin M French, Georgia Inst of Technology and Emory Univ, Atlanta, GA; Andres J Garcia, Georgia Inst of Technology, Atlanta, GA; Michael E Davis, Georgia Inst of Technology and Emory Univ, Atlanta, GA

Ischemic heart disease is the leading cause of death in the United States. The ideal therapy would include regeneration of functional myocardial cells as well as vascularization of the regenerated cardiac tissue. In this context, c-kit+ cardiac progenitor cells (CPCs) isolated from the heart are an exciting stem cell population as they have been shown to have potential to differentiate into cells of myocardial as well as vascular (endothelial, vascular smooth muscle) lineages. Being an autologous adult stem cell source, they provide advantages of alleviation of immune-rejection and disease transmission risks and are free from ethical concerns. Driving the vascular differentiation of CPCs can enable them to be used for angiogenic cell therapy. The objective of this project is to direct CPCs to the endothelial lineage via stimulation with VEGF immobilized in a PEG-maleimide (PEG-MAL) hydrogel scaffold for better retention of grafted cells. This hydrogel has protease-cleavable sites which should enable the hydrogel to be degraded while allowing for tube formation. CPCs are encapsulated in PEG-MAL hydrogel constructs presenting 100 ng VEGF/mL hydrogel. CPCs encapsulated in these PEG-MAL hydrogels maintain high viability for up to 14 days. In hydrogels presenting immobilized VEGF, successful biochemical stimulation of the encapsulated CPCs is evidenced by downstream ERK phosphorylation, likely through VEGFR2. These immobilized-VEGF treated CPCs also demonstrate greater RNA expression

of endothelial markers 7 days post-encapsulation. CPCs in VEGF presenting gels show a trend toward increasing formation of vascular structures in comparison to cells encapsulated in empty hydrogels. Together, this preliminary evidence suggests that c-kit+ cardiac progenitor cells can be driven toward the endothelial lineage when stimulated with immobilized VEGF, and may adapt a vascular phenotype. This system has the potential to be used for therapeutic angiogenesis as the PEG hydrogel scaffold should enable controlled delivery of the CPCs in vivo and is injectable and biodegradable.

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Cardiac TIMP-4 Dictates cardiomyocyte contractility and differentiation of embryonic stem cells into cardiomyocytes: Road to Therapy

Pankaj Chaturvedi, Anuradha Kalani, Anastasia Familtseva, Pradip Kumar Kamat, Naira Metriveli, Suresh C Tyagi, Univ of Louisville, Louisville, KY

The remarkable nature of cardiomyocytes for contractility is attributed to the extracellular matrix which is maintained by the balance between MMPs (Matrix Metalloproteases) and TIMPs (Tissue Inhibitors of Matrix Metalloproteases). Any deviation from this delicate balance of MMP/TIMP is a hallmark of cardiovascular pathologies including myocardial infarction (MI). TIMP4, which is the least studied molecule, is deficient in failing hearts and mice lacking TIMP4 show poor regeneration capacity after MI. Therefore, we hypothesize that TIMP4 helps in cardiac regeneration by alleviating contractility and inducing the differentiation of cardiac progenitor cells into cardiomyocytes. To validate this hypothesis, we transfected cardiomyocytes with TIMP4 and TIMP4-siRNA and observed that there was increase in contractility in the TIMP4 transfected cardiomyocytes as compared to siRNA-TIMP4 transfected cardiomyocytes. To explain this we looked into the calcium channel genes and found increase in the expression of serca2a (sarcoplasmic reticulum calcium ATPase2a) in the TIMP4 transformed myocytes. Serca2a is tightly regulated by mir122a and we found decrease in the expression of mir122a in the TIMP4 transfected cells as compared to the TIMP4-siRNA cells. To observe the effect of TIMP4 in differentiation of cardiac progenitor cells we treated mouse embryonic stem cells with cardiac extract and cardiac extract minus TIMP4 (using TIMP4 monoclonal antibody). The cells treated with cardiac extract showed cardiac phenotype in terms of Ckit+, GATA4+ and Nkx2.5 expression. This is a novel report on the influence of TIMP4 on contractility and inducing the differentiation of stem cells to cardiomyocytes. In view of the failure of MMP9 inhibitors for cardiac therapy in clinical trials, TIMP4 provides and alternative approach, being an indigenous molecule, a natural inhibitor of MMP9 and efficient ROS scavenger.

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Subcutaneous and Visceral Adipose-Derived Stem Cells Have Similar Biological Properties and Both Improve Cardiac Function of Infarcted Rat Hearts

Chao Chi, Dept of Cardiac Surgery, The First Affiliated Hosp of Harbin Medical Univ, Harbin, China; Bo Xiang, Jixian Deng, Fei Wang, Canada Natl Res Council, Winnipeg, MB, Canada; Kanmani Natarajan, Dept of Physics and Astronomy, Univ of Manitoba, Winnipeg, MB, Canada; Hung-Yu Lin, Canada Natl Res Council, Winnipeg, MB, Canada; Hongyu Liu, Dept of Cardiac Surgery, The First Affiliated Hosp of Harbin Medical Univ, Harbin, China; Francis Lin, Dept of Physics and Astronomy, Univ of Manitoba, Winnipeg, MB, Canada; Darren H. Freed, Univ of Alberta, Edmonton, AB, Canada; Rakesh C. Arora, St. Boniface Res Ctr, Univ of Manitoba, Winnipeg, MB, Canada; Ganghong Tian, Canada Natl Res Council, Winnipeg, MB, Canada

Background: Adipose stem cells (ASC) from subcutaneous and visceral adipose tissues have been studied individually. However, it is unclear whether ASC from the two sources have different biological properties and, more importantly, whether one sub-type of ASC is more effective in treatment of CHF. This study

was designed to address these concerns. Methods: Morphology, yield, proliferation, surface markers, and cytokine secretion of rat subcutaneous ASC (S-ASC) and visceral ASC (V-ASC) were analyzed. A rat model of myocardial infarction (MI) was established by occlusion of the LAD. 7 days after MI, S-ASC (n = 11), V-ASC (n = 11), and cell culture medium (Control, n = 7) were injected into the infarct rim, respectively. Cardiac function of the infarcted hearts was monitored with MRI for 6 months. Results: Both S-ASC and V-ASC exhibited a fibroblast-like morphology and expressed stromal cell markers (CD29, CD90 and CD105). No significant expression of hematopoietic markers (CD11b, CD34 and CD45) was found. Under appropriate conditions, both cells could differentiate to adipocyte- and osteocyte-like cells. Both of them expressed a significant level of HGF, IGF-1 and VEGF. As to their differences, V-ASC had approximately 3-times greater cell yield and a lower colony-formation rate (9.8±1.0% vs.13.5±2.6%) relative to S-ASC. In contrast, S-ASC showed a significantly greater growth rate (Doubling Time: 17.9 h vs. 26.0 h) relative to V-ASC. Both S-ASC and V-ASC-treated hearts showed a significantly greater left ventricular ejection fraction (LVEF, 58.3% and 56.7%) than the control group (LVEF, 47.2%) at end of 6 months. LVEF between the two ASC-treated groups was not significantly different. Finally, the implanted stem cells were readily detected in vivo with MRI for at least 6 months. Myocardial tissue sections showed existence of ASC and their locations matched with MRI signals. Conclusions: S-ASC and V-ASC share several biological characteristics. Both provide comparable significant improvement on cardiac function. Moreover, these implanted cells can be reliably tracked for at least 6 months using MRI. We conclude that the S-ASC and V-ASC are equally effective for treatment of heart failure.

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Profilin-1 Phosphorylation Regulates Post-ischemic Cardiac Repair By Induction Of Vascular Stem Cell Homing

Yi Fan, Univ of Pennsylvania, Philadelphia, PA

Neovascularization, the formation of new blood vessels, is fundamental to cardiac repair and regeneration in ischemic heart disease. After myocardial infarction (MI), vascular stem cells (VSC) are mobilized from bone marrow and recruited to the ischemic site, de novo generating new blood vessels to promote cardiac recovery. Our previous studies have revealed that phosphorylation of profilin-1 (Pfn-1) induces endothelial cell migration and sprouting angiogenesis. Here, we show that Pfn-1 phosphorylation regulates VSC homing to ischemic site and cardiac repair after MI through protease expression. Vascular lineage-specific knock-in of phosphorylation-dead Pfn-1(Y129F) mutant in mice show that Pfn-1 phosphorylation is critical for ischemia-induced neovascularization and cardiac function recovery after MI. Deficiency in Pfn-1 phosphorylation inhibits VSC homing to the ischemic hindlimb, suggesting a critical role of Pfn-1 phosphorylation in VSC recruitment. Mechanistic studies show that Pfn-1 phosphorylation is required for vascular endothelial growth factor (VEGF)-A-induced expression of metalloproteinase (MMP) -2 and -9 and cell migration in VSC. Therefore, these studies identify a critical role of Pfn-1 phosphorylation in VSC homing and neovascularization after MI, and suggest that Pfn-1 phosphorylation may represent as a therapeutic target for treating ischemic heart disease.

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Cytoprotective Effect of Growth Hormone Releasing Hormone Agonist in Cardiac Stem Cells

Victoria Florea, Sonia S Majid, Rosemeire M Kanashiro-Takeuchi, Norman L Block, Andrew V Schally, Joshua M Hare, Claudia O Rodrigues, Univ of Miami, Miami, FL

Background: Our group has previously shown that growth hormone releasing hormone receptor agonists (GHRHR-A) improve cardiac performance in heart failure models and reverse remodeling. This effect was associated with an

increase in the number of c-kit+ cardiac stem cells (CSCs), suggesting that this agonist might have an effect on these cells. **Methods and Results:** We investigated the expression of GHRH receptor (GHRHR) in CSCs of different species by flow cytometry analysis. GHRH-R is expressed in 96-98% of CSCs isolated from mouse, rat and porcine. Results were compared to GHRHR expression in HeLa and MCF7, and T47D cell lines, positive and negative controls, respectively. To determine if GHRHR activation can improve CSCs self-renewal, we tested the effect of agonists on porcine CSCs proliferation. The rate of cell division was increased 2-fold with JI38 (GHRHR-A) treatment (3.4 ± 0.7) vs. vehicle control (1.7 ± 0.2) ($p<0.05$). Pre-treatment of CSCs with the GHRHR antagonist MIA-602, showed a trend toward reversal of the JI38 agonistic effect on proliferation rate (2.2 ± 0.6). These studies were further extended to other GHRHR agonists. In addition to JI38, MR356 and MR409, both of which showed significant increase in CSCs proliferation relative to vehicle control, by 20 ± 5.7%, 37 ± 8.5% and 36 ± 12.2%, respectively ($p<0.05$). The protective effect of JI38 on porcine CSCs survival was determined under oxidative stress generated by hydrogen peroxide exposure. Pre-treatment of CSCs with JI38 prior to peroxide exposure significantly reduced cell death by 33 ± 2.2% ($p<0.02$). Similar effects were observed for MR356, which decreased cell death by 12 ± 8.6% ($p<0.03$). Furthermore, we found that the effect of GHRHR-A on CSCs proliferation was completely reversed by inhibitors of the ERK, PI3K and Akt pathways ($p<0.05$). **Conclusion:** These findings confirm for the first time the expression of GHRHR in CSCs. GHRHR-A promotes CSCs proliferation and enhance survival. GHRHR-A effects on CSCs proliferation are mediated through activation of ERK, PI3K and AKT pathways. Accordingly, activation of GHRHR signaling pathways represents a novel therapeutic approach to protect and stimulate endogenous CSC population, promoting cardiac repair.

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Greater Southeast Affiliate (Alabama, Florida, Georgia, Louisiana, Mississippi, Puerto Rico & Tennessee)

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Plasminogen Augments Cxcl12/cxcr4-regulated Stem Cell Homing And Contributes To Cardiac Repair After Myocardial Infarction.

Yanqing Gong, Perelman Sch of Med at The Univ of Pennsylvania, Philadelphia, PA; Yujing Zhao, Dept of Molecular Cardiology, Cleveland, OH; Yi Fan, Perelman Sch of Med at The Univ of Pennsylvania, Philadelphia, PA; Jane Hoover-Plow, Dept of Molecular Cardiology, Cleveland, OH

Myocardial infarction (MI) is a leading cause of morbidity and mortality worldwide. Bone marrow (BM)-derived stem cells promote tissue repair and regeneration after MI. Thrombolytic treatment with plasminogen (Plg) activators significantly improves the clinical outcome in MI by restoration of cardiac perfusion. In addition to its canonical function, Plg is critical for cardiac repair, wound healing and liver injury, however, the mechanism for Plg-regulated tissue repair remains unclear. Here, we show a novel role of Plg in stem cell-mediated neovascularization and cardiac repair after MI. Our data show that Granulocyte colony-stimulating factor (G-CSF), a stem cell mobilizer, significantly increased neovascularization and decreased infarct size in the infarct area, and improved ejection fraction and LV internal diameter by echocardiogram in wild-type mice. No improvement in tissue repair and heart function was observed in Plg deficient (Plg-/-) mice indicating that Plg is required for stem cell-regulated cardiac repair after MI. In vivo tracking of GFP-expressing BM cells after BM transplantation revealed that in Plg-/- mice, recruitment of BM-derived stem cells (GFP+c-kit+ cells) to the infarcted heart and stem cell-derived vessels and arteries are dramatically decreased (by 11 fold) suggesting that Plg may regulate stem cell homing to the lesion sites and subsequently contribute stem cell-mediated tissue regeneration. Mechanistic studies show that Plg up-regulated CXCR4 expression on stem cell in vivo and in vitro, suggesting Plg may promotes stem cell homing by induction of CXCR4 expression in stem cells. Stem cell

migration was enhanced by endogenous Plg in vitro, however, AMD3100, a CXCR4 antagonist, significantly inhibited Plg-regulated stem cell migration. Furthermore, lentiviral reconstitution of CXCR4 expression in BM cells rescued stem cell homing to the infarcted heart in Plg-/- mice, indicating that Plg mediates stem cell homing through regulating CXCR4 expression. These findings identified a novel role of Plg in cardiac repair by promoting stem cell homing to the injured heart after MI. Thus, targeting Plg may offer a new therapeutic strategy for strengthening stem cell-mediated cardiac repair and regeneration after MI.

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Translocation Of Myomirs Via Gap Junction Channels Prevents Cancer Cell Growth

Laura Graciotti, Toru Hosoda, Marcello Rota, Giulia Borghetti, Sergio Signore, Piero Anversa, Annarosa Leri, BWH, Dept of Anesthesia and Regenerative Med, Boston, MA

The adult heart is resistant to cancer formation and the metastatic invasion of distant neoplasms. This biological advantage may be dictated by the molecular properties of myocytes that constitutes 90% of the myocardium. We raised the possibility that microRNAs (miRs) highly expressed in myocytes (myomirs) may translocate via gap junctions to neighboring cancer cells, preventing their growth or inhibiting their survival. First, we established whether overexpression of myomirs interferes with the proliferation and death of MCF7 human breast cancer cells. Infection of MCF7 with lentiviruses carrying miR-1, miR-133a and miR-499 (miR-MCF7) resulted in a 5-fold decrease in Ki67 labeling and a 20% increase in the fraction of cells arrested at G0/G1. In contrast, TdT-positive apoptotic cells averaged 0.5% and did not differ in miR-MCF7 and control cells. To mimic the in vivo condition, EGFP-labeled MCF7 were co-cultured with myocytes and, 4 days later, the expression of myomirs was measured in FACS-sorted MCF7. With respect to baseline, co-cultured MCF7 showed 100-fold, 16-fold, and 27-fold increase in the expression of miR-1, miR-133a and miR-499, respectively. Co-culture of myocytes and MCF7 led to the formation of gap junctions made of connexin 43 (Cx43) and connexin 45 (Cx45). Silencing of Cx43 and Cx45 decreased significantly the expression of myomirs in co-cultured MCF7. Importantly, proximity of MCF7 to myocytes reduced markedly the growth rate of the cancer cells. Subsequently, 1 x 106 MCF7 or miR-MCF7 were injected subcutaneously in NOD-scid mice. At 5 weeks, the tumors developed from miR-MCF7 were 70% smaller than those originated from control MCF7. Two doses of breast cancer cells were injected intramyocardially to establish their in situ tumorigenic effects. Tumor formation was found in all hearts that received 1 x 106 MCF7. Conversely, mice injected with 1 x 105 cells did not show macroscopic evidence of neoplastic lesions. The lack of tumor development in the latter case is consistent with the ability of the heart to prevent neoplasm development when cancer cell colonization is not massive. Our findings document that miR-1, miR-133a and miR-499 translocate from myocytes to cancer cells via gap junctions, inhibiting tumor growth in vitro and in vivo.

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Heparanase-overexpressed Mesenchymal Stem Cells Enhances Angiogenesis Via Hypoxia-inducible Factor-2α/Vascular Endothelial Growth Factor Receptor-2 Pathway

Xin Yang Hu, Ling Zhang, Jing Jin, Huiqiang Cheng, Han Chen, Jian-an Wang, Second Affiliated Hosp, Zhejiang Univ Coll of Med, Hangzhou, China

Background: Heparanase is an endoglycosidase involved in the cleavage of the heparin sulfate and plays important role in tumor metastasis and angiogenesis. Here we showed heparanase – overexpressed mesenchymal stem cells (MSCs) promoted angiogenesis in rat model after femoral artery ligation, and its underlying mechanism was via endothelial cells migration by Hypoxia-Inducible Factor-2α (HIF-2α) / vascular endothelial growth factor receptor-2 (Flk-1) pathway and collateral formation.

Methods: MSCs were transduced with heparanase ($MSC^{heparanase}$), control cells were either nontransduced (MSC^{WT}) or transduced with empty vector (MSC^{Null}). The effect of human umbilical vein endothelial cells (HUVECs) migration, tube formation and the role for heparanase were determined by transwell, matrigel and arterial rings asaay after co-culture with $MSC^{heparanase}$, MSC^{Null} and MSC^{WT} conditioned medium. Moreover, right femoral artery was occluded in rats receiving PBS or MSC^{Null} or $MSC^{heparanase}$, blood flow recovery was assessed by laser-Doppler imaging (LDI) before surgery, immediately after operation, as well as 3, 7 and 14 days after occlusion, capillary density was measured at 14 days after occlusion.
Results: *In vivo*, the group receiving $MSC^{heparanase}$ had a fast recovery of blood flow at 7 days after occlusion and more capillary densities at 14 days after occlusion. *In vitro,* HUVECs treated with $MSC^{heparanase}$ conditioned medium exhibited increased formation of capillary-like structures and promoted migration, compared with HUVECs treated with MSC^{Null} conditioned medium. All effects of $MSC^{heparanase}$ conditioned medium were abolished by knockdown of HIF-2α and flk-1 with a selective shRNA and were absent in HUVECs. Mechanism studies revealed that heparanase increased the cellular accumulation of HIF-2α protein and affects its nuclear translocation, which regulates flk-1 and its downstream proteins: p38 and HSP27 expression.
Conclusions: These findings identify heparanse as a key regulator in MSCs induced angiogenesis and collateral formation. Heparanse up-regulates HIF-2α and mediates Flk-1/p38/HSP27 pathway, which is necessary for angiogenesis and collateral artery network.

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Comparison Of Transplantation Of Bone Marrow-derived Stem Cells, Adipose-derived Stem Cells And Endometrium- Derived Stem Cells In The Infarcted Heart

Xin Yang Hu, Kan Wang, Jian-an Wang, Second Affiliated Hosp, Zhejiang Univ Coll of Med, Hangzhou, China

Background: A variety of adult stem cells have been transplanted into the infarcted heart to cure myocardial infarction(MI), however, comparative studies are lacking to show more suitable source of cells for transplantation. Mesenchymal stem cells hold promise for myocardial regeneration therapy. Derivation of these cells from the endometrium tissue might be easier compared to bone marrow and adipose tissue. However,the in vivo fate of endometrium stem cells (EnSCs) in the infarcted heart has never been compared directly to mesenchymal cells derived from bone marrow(BMMSCs) and adipose tissue(AdMSCs).
Methods: EnSCs, AdMSCs and BMMSCs were isolated from healthy donors were characterized using flow cytometry for surface markers identification and microscopy for cell morphology. They were characterized with β-actin promoter driving firefly luciferase and green fluorescent protein (Fluc-GFP) double fusion reporter gene, and were characterized using flow cytometry, bioluminescence imaging (BLI) and luminometry. Cell proliferation was tested by CCK-8 kit, colony forming unit(CFU) was stained by crystal violet staining and apoptosis ratio were detected by TUNEL assay. Rat (n=8/group) underwent myocardial infarction followed by intramyocardial injection of 5×105 EnSCs, AdMSCs and BMMSCs, or saline (negative control). Cell survival was measured using BLI for 6 weeks and cardiac function was monitored by echocardiography and hemodynamics analysis. Ventricular morphology was assessed using histology.
Results: EnSCs, AdMSCs and BMMSCs were CD29+, CD90+, CD105+, shared similar morphology, but EnSCs had best proliferation, colony-forming and anti-apoptosis activity of 3 types of MSCs. Cells expressed Fluc reporter genes in a number-dependent fashion, as confirmed by luminometry. After cardiac transplantation, transplantation of EnSCs was better capable of preserving ventricular function and dimensions than others, as confirmed by echo test, PV-loops and histology.
Conclusions: This is the first study comparing the in in vitro and in vivo behavior of 3 types of MSCs in the infarcted heart. AdMSCs and BMMSCs do not tolerate well in the cardiac environment, resulting in more cell death andworse cardiac function than EnSCs groups.

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Mir-211 Regulates Bone Marrow Mesenchymal Stem Cells Migration Through Stat5a

Xin Yang Hu, Panpan Chen, Yan Wu, Wei Zhu, Jian-an Wang, Second Affiliated Hosp, Zhejiang Univ Coll of Med, Hangzhou, China

Background: Efficacy of intravenous mesenchymal stem cells (MSCs) administration for myocardial infarction (MI) is limited by low cell migration to the damaged myocardium. Our previous study demostrated that migration ability of MSCs enhanced by hypoxia preconditioning (HPC). miRNA microarray displayed that miR-211 exhibited most significant change between HPC and normoxia cultured MSCs. The aim of this study is to study whether and how miR-211 regulate MSCs migration.
Methods: In vitro, transwell assay were used to assess the migration ability of MSC moduated by miR-211 using overexpressing and knockdown lentivirus. The target gene of miR-211 predicted by Targetscan were verified by PCR, western blot and lucifurase assay. Chromatin immunoprecitation (ChiP) were used to explore the transcription factors that regulate the expression of miR-211. To evaluate the effect of miR-211 on MSCs migration in vivo, miR-211-mimic and miR-211-shRNA male MSCs were intravenously delivered 24h after MI, the engraft cells were detected by RT-PCR of SRY gene.
Results: Quatitative RT-PCR showed that miR-211 expression of MSCs upregulated by HPC. MiR-211 mimic improved MSCs migration by 31.03% ($p<0.05$), however, knockdown miR-211 using shRNA attenuated MSCs migration ability significantly. Signal transducer and activator of transcription 5A (STAT5A) was predicted as one of miR-211 target genes, PCR and Western blot showed miR-211 overexpression dramatically decreased STAT5A expression, while miR-211 knockdown upregulated STAT5A. The luciferase assay showed the similar results. Transwell assay showed that STAT5A knockdown reverse the inhibition of MSCs migration induced by miR-211-shRNA. Intrestingly, ChiP assay showed that STAT5A can combine to the promoter of miR-211, which lead to the regulation of miR-211 transcription. In vivo data showed that MiR-211 overexpression enhanced MSCs homing to ischemic myocardium, and miR-211 overexprssing MSCs improved cardiac function 28days post-MI. However, miR-211 knockdown decreased MSCs homing and hampered cardiac function recovery.
Conclusions: These results indicate that miR-211 has important role in regulating MSCs migration through targeting STAT5A, meanwhile STAT5A regulated miR-211 transcription.

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Pericardial Grafting Of Cardiac Progenitor Cells In The Three-dimensional Thick Scaffold Improves Cardiac Function After Myocardial Infarction In Mice.

Naomichi Kondo, Dept of Cardiovascular Science and Med, Chiba Univ Graduate Sch of Med, Chiba, Japan; Toshio Nagai, Mei-Lan Liu, Toshinao Takahashi, Masato Kanda, Dept of Cardiovascular Science and Med, Chiba Univ Graduate Sch of Med, chiba, Japan; Katsuhisa Matsuura, Dept of Cardiology and Inst of Advanced Biomedical Engineering and Science, Tokyo, Japan; Issei Komuro, Dept of Cardiovascular Med, The Univ of Tokyo Graduate Sch of Med, Tokyo, Japan; Yoshio Kobayashi, Dept of Cardiovascular Science and Med, Chiba Univ Graduate Sch of Med, Chiba, Japan

Cardiac progenitor cell (CPC) therapy for heart disease has been examined enthusiastically. However, optimal scaffolds which maintain the transplanted cells are still elusive. We used clonally expanded stem cell antigen 1-positive CPCs from adult mice and produced a three-dimensional thick scaffold (CPC-scaffold), in which CPCs were cultivated up to 2 month with self-assembling peptide RADA16-I. Addition of designer self-assembling peptide containing the active motifs of 2-unit RGD binding sequence and IGF-1 promoted three-dimensional spreading and viability of CPCs. After making myocardial infarction (MI) with left coronary artery ligation in mice, we transplanted CPC-scaffold on the surface of infarction area and confined it inside of the pericardial space by closing parietal pericardium. Four weeks after transplantation, echocardiography showed that FS of treatment group (16±10%, n=17) was higher than that of control (MI only) group (10±6.8%, n=19) ($P<0.05$) and that LVDd of treatment group (5.4±1.0mm, n=17) was smaller than that of control group (6.2±1.1mm, n=19)

(P<0.05). Infarction area was significantly decreased in treatment group (46±21%, n=17), compared to control group (59±19%, n=18) (P<0.05). Immunohistochemical staining for von-Willebrand factor (vWF) showed that the number of vWF-positive capillaries per mm2 in treatment group (16.8±3.2, n=5) was higher than that of control group(8.9±3.4, n=5) (P<0.05). There were many of CD31-positive capillaries with or without α-smooth muscle cell actin-expressing perivascular cells in the graft area. By using fluorescent-conjugated avidin, biotin-labeled scaffold was globally detected in the graft area 1 week after transplantation, but sparsely 4weeks after, suggesting that the transplanted scaffold was biodegradable. To examine whether transplanted CPCs remain in the scaffold, we labeled CPCs with red fluorescence protein (RFP). RFP+CPCs were observed in the graft area 4 weeks after transplantation of RFP+CPC-scaffold. FISH analysis showed that sex-mismatched CPCs were globally detected in the graft area on the surface of the heart. Therefore pericardial grafting of well-vascularized and cellularized CPC-scaffold was a useful method to improve cardiac function after MI.

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Neuregulin Administration Stimulates Cardiomyocyte Proliferation In Neonatal Mice And In Myocardium From Human Infants

Brian Polizzotti PhD, Balakrishnan Ganapathy, Children's Hosp Boston, Boston, MA; Stuart Walsh, Bayer Healthcare, Wuppertal, Germany; Sangita Choudhury, Children's Hosp Boston, Boston, MA; David Bennett, Beth Israel Deaconess Medical Ctr, Boston, MA; Bernhard Haubner, Joseph Penninger, Inst of Molecular Biotechnology of the Austrian Acad of Sciences, Vienna, Austria; **Bernhard Kuhn**, Children's Hosp Boston, Boston, MA

Background:
Regenerative strategies have the potential to transform heart failure therapies. Cell transplantation strategies have shown effectiveness and safety in clinical trials. A complementary strategy is based on directly stimulating the proliferation of endogenous cardiomyocytes. We have demonstrated that administration of the growth factor neuregulin (rNRG1), which has passed phase 2 testing, stimulates cardiomyocyte proliferation and improves myocardial repair in adult animal models. Since cardiomyocyte proliferation is active during myocardial growth, we hypothesized that NRG1 may be more effective in growing animals and humans.
Methods:
We developed a myocardial injury model (cryoinjury) in neonatal mice that recapitulates scar formation seen in infants with congenital heart disease (CHD). In contrast with ligation of the left anterior descending coronary artery (LAD), cryoinjury reduced cardiomyocyte cell cycle activity. We used this model to evaluate the effectiveness of rNRG1 administration. To assess the ability of rNRG1 to stimulate human cardiomyocyte proliferation, we developed an organotypic culture system for myocardium from infants with CHD.
Results:
Administration of NRG1 resulted in a 2-fold increase in cardiomyocyte cell cycle activity at 10 days post injury (dpi). After neonatal cryoinjury in mice, administration of rNRG1 from birth to 30 days of life significantly improved the ejection fraction, which was sustained for 30 days after the cessation of rNRG1 administration. In contrast, administration of NRG1 four to 34 days after cryoinjury transiently reduced scar formation, and transiently improved myocardial structure and function. In myocardium from infants with Tetralogy of Fallot (ToF) less than 6 months of age (n = 6), rNRG1 induced cardiomyocyte proliferation.
Conclusions:
These results indicate that stimulating cardiomyocyte proliferation may be clinically effective in human infants with heart disease, a population in whom a significant and unmet need exists.

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ABSTRACTS

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Generation of a Mouse ESC Reporter Line to Identify Purkinje-like Conduction System Cells

Karen Maass, Jia Lu, Fiona See, Guoxin Kang, Camila Delgado, Glenn I Fishman, NYU Sch of Med, NYC, NY

We have previously demonstrated that contactin2 (Cntn2) protein is enriched in Purkinje cells of the adult cardiac conduction system (CCS). Here we present a mouse embryonic stem cell (mESC) reporter line to identify Purkinje-like cardiomyocytes in vitro. Methods and Results: Dual reporter Purkinje cell mESC were generated from Cntn2-eGFP blastocysts, transduced with αMHC-mCherry lentivirus. The mESC expressed markers of pluripotency (Oct3/4; Sox2, Nanog) and differentiated into all three germ layers (Nestin, a-fetoprotein, Brachyury). Cultures differentiated under serum-free, cardiogenic conditions were enriched in cardiomyocytes (n=5), consisting of single positive aMHC-mCherry [57.76±3.7%] and double positive αMHC-mCherry/Cntn2-eGFP cells [1.9±0.9%]. Compared with eGFP negative cardiomyocytes (n=15), double positive cells (n=11) revealed electrophysiological characteristics consistent with Purkinje-like derivatives, including increased peak sodium currents (180.3±20.5 pA/pF vs. =-118.0±21.2pA/pF), faster upstroke velocities (133.7±12.3 V/s vs.60.0±13.2V/s) and elongated action potential duration (APD90=170.2±17.5ms vs. APD90=120.6±17.3ms). Calcium imaging demonstrated chronotrop regulation and spontaneous Ca2+ oscillations in mCherry+eGFP+ cells. Immunofluorecence analysis demonstrated endogenous expression of Purkinje cell markers, including Cntn2, Cx40 and Troponin-T, in double positive cells. Quantitative real-time PCR analysis of double positive cells verified cardiomyocyte-specific transcript expression [Mlc2v: 3.8x106-fold ($p<0.05$); Nkx2.5: 178-fold ($p<0.05$) compared to double negative cells]. Moreover, double positive cells expressed significantly elevated levels of CCS-specific transcripts compared to mCherry-single positive cells [Cntn2: 31-fold ($p<0.001$); Cx40: 878 -fold ($p<0.01$); Cav3.1: 11-fold ($p<0.05$); HCN4: 7-fold ($p<0.001$)]. Conclusion: The dual reporter Purkinje cell mESC reporter line permits identification and enrichment of ventricular CCS derivatives.

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The Presence of Coronary Artery Disease Influences Number and Function of Stem Cells in the Bone Marrow

Evelien Nollet, Univ of Antwerp, Edegem, Belgium; Dina De Bock, Inez R Rodrigus, Antwerp Univ Hosp, Edegem, Belgium; Vicky Y Hoymans, Univ of Antwerp, Edegem, Belgium; Christiaan J Vrints, Emeline M Van Craenenbroeck, Antwerp Univ Hosp, Edegem, Belgium

Purpose. Despite the observed therapeutic benefits of autologous bone marrow (BM)-derived stem cell transplantation in patients with ischemic heart disease, the efficacy of this approach could be hampered by BM dysfunction. We investigated whether BM cellularity and function is affected by coronary artery disease (CAD).
Methods & Results. BM samples were obtained peri-operatively from 26 CAD patients, undergoing coronary artery bypass surgery (LVEF 54±16%), and 6 controls, undergoing mitral valve surgery (LVEF 50±12%; age 59±10yrs). CAD patients were stratified according to their Syntax score (mild ≤15, age 61±10yrs; and moderate CAD >15, age 63±8yrs; stratification based on median score), which is used to assess complexity of coronary lesions. In vitro functional analysis of isolated BM-derived mononuclear cells (BM-MNC) revealed a significant impairment of migratory capacity towards SDF-1α and VEGF in patients with moderate CAD (25.71±7.3%) compared to controls (33.82±8.3%; p=0.042) and patients with mild CAD (34.76±7.8%; p=0.007). Hematopoetic stem cells (HSC, CD45dimCD34+SSClow) were reduced in patients with moderate CAD (8178±5530 HSC/106 BM-MNC; p=0.014) and mild CAD (10655±5489 HSC/106 BM-MNC; p=0.054) compared to controls (16220±6126 HSC/106 BM-MNC). An inverse correlation was found between age and the number of granulocyte-macrophage colony forming units (r= -0.408;

p=0.048), burst forming units erythroid (r= -0.458; p=0.028) and HSC (r=-0.356; p=0.046). Furthermore, our data revealed a relation between reduced renal function (CKD-EPI eGFR, 81.2±19 ml/min) and reduced number of HSC (r=0.480; p=0.011) and endothelial progenitor cells (EPC, CD45dimCD34+KDR+; r=0.522; p=0.008).
Conclusions. Migratory capacity of BM-MNC and the number of HSC are reduced in patients with CAD, which is more pronounced in more complex CAD. In addition, age and renal function emerge as relevant determinants on BM function and stem cell populations. Therefore, these factors should be taken into account when assessing benefits of autologous stem cell therapy.

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Small Molecule Induced Caveolin Regulate GPCR Signaling In IPS cells Survival And Differentiation

ZEESHAN PASHA, MUHAMMAD ASHRAF, UNIVERSITY OF ILLINOIS AT CHICAGO, CHICAGO, IL

Background: Previously we reported the use of a small molecule in upregulating cardiac genes by GPCR signaling. We further investigated the small molecule mediated effect involve caveolin and critical miRs that modulate the signaling pathway for increased survival reduced apoptosis and cardiac regeneration in ischemic myocardium. Methods and Results: IPs were treated with a small molecule (ZP-G;20uM) for three to five days. Increased cell survival and proliferative activity ($p<0.01$ vs non treated IPS) with reduced cytochrome c release in cytosol was observed in small molecule treated IPS. Given the upregulation of cardiac genes, ultrastructure feature of small molecule treated IPS cells showed caveolin formation on transmission electron microscopy. miR Microarray analysis of treated IPS cells showed upregulation of miR 705,149, cardiac specific miR -133,762 and down regulation of miR-16, miR 34a, miR-290/292-5p modulating the downstream targets Chemokines, VEGFA, Vesicles associated membrane protein (Vamp), Cdk, E2f, Wnt3a and GPCR receptors. mRNA Microarray further validate 2 fold downregulation of Dnmt1,3b, Max gene associated protein, CDK2, NFkB repressing factor with concomitant 2-3 fold upregulation of caveolin, chemokines and integrins. Western blot analysis displayed significant upregulation of Gα protein, phospho Akt, and Bcl-2 levels as compared to nontreated IPS cells. Small molecule mediated effect was abolished by treatment of IPS cells with GPCR blocker. No tumor formation when treated IPS cells were injected in nude mouse. For Invivo study, female mice with myocardial infarction after permanent LAD ligation were transplanted with either DMEM (Gp1),or 2×105 non treated IPS (Gp 2), or treated IPS (Gp 3). At 4-week of engraftment of IPS cell derived cardiac progenitors (Gp 3) showed reduced apoptosis, extensive survival and myogenesis with attenuation of infarct size and improved heart function as compared to other groups.
Conclusion:This study provides the first evidence that small molecule differentiate IPS to cardiac progenitors and regenerate the ischemic myocardium by targeting downstream targets that regulate the transcription of critical genes and proteins expression to facilitate receptor- tissue- cell signaling.

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A Fibroblast Population Shares A Developmental Origin With Cardiovascular Lineages

Sara Ranjbarvaziri, Shah Ali, Mahmood Talkhabi, Peng Zhao, UCLA, Los Angeles, CA; Young-Jae Nam, Univ of Texas Southwestern Medical Ctr, Dallas, TX; Reza Ardehali, UCLA, Los Angeles, CA

Rationale: The traditional definition of “cardiovascular” lineages describes the eponymous cell types - cardiomyoctes, endothelial cells, and smooth muscle cells - that arise from a common mesodermal progenitor cell during heart development. Fibroblasts are an abundant mesenchymal population in the

mammalian heart which may have multiple, discrete developmental origins. Mesp1 represents the earliest marker of cardiovascular progenitors, contributing to the majority of cardiac lineages. To date no link between Mesp1 and fibroblast generation has been reported.
Objective: We hypothesized progenitor cells expressing Mesp1 can also give rise to cardiac fibroblasts during heart development.
Methods and Results: We generated Mesp1cre/+;R26RmTmG reporter mice where Cre-mediated recombination results in GFP activation in all Mesp1 expressing cells and their progeny. To explore their developmental potential, we isolated GFP+ cells from E7.5 Mesp1cre/+;R26RmTmG mouse. In vitro culture and transplantation studies into SCID mouse kidney capsule as wells as chick embryos showed fibroblastic adoption. Results showed that at E9.5 Mesp1+ and Mesp1- progenitors contributed to the proepicardium organ and later at E11.5 they formed epicardium. Analysis of adult hearts demonstrated that the majority of cardiac fibroblasts are derived from Mesp1 expressing cells. Immunohistochemical analysis of heart sections demonstrated expression of fibroblast markers (including DDR2, PDGFRα and Col1) in cells derived from both Mesp1+ and Mesp1- progenitors. Additionally, we investigated whether the two distinct fibroblast populations have different potency towards reprogramming to cardiomyocytes. Results showed no significant difference between Mesp1 and non-Mesp1 isolated fibroblasts to convert to cardiomyocyte fate.
Conclusions: Our data demonstrates that cardiovascular progenitors expressing Mesp1 contribute to the proepicardium. These cells, as cardiovascular progenitors, also give rise to the highest portion of cardiac fibroblasts in the mouse heart.

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Paracrine miRNA-Crosstalk Between Human CD34+ Stem Cells and Selective Myocardial Cells Via Therapeutic Exosomes Promotes Repair of the Ischemic Heart

Susmita Sahoo, David Kim, Sol Misener, Christine E Kamide, Douglas E Vaughan, Northwestern Univ, Chicago, IL; Douglas W Losordo, NeoStem Inc., Chicago, IL

Introduction: Earlier, in a first study of its kind, we have demonstrated a novel mechanism that therapeutically significant human CD34+ stem cells secrete membrane bound nano-vesicles called exosomes (CD34Exo). CD34Exo are angiogenic and constitute a critical component of the pro-angiogenic paracrine activity of the cells. Further, when transplanted locally, cell-free CD34Exo induce ischemic tissue repair in a murine hindlimb ischemia model.
Here, we hypothesize that exosomes released via paracrine secretion from human CD34+ cells mediate myocardial repair by direct transfer of microRNAs to target cells in the heart.
Methods and Results: When injected into mouse ischemic myocardium, cell-free CD34Exo replicated the therapeutic activity of human CD34+ cells by significantly improving ischemia (ejection fraction, 42±4 v 22±6%; capillary density, 113±7 v 66±6/HPF; fibrosis, 27±2 v 48±7%; $p<0.05$, n=7-12) compared with PBS control. Interestingly, confocal imaging and flow cytometry analyses of the exosomes-injected ischemic myocardial tissue revealed that CD34Exo was selectively internalized into endothelial cells and cardiomyocytes. CD34Exo, which is enriched with miR126, induced the expression of miR126 and several pro-angiogenic mRNAs in the exosomes-treated ischemic myocardium, but did not affect the endogenous synthesis of miR126. CD34Exo lacking miR126 had decreased angiogenic activity in vitro and decreased proangiogenic gene expression in vivo indicating that miR126 is important for CD34Exo function. Imaging using fluorescent miR126 confirms that CD34Exo directly transferred miR126 and possibly other yet to be identified moieties from its cargo, selectively to endothelial cells and cardiomyocytes in the ischemic heart.
Conclusion: Our results reveal a novel molecular and trafficking mechanism of CD34Exo that may be responsible for intercellular transfer of genetic information such as miRNAs from human

CD34+ stem cells, selectively to endothelial cells and cardiomyocytes inducing changes in gene expression, angiogenesis and myocardial recovery. Exosomes-shuttled miRNAs may signify amplification of stem cell function and may explain the therapeutic benefits associated with human CD34+ cell therapy.

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Clonal Analysis Of Multipotent Cardiovascular Progenitors That Generate Cardiomyocytes During Development

Konstantina Ioanna Sereti, Paniz Kamran Rashani, Peng Zhao, Reza Ardehali, UCLA, Los Angeles, CA

It has been proposed that cardiac development in lower vertebrates is driven by the proliferation of cardiomyocytes. Similarly, cycling myocytes have been suggested to direct cardiac regeneration in neonatal mice after injury. Although, the role of cardiomyocyte proliferation in cardiac tissue generation during development has been well documented, the extent of this contribution as well as the role of other cell types, such as progenitor cells, still remains controversial. Here we used a novel stochastic four-color Cre-dependent reporter system (Rainbow) that allows labeling at a single cell level and retrospective analysis of the progeny. Cardiac progenitors expressing Mesp1 or Nkx2.5 were shown to be a source of cardiomyocytes during embryonic development while the onset of αMHC expression marked the developmental stage where the capacity of cardiac cells to proliferate diminishes significantly. Through direct clonal analysis we provide strong evidence supporting that cardiac progenitors, as opposed to mature cardiomyocytes, are the main source of cardiomyocytes during cardiac development. Moreover, we have identified quadri-, tri-, bi, and uni-potent progenitors that at a single cell level can generate cardiomyocytes, fibroblasts, endothelial and smooth muscle cells. Although existing cardiomyocytes undergo limited proliferation, our data indicates that it is mainly the progenitors that contribute to heart development. Furthermore, we show that the limited proliferation capacity of cardiomyocytes observed during normal development was enhanced following neonatal cardiac injury allowing almost complete regeneration of the scared tissue. However, this ability was largely absent in adult injured hearts. Detailed characterization of dividing cardiomyocytes and proliferating progenitors would greatly benefit the development of novel therapeutic options for cardiovascular diseases.

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Pitx2 Promotes Murine Myocardial Regeneration after Myocardial Injury

GE TAO, Elzbieta Klysik, Yuka Morikawa, James F. Martin, Baylor Coll of Med, Houston, TX

Myocardial infarction is the leading cause of morbidity and mortality in the United States. Compromised myocardial function, due to the lack of self-renewal capacity in mature hearts, is a major reason for heart failure. Available therapies can only ameliorate, but not reverse the loss of functional myocardium. With heart transplantation as the only available cure, design of an effective regenerative therapy has become imperative for cardiovascular research. To repopulate the heart with de novo cardiomyocytes, most attempts have been based on the transplantation of cardiac, non-cardiac stem cells or their derivatives, however a more profound knowledge of stem cells is required for achieving significant progress. Meanwhile, triggering endogenous regenerative capacity is a compelling strategy for cardiac repair. It has been reported that proliferation of pre-existing cardiomyocytes strongly contributes to regeneration. Thus, efforts have been made to reintroduce mature cardiomyocytes into mitotic cycle. The mechanisms underlying the proliferation of cardiomyocytes during development and their homeostasis during adulthood are not fully understood, but likely require tight regulation of transcription factors in

specific cell types. We have previously shown that the mouse Hippo kinase cascade is a major heart-size control pathway during development. In addition, activation of Yap, a transcriptional cofactor inhibited by Hippo, by genetically disrupting Hippo signaling is sufficient to induce juvenile and adult myocardial regeneration after surgery-induced myocardial infarction. Here we identified the paired-like homeodomain transcription factor 2 (pitx2) as a potential downstream target and cofactor of Yap in mouse heart. Our data indicates that Pitx2 expression is induced by myocardial injury, and is required for neonatal myocardial regeneration in a postnatal day 1 (P1) apex resection model. Further studies show that over-expression of pitx2 in adult cardiomyocytes is sufficient to promote the restoration of myocardial structure and function after myocardial infarction. Together, we show that pitx2 is a new manipulator of myocardial regeneration and could serve as a novel therapeutic target in cardiac regenerative therapy.

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A Microrna Pathway That Promotes Cardiomyocyte Proliferation And Cardiac Regeneration By Inhibiting Hippo Signaling

Ying Tian, Temple Univ, Philadelphia, PA; Li Chen, Tao Wang, Ning Zhou, Jun Kong, Ying Liu, Melinda Snitow, Michael Morley, Deqiang Li, Nataliya Petrenko, Minmin Lu, Kathleen Stewart, Edward Morrisey, Univ of Pennsylvania, Philadelphia, PA

In contrast to lower vertebrates, the mammalian heart has limited capacity to regenerate after injury in part due to ineffective reactivation of cardiomyocyte proliferation. While evidence exists for a low level of cardiomyocyte proliferation in the adult heart, it remains unclear whether increasing the rate could be used to therapeutically promote cardiac regeneration. In this study, we show that the microRNA cluster miR302-367 is important for cardiomyocyte proliferation during development and is sufficient to induce cardiomyocyte proliferation in the adult and promote cardiac regeneration. Loss of miR302-367 leads to decreased cardiomyocyte proliferation during development. In contrast, increased miR302-367 expression leads to a profound increase in cardiomyocyte proliferation, in part through repression of the Hippo signal transduction pathway. Postnatal re-expression of miR302-367 leads to reactivation of the cell cycle in cardiomyocytes resulting in reduced scar formation after infarction. However, long-term expression of miR302-367 leads to cardiomyocyte de-differentiation and dysfunction, suggesting that persistent reactivation of the cell cycle in postnatal cardiomyocytes is not desirable. Importantly, this limitation can be overcome by transient systemic application of miR302-367 mimics, leading to increased cardiomyocyte proliferation and mass, decreased fibrosis, and improved function after injury. Our data demonstrate the ability of microRNA based therapeutic approaches to promote cardiac repair and regeneration through the transient activation of cardiomyocyte proliferation.

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A miRNA-Hippo Pathway Promotes Cardiac Conduction System Regeneration

Jun Wang, James F Martin, Baylor Coll of Med, Houston, TX

The cardiac conduction system (CCS) is required for initiating and maintaining regular rhythmic heartbeats and CCS defects can give rise to cardiac arrhythmia, a leading cause for morbidity worldwide. Given the poor self-repair potential in the adult human CCS, it is critical to elucidate the molecular mechanisms limiting CCS regeneration to facilitate developing efficient cardiovascular therapies. microRNAs (miRs) are small non-coding RNAs that repress gene expression post-transcriptionally. The miR-17-92 cluster can induce cardiomyocyte proliferation and regeneration. Hippo signaling, an ancient organ size control pathway, represses cardiomyocyte proliferation and regeneration. Here we found that both miR-17-92 and Hippo signaling were active in CCS. Disruption of either miR-17-92 or Hippo signaling

in heart gave rise to cardiac arrhythmias in mice. Notably, miR-17-92 regulates Hippo signaling through repressing Lats2, a core Hippo pathway component. In miR-17-92 null mutant hearts, up-regulated Lats2 led to increased Hippo pathway activity. Moreover, we performed chromatin immunoprecipitation deep sequencing (ChIP-Seq) using YAP, the Hippo signaling effector, which suggested that Hippo signaling regulates genes involved in CCS homeostasis. Together, we propose a novel miR-Hippo genetic pathway that promotes CCS regeneration.

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Robust Cardioprotection Afforded by a Previously Unrecognized Link between the Unfolded Protein Response and the Hexosamine Biosynthetic Pathway

Zhao V. Wang, Yingfeng Deng, Ningguo Gao, Zully Pedrozo, Dan Li, Cyndo Morales, Alfredo Criollo, Xiang Luo, Wei Tan, Nan Jiang, Mark Lehrman, Beverly Rothermel, Ann-Hwee Lee, Sergio Lavandero, Pradeep Mammen, Anwarul Ferdous, Thomas Gillette, Philipp Scherer, Joseph Hill, UT Southwestern Medical Ctr, Dallas, TX

Background: The hexosamine biosynthetic pathway (HBP) generates UDP-GlcNAc (uridine diphosphate N-acetylglucosamine) for glycan synthesis and O-linked GlcNAc (O-GlcNAc) protein modifications. Despite the established role of the HBP in glucose metabolism and multiple diseases, regulation of the HBP remains largely undefined. **Methods & Results:** Here, we show that spliced Xbp1 (Xbp1s), the most conserved signal transducer of the unfolded protein response (UPR), is a direct transcriptional activator of the HBP. We demonstrate that the UPR triggers activation of the HBP by means of Xbp1s-dependent transcription of genes coding for key, rate-limiting enzymes. We establish that this previously unrecognized UPR-HBP axis is triggered in a variety of stress conditions known to promote O-GlcNAc modification. We go on to demonstrate that Xbp1s, acutely stimulated by ischemia/reperfusion (I/R) in heart, confers robust cardioprotection against I/R injury. We also show that HBP induction is required for this cardioprotective response. Mechanistically, HBP may mediate the adaptive branch of the UPR by activating autophagy and ER-associated degradation. **Conclusion:** These studies reveal that Xbp1s couples the UPR to the HBP, promoting robust cardioprotection during I/R.

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Deletion Of E2f1 Promotes Bone marrow Progenitor Cell Differentiation And Ischemic Cardiac Repair

Shiyue Xu, Feinberg Cardiovascular Res institute,Northwestern Univ Feinberg Sch of Med, Chicago, IL

Background: We have previously shown that knockout of E2F1 in mice enhances angiogenesis following induction of hind limb ischemia. Recent studies suggest that suppression of E2F1 enhances oxidative phosphorylation in a variety of cell types. Since an increase in oxidative phosphorylation in stem/progenitor cells is often associated with cell differentiation, we hypothesize that E2F1-deficiency may promote bone marrow (BM) progenitor cell differentiation thereby impact on ischemic cardiac repair. Methods and Results: We cultured bone marrow (BM) Lin- progenitor cells under hypoxic and normxic conditions for 24 h, then measured the expression of metabolism associated genes and evaluated cell proliferation and differentiation. We also performed adoptive BM transplantation to reconstitute BM of WT mice with E2F1-/- or WT BM, followed by surgical induction of myocardial infarction (MI), to compare the role of BM E2F1 in the cardiac repair in vivo. Notably, we found that the expression levels of pyruvate dehydrogenase kinase (PDK)-4 and PDK2, two critical inhibitors of mitochondrial oxidative phosphorylation, in the E2F1-/- BM Lin- progenitor cells is markedly lower than those in WT cells ($P<0.01$, $n=4$). After culture in the EPC

differentiation medium for 7 days, the expression levels of EPC markers, CD31 and KDR , were significantly higher in E2F1-/- cells than in WT control cells (P<0.05, n=3). Although there was no significant difference in the proliferating rate between WT and E2F1-/- BM Lin- progenitor cells cultured in normoxia, when cultured under hypoxic condition the proliferating rate of E2F1-/- cells were markedly greater than that of WT cells (P<0.05, n=3). Consistently, the infarcted size in mice transplanted with E2F1-/-eGFPTg BM was significantly smaller than in mice transplanted with WTeGFPTg BM (P<0.01, n=5). Conclusions: Genetic deletion of E2F1 in the BM progenitor cells enhances oxidative metabolism that may result in enhanced differentiation towards endothelial lineage and increased proliferation in the ischemic/hypoxic tissue environment. Therefore, inhibition of E2F1 in BM progenitor cells may improve the recovery from cardiac ischemic injury.

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Enhance Cd34+ Cell Therapy For Limb Ischemia Using Novel Bioactive Peptide Amphiphiles Nanofibers

Yang Yu, Feinberg Cardiovascular Res Inst, Chicago, IL; Eduard Sleep, Dept of Med, Northwestern Univ, Chicago, IL; Junlan Zhou, Douglas W Losordo, Feinberg Cardiovascular Res Inst, Chicago, IL; Samuel I Stupp, Inst for Bionanotechnology in Med, Northwestern Univ, Chicago, IL; Gangjian Qin, Feinberg Cardiovascular Res Inst, Chicago, IL

Background_ CD34+ cells have shown unprecedented promise for ischemic tissue repair in recent clinical trials; however, their recruitment, retention, and survival in the injured tissue remain one of the major barriers to the success of this novel therapeutic strategy. Methods and Results_ In this study, we have developed a self-assembling bioactive peptide amphiphile (PA) nanofiber that displays the integrin-recognized cell adhesion epitope RGDS and tested this novel scaffold material for therapeutic delivery, retention, and functional support of human CD34+ cells in a mouse model of critical hind-limb ischemia (CLI). In culture, PA nanofibers, but not non-bioactive nanostructures or nanostructures with scrambled DGRS epitope, significantly enhanced the adhesion and survival of human CD34+ cells under a number of cellular stresses. In CLI nude mice, intramuscular injection of human CD34+ cells contained in the RGDS nanostructures led to a significantly better blood perfusion, motor ability, limb salvage, and treadmill endurance when compared with those of CD34+ cells contained in DGRS nanostructures, cells alone or RGDS alone. Histology analyses revealed a better viability and retention of CD34+ cells in the limb tissue of the RGDS nanostructures/CD34+ cells group than in the RGDS nanostructures alone or cells alone group. In consistent with the improved functional recovery, the density of capillaries in the ischemic limb of RGDS nanostructures/CD34+ cells group was significantly greater than in other groups. Conclusions_ Our data demonstrate that integrin-specific binding epitope RGDS nanostructure enhances viability, adhesion, and angiogenic capability of human CD34+ cells. This pre-clinical study provides a rationale for further investigation of combining RGDS PAs with human CD34+ cells to enhance the effectiveness of cell therapy for ischemic limb disease.

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TNFR2-Bmx Signaling in Cardiac Stem Cells and Cardiac Repair

Huanjiao Jenny Zhou, Qunhua Huang, Yale Univ, New Haven, CT; Wang Min, Yale Univ Sch of Med, New Haven, CT

While cytokine TNF via TNFR1 induces inflammation and apoptosis, it through its second receptor TNFR2 induces cell survival and migration by activating bone marrow non-receptor tyrosine kinase Bmx. Since Bmx has

been implicated in self-renewal of stem cells, we hypothesize that TNF via TNFR2 activates Bmx in cardiac stem cells (CSCs) to mediate cardiac repair. We show that in human cardiac tissue affected by ischemia heart disease (IHD), TNFR2 is expressed on intrinsic CSCs, identified as c-kit(+) /CD45(-) /VEGFR2(-) interstitial round cells, which are activated as determined by entry to cell cycle and expression of Lin-28. Wild-type mouse heart organ cultures subjected to hypoxic conditions both increase cardiac TNF expression and show induced TNFR2 and Lin-28 expression in c-kit(+) CSCs that have entered cell cycle. These CSC responses are enhanced by exogenous TNF. TNFR2(-/-) mouse heart organ cultures subjected to hypoxia increase cardiac TNF but fail to induce CSC activation. Similarly, c-kit(+) CSCs isolated from mouse hearts exposed to hypoxia or TNF show induction of Lin-28, TNFR2, cell cycle entry, and cardiogenic marker, α-sarcomeric actin (α-SA), responses more pronounced by hypoxia in combination with TNF. Knockdown of Lin-28 by siRNA results in reduced levels of TNFR2 expression, cell cycle entry, and diminished expression of α-SA (references: Stem Cells 2013;31:1881-1892). In the present study, we detect the c-kit(+)Lin28(+) CSCs populations in a mouse coronary artery ligation ischemic model. Furthermore, the c-kit(+) CSCs are reduced in TNFR2-KO and Bmx-KO mice. Mechanistically, we show a crosstalk between the TNFR2-Bmx and the c-Kit signaling pathways to mediate CSC proliferation, survival and migration. These observations suggest that TNFR2-Bmx signaling in c-kit(+) CSCs induces cardiac repair, providing a potential strategy to stimulate cardiac regeneration by TNFR2-specific agonists.

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Uroetensin II Protects Cardiomyocytes from Apoptosis Induced by Oxidative Stress through Cystathionine-γ-Lyase (CSE)/Hydrogen Sulfide Pathway

Hui Gong, Zhidan Chen, Xiaoyi Zhang, Fudan Univ, Shanghai, China; Jie Zhang, Jiaotong Univ, Shanghai, China; Yang Li, Yong Ye, Guoping Zhang, Chunjie Yang, Yunzeng Zou, Fudan Univ, Shanghai, China

Plasma UII has been observed to be raised in patients with acute myocardial infarction, a lower UII response is associated with more severe injury of myocardium, suggesting a possible cardioprotective role for this peptide. In the present study, we studied plasma UII concentration of thirty patients admitted to the Cardiology Department with acute myocardial infartion.The results showed that plasma UII was sharply increased in patients compared to that in health control within one week after admission. We then explore whether UII could protect cardiomyocytes from injury induced by oxidative stress. Cultured cardiomyocyte were treated with H2O2 to induce oxidative stress, and the influence of UII on H2O2-induced apoptosis was observed. The results showed that UII pretreatment significantly reduced the number of TUNEL-positive cardiomyocytes induced by H2O2, and it partly abolished the upregulation of pro-apoptotic protein Bax and the down-regulation of anti-apoptotic protein Bcl-2. siRNA targeted to urotensin receptor (UT) greatly inhibited these effects. H2S has been reported to exert protective effect on cardiomyocytes, we detected the effect of UII on H2S production and CSE (Major H2S-producing enzyme) expressions in cardiomyocytes exposed to H2O2.The present data revealed that UII increased the H2S production by enhancing the expression of CSE by activating the ERK signaling in cardiomyocytes exposed to H2O2. Si-CSE or ERK inhibitor not only greatly inhibited the upregulation of CSE or the phosphorylation of ERK induced by UII but also reversed UII-induced-upregulation of H2S production and anti-apoptosis in cadiomyocytes exposed to H2O2.

In conclusion, UII rapidly promoted the phosphorylation of ERK, increased CSE exression and induced H2S production, which in turn enhanced the p-ERK level to protect cardiomyocytes from apoptosis under ischemic or oxidative stress. The increased plasma UII level in patients may be critical for cardiac protection in patients at early-phase of acute myocardial infarction.

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Novel Sites of Angiotension II Type 1 Receptor are Identified for Direct Activation by Mechanical Stretch Independent of Angiotension II

Hui Gong, Guoliang Jiang, Chunjie Yang, Shijun Wang, Zhidan Chen, Guoping Zhang, Yunzeng Zou, Fudan Univ, Shanghai, China

The angiotensin II type 1 receptor (AT1R) has a crucial role in cardiac hypertrophy induced by pressure overload. In the previous study, we found a novel mechanism for mechanical stress-induced AT1R activation without the involvement of Ang II. However, few reports focus on how AT1R senses mechanical stress and translates it into biochemical signals inside the cells to induce cardiomyocyte hypertrophy. Here, we constructed different site-directed mutagenesis of AT1R and transfected them to COS7 cells and ATG–/– (Angiotensinogen knockout) cardiomyocytes, respectively, to observe the activation of downstream signaling to identify functional site of AT1R. The results showed AT1R-WT, AT1R-K199Q, AT1R-L212F,AT1R-Q257A and AT1R-C289A plasmids or adenovirus were overexpressed at high level in plasma membrane of COS7 or cardiomyocytes respectively. There was no obvious difference in subcellular expression of wt-AT1R and all the mut-AT1Rs. The further study revealed that Ang II-induced-phosphorylation of ERK, Jak2 and the redistribution of Gαq11 were dramatically decreased in COS7 cells expressing AT1R-K199Q or AT1R-Q257A, while these effects induced by mechanical stretch were greatly suppressed in COS7 cells expressing AT1R-L212F,AT1R-Q257A or AT1R-C289A compared to these in COS7 cells expressing AT1R-WT. We then transfected the adenovirus of wt-AT1R or different mut-AT1Rs to ATG–/– cardiomyocytes to exclude the influence of endogenous Ang II. The results were consistent with these results in COS7 cells. Moreover, ATG–/– cardiomyocytes overexpressing AT1R-K199Q or AT1R-Q257A parlty abolished hypertrophic response induce by Ang II, while the cardiomyocytes overexpressing AT1R-L212F,AT1R-Q257A or AT1R-C289A greatly inhibited the hypertrophic response induced by mechanical stretch. The present study indicated that Leu212, Gln257 and Cys289 are critical sites for AT1R activation by mechanical stretch without Ang II but Lys199 and Gln257 play important role in AT1R activation with Ang II.

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High glucose-induced mitophagy limits Cardiomyocyte Injury

Qiangrong Liang, Satoru Kobayashi, Yuan Huang, New York Inst of Technology Coll of Osteopathic Med, Old Westbury, NY

Cardiovascular complications are the major cause of death among diabetic patients. Mitochondrial dysfunction has been suggested to play an important role in the pathogenesis of diabetic cardiomyopathy. Therefore, timely elimination of dysfunctional mitochondria is essential for maintaining a healthy mitochondrial network and limiting diabetic cardiac injury. This function is performed by mitophagy, a process that selectively degrades mitochondria through the autophagy-lysosome pathway. In the present study, we investigated the functional role of mitophagy in cardiomyocytes treated with high glucose, an independent risk factor for heart failure in diabetic patients. We found that mitophagy in neonatal rat ventricular myocytes was increased by high glucose (30 mM) as indicated by a novel dual fluorescent reporter as well as the co-localization of mitochondrial protein Tom20 and lysosome-associated membrane protein 1 (LAMP1). Parkin is an E3-ubiquitin ligase that positively regulates mitophagy. Parkin overexpression enhanced high glucose-induced mitophagy and attenuated cardiomyocyte injury as measured by the levels of oxidative injury, mitochondrial damage, ROS generation and cardiomyocyte death. Conversely, Parkin knockdown potentiated the toxic effects of high glucose, suggesting that mitophagy is cardioprotective under hyperglycemic conditions. In addition, high glucose increased mitochondrial fragmentation. By overexpressing or knocking down Drp1, a key regulator of mitochondrial fission, we showed that mitochondrial fragmentation was coupled with mitophagy and negatively related to the toxic effects of high glucose. Together, these findings demonstrate that high glucose induces mitochondrial fragmentation and mitophagy,

which are adaptive responses that protect cardiomyocytes against high glucose toxicity. Future studies are warranted to determine if mitophagy plays a similar role in diabetic cardiac damage in vivo.

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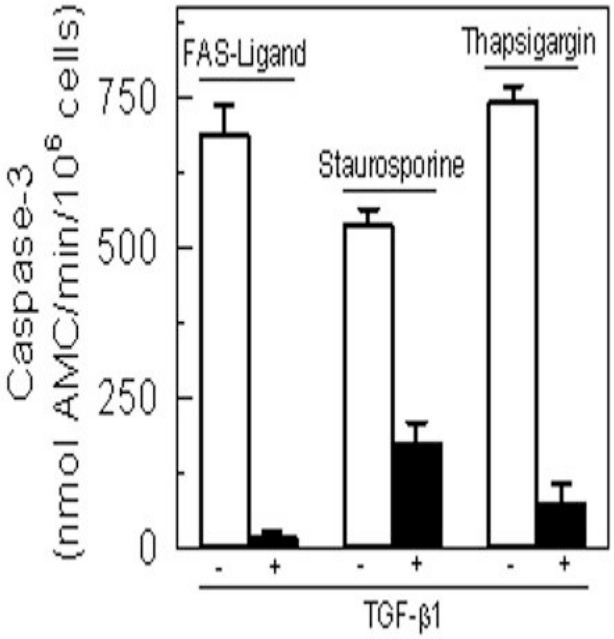
Transforming Growth Factor-β1 Increases Resistance of Fibroblasts to Apoptotic Cell Death

Ulugbek Negmadjanov, Zarko Godic, Mahek Mirza, Larisa Emelyanova, Farhan Rizvi, Ekhson Holmuhamedov, Arshad Jahangir, Ctr for Integrative Res on Cardiovascular Aging (CIRCA), Aurora UW Medical Group, Milwaukee, WI

Introduction: Cardiac injury results in the death of cardiac myocytes and subsequent scar formation through extracellular matrix (ECM) deposition by fibroblasts (FB) and myofibroblasts (myoFB). Excessive fibrosis results in pathological scarring that predisposes to arrhythmogenesis and heart failure, particularly in the elderly. Strategies to limit adverse ECM remodeling are urgently needed to curtail the growing epidemic of atrial fibrillation and heart failure in the aging population. Persistence of myoFB and resistance to apoptotic cell death has been proposed to underlie the mechanism of excessive fibrosis, yet is not fully characterized.
Methods: Cultured NIH/3T3 cells (control and TGF-β1 treated) have been challenged with activators of extrinsic (FAS-Ligand, 1 μg/mL) or intrinsic (Thapsigargin 10 μM and Staurosporine 5 μM) apoptotic pathways and Caspase-3 activity was measured in cellular lysate.
Results: FAS-L exposure induced ~40-fold suppression of Caspase-3 activity in TGF-β1 treated cells as compared with control (17±12 vs 686±5 nmol AMC/min/106 cells, respectively). Similarly, Staurosporine activated Caspase-3 in TGF-β1 treated cells ~3-fold (171±38 vs 536±29 nmol AMC/min/106 cells), and Thapsigargin ~10-fold (73±33 vs 742±8 nmol AMC/min/106 cells).
Conclusion: TGF-β1 treatment increased the sensitivity of NIH/3T3 cells toward extrinsic and intrinsic apoptotic stimuli. Although, TGF-β1 treatment increased overall resistance of NIH/3T3 cells to apoptosis, the responsiveness of cells to extrinsic vs intrinsic pathways was differentially affected. This data support the hypothesis that persistence of myoFB results in pathological scarring.



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Duo-impairment of the Ubiquitin-Proteasome System and Autophagy by Ablation of COP9 Signalosome Subunit 8 Activates a Programmed Necrosis Pathway Mediated by RIP1-RIP3 Kinases but not Cyclophilin D-regulated Mitochondrial Membrane Permeability

Changhua Wang, Andrea Jahn, Huabo Su, Xuejun Wang, Univ of South Dakota Sanford Medical Sch, Vermillion, SD

Programmed cell death includes apoptosis and programmed necrosis (AKA, necroptosis). A well-recognized feature of necrosis is the loss of

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membrane integrity of the dying cell. Cells undergoing apoptosis, however, maintain their membrane integrity. We previously reported massive cardiomyocyte (CM) necrosis, before increased apoptosis is discernible, in mouse hearts with cardiomyocyte-restricted knockout of the COP9 signalosome subunit 8 (CR-Csn8KO). This is associated with defective autophagosome maturation and ubiquitin-proteasome system (UPS) dysfunction. Consequently, mice with perinatal CR-Csn8KO develop rapidly dilated cardiomyopathy (DCM) and die prematurely. The present study sought to search for the mechanisms underlying the CM necrosis. Here our immunoprecipitation revealed significant increases of RIP1-interacted RIP3 in CR-Csn8KO myocardium, indicative of activation of the RIP1-RIP3 pathway. The RIP1-RIP3 pathway is known to mediate necroptosis. To further test whether RIP1 activation plays an essential role in CM necrosis in CR-Csn8KO mice, we treated the mice with a RIP1 kinase specific inhibitor necrostatin-1 (Nec-1) or vehicle control via osmotic mini-pump implanted in the peritoneal cavity at 2 weeks of age. Nec-1 significantly suppressed CM necrosis as measured by the positivity of Evan's blue dye (EBD) uptake ($p<0.00001$), prevented left ventricle dilatation ($p<0.05$) at 3 weeks, and delayed premature death of the CR-Csn8KO mice ($p=0.0072$). These results demonstrate that CM necrosis in CR-Csn8KO mice is necroptosis in which the RIP1-RIP3 kinases-mediated pathway plays a major pathogenic role, and that CM necroptosis is the primary cause of DCM and mouse premature death. Increased mitochondrial membrane permeability, which can be suppressed by inhibition of cyclophilin D, has been shown to play a critical mediating role in myocytes necrosis. Hence, we cross-bred CR-Csn8KO mice with cyclophilin D knockout mice and obtained the Kaplan-Meier survival curve. Unexpectedly, cyclophilin D deficiency exacerbated the premature death of CR-Csn8KO mice ($p=0.007$), indicating that cyclophilin D regulated mitochondrial membrane permeability does not play an important role in CM necrosis in CR-Csn8KO mouse hearts.

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Rnd3/RhoE Haploinsufficient Mice are Hypersensitive to Pressure Overload and Develop Apoptotic Cardiomyopathy with Heart Failure

Xiaojing Yue, Xiangsheng Yang, Xi Lin, Tingli Yang, Texas A&M Univerisity Health Science Ctr, Houston, TX; Xin Yi, Renmin Hosp of Wuhan Univ, Wuhan, China; Yuan Dai, Jiang Chang, Texas A&M Univerisity Health Science Ctr, Houston, TX

Rationale: Rho family guanosine triphosphatase (GTPase) 3 (Rnd3, also called RhoE), a member of the small Rho GTPase family, has been suggested to regulate cell actin cytoskeleton dynamics, cell migration, and apoptosis through the Rho kinase-dependent signaling pathway. The biological function of Rnd3 in the heart is unknown. The downregulation of small GTPase Rnd3 transcripts was found in patients with end-stage heart failure. The pathological significance of Rnd3 loss in the transition to heart failure remains unexplored.

Objective: To investigate the functional consequence of Rnd3 downregulation and the associated molecular mechanism,

Methods and Results: we generated $Rnd3^{+/-}$ haploinsufficient mice to mimic the downregulation of Rnd3 observed in the failing human heart. $Rnd3^{+/-}$ mice were viable; however, the mice developed heart failure after pressure overload by transverse aortic constriction (TAC). Remarkable apoptosis, increased caspase-3 activity, and elevated Rho kinase activity were detected in the $Rnd3^{+/-}$ haploinsufficient animal hearts. Pharmacological inhibition of Rho kinase by Fasudil treatment partially improved $Rnd3^{+/-}$ mouse cardiac functions and attenuated myocardial apoptosis. To determine if Rho associated coiled-coil kinase 1 (ROCK1) was responsible for Rnd3 deficiency-mediated apoptotic cardiomyopathy, we established a double knockout mouse line, the Rnd3 haploinsufficient mice with ROCK1-null background ($Rnd3^{+/-/ROCK1-/-}$). Again, genetic deletion of *ROCK1* partially but not completely rescued Rnd3 deficiency-mediated heart failure phenotype.

Conclusion: Downregulation of Rnd3 correlates with cardiac loss of function as in heart failure patients. Hyperactivation of Rho kinase activity is responsible in part for the apoptotic cardiomyopathy development. Further investigation of ROCK1-independent mechanisms in Rnd3-mediated cardiac remodeling should be the focus for future study.

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Role of Arterial AT1 Receptor on the Regulation of GRK4 in Hypertension

Ken Chen, Li Liu, Caiyu Chen, Daping Hosp, The Third Military Medical Univ, Chongqing, China; Pedro A Jose, Div of Nephrology, Dept of Med, Univ of Maryland Sch of Med, Baltimore, MD; Chunyu Zeng, Daping Hosp, The Third Military Medical Univ, Chongqing, China

G protein-coupled receptor kinase 4 (GRK4) gene variants or increased GRK4 expression, via impairment of renal dopamine receptor and enhancement of renin angiotensin system function, impairs renal sodium excretion, resulting in sodium retention and an increase in blood pressure. Increased aortic stiffness, a risk factor in cardiovascular disease, may be related to increased activity of the renin-angiotensin system Whether or not GRK4 and the angiotensin type 1 receptor (AT1R) interact in the aorta is not known. We now report that GRK4 is expressed in vascular smooth muscle cells (VSMCs) of the aorta. Exogenous expression of the GRK4 variant 142V in aortic A10 cells increased AT1R protein expression and AT1R-mediated increase in intracellular calcium concentration. The increased AT1R expression was caused by an increase in AT1R mRNA expression via NF-kappa B, because blockade of NF-kappa B abolished those effects of GRK4 A142V. As compared with control (vector-transfected) cells, cells expressing 142V had higher NF-kappa B activity and more NF-kappa B bound to AT1R promoter. The increased AT1R expression in cells expressing GRK4 142V was associated with decreased AT1R degradation, which was ascribed to the lower AT1R phosphorylation. There was direct interaction between GRK4 and AT1R in A10 cells which was decreased by GRK4 that could have caused the lower AT1R phosphorylation and degradation. The regulation of GRK4 of AT1R expression was confirmed in GRK4142V transgenic mice, the AT1R expression was higher, while AT1R phosphorylation was lower in aorta in GRK4 142V than control mice. Angiotensin II-mediated vasoconstriction was higher in A142V mice. This study provides a mechanism that GRK4, via regulation of arterial AT1R expression and function, engaged into the pathogenesis of hypertension.

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Sex Differences in β-Adrenergic Responsiveness of Excitation-Contraction Coupling in Isolated Rabbit Hearts

Gregory Hoeker, Ashleigh Hood, Rodolphe Katra, Univ of Alabama Birmingham, Birmingham, AL; Steven Poelzing, Virginia Tech, Roanoke, VA; Steven Pogwizd, Univ of Alabama Birmingham, Birmingham, AL

Introduction: Sex differences in β-adrenergic receptor (β-AR) responsiveness are associated with female cardioprotection. We hypothesize that female (F) rabbits have reduced responsiveness to β-AR stimulation vs males (M), and that the degree and type of sex differences vary with the β-AR subtypes that are activated.
Methods: Ventricular action potentials (AP) and intracellular calcium transients (CaT) were optically mapped from the epicardial surface of rabbit hearts during 3 Hz pacing. Spontaneous calcium release (SCR) and ectopic activity were elicited at 1, 3, and 5.5 Hz. β-responsiveness was assessed with the nonselective β-agonist isoproterenol (Iso, 1-316 nM), or β2-AR selective agonist zinterol (Zin, 10 nM).
Results: At baseline, the time constant of CaT decay (τ) was faster in F than M (54.0±1.7 vs 62.1±3.0 ms; n=14, 14; $p < 0.05$), with no sex difference in CaT duration (CaD80). AP duration (APD90) was shorter in F than M (202.5±5.0 vs 218.2±5.7 ms; $p < 0.05$). Iso decreased τ, CaD80, and APD90 in a dose-dependent manner in both sexes (n = 5, 5 for F, M). Iso decreased τ to a lesser extent in F than M for 1 and 32-316 nM Iso (F = 11-32 ms, M = 23-48 ms; $p < 0.05$). The Iso-induced decrease in CaD80 was not significantly different in F than M at any dose. The Iso-induced decrease in APD90 was significantly less in F than M only at 316 nM Iso (75.5±8.7 ms vs 103.9±6.2 ms, $p < 0.05$). In contrast, there were no sex differences

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in the response to Zin for τ, CaD80, or APD90 (n = 6, 6 for F, M). Zin decreased τ by 7.2±2.0 ms in F vs 12.7±3.7 ms in M; CaD80 by 18.0±5.3% in F vs 21.1±8.0 ms in M; and APD90 by 24.9±8.5 ms in F vs 21.9±8.9 ms in M. SCR was observed in 50% (6/12) of hearts treated with Zin, whereas Iso elicited SCR in all hearts (10/10) with a dose threshold of 32 nM. No ectopic beats were observed with Zin (0/36 trials in 12 hearts). With Iso, ectopic activity was less frequent in F hearts (16%, 12/75 trials in 5 hearts) than in M hearts (41%, 26/68 trials in 5 hearts, $p < 0.05$).
Conclusions: These results suggest that sex differences in AP and CaT depend on the dose of the agonist used and the β-AR subtypes that are activated. Elucidating nuances of sex differences in β-AR subtype physiology will provide a better understanding of the mechanisms of reduced β-responsiveness in F and its cardioprotective effects.

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Glycogen Depletion During Hypoxia Results in Enhanced AMPK Phosphorylation and Diminished Smooth Muscle Contractility in Human Saphenous Vein

Lakshman Segar, Rajkumar Pyla, Prahalathan Pichavaram, Arwa Fairaq, Univ of Georgia Coll of Pharmacy, Augusta, GA; Mark Kozak, Penn State Coll of Med, Hershey, PA; Vinayak Kamath, Vijay Patel, Georgia Regents Univ, Augusta, GA

Objectives: Hypoxia promotes vasodilation of coronary arteries as a protective response to improve blood flow to the myocardium. Although diminished formation of ATP and enhanced phosphorylation/activation of AMP-activated protein kinase (AMPK) in vascular smooth muscle are known to mediate hypoxia-induced vasorelaxation, the contribution of glycogen toward AMPK phosphorylation and contractility remains unclear. Methods: Using surgically-removed endothelium-denuded human saphenous vein segments ex vivo, the present study has examined serotonin (5-HT)-induced smooth muscle contractility by isometric tension measurements and AMPK phosphorylation by immunoblot analysis under normoxic (95% O2/5% CO2) and hypoxic conditions (95% N2/5% CO2, 30 min). Results: Under normoxic glycogen-enriched conditions, the maximal contractile response (Emax) and sensitivity (pEC50) to 5-HT-induced contractility were 145 ± 3% and 6.7 ± 0.2, respectively (n = 4). Induction of hypoxia diminished the glycogen content by ~95% (3.7 ± 1 under hypoxia versus 73.6 ± 5 μg/mg protein under normoxia; n = 2). Importantly, glycogen depletion led to diminution in 5-HT-induced maximal contractility to 37 ± 11% with an accompanying exaggerated increase in AMPK phosphorylation, compared with normoxic conditions (n = 4). Inclusion of exogenous D-glucose (5.5 mM) prevented the exaggerated increase in AMPK phosphorylation thereby restoring 5-HT-induced maximal contractility to 125 ± 6% (n = 4). Parallel studies that included either L-glucose or 2-deoxy-D-glucose (non-metabolizable forms of glucose) did not show any changes in contractility or AMPK phosphorylation. Conclusion: The present findings suggest that depletion of smooth muscle glycogen during hypoxia may limit the availability of intracellular glucose in vein grafts, thereby enhancing AMPK phosphorylation to promote vasorelaxation.

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Spatiotemporal Regulation of β Adrenergic Signaling In Heart failure

Yang K Xiang, Federica Barbagallo, Bing Xu, Qin Fu, Univ of California at Davis, Davis, CA

Our long-term goal is to understand mechanisms that govern spatiotemporal regulation of cAMP/PKA signaling in cardiac myocytes under physiological and pathophysiological conditions, and their implication in cardiac disease therapy. Here we use a series of biosensors to measure cAMP/PKA activity under βAR subtype regulation. In failing cardiac myocytes, the cAMP and PKA activity are shifted from the plasma

membrane to the intracellular sarcoplasmic reticulum and the myofilaments. Meanwhile, β2AR displays an increased role in signaling to the myofilaments in failing myocytes when compared to the control myocytes. Moreover, we show that an increased βAR association with phosphodiesterases promotes the alteration in spatiotemporal propagation of cAMP/PKA signaling in failing myocytes. These observations and the underlying mechanisms and functional implications will be discussed.

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Electrophysiological Consequences of AAV9 mediated SERCA2a Gene Transfer to Normal Rat Myocardium

Chaoqin Xie, Dongtak Jeong, Erik Kohlbrenner, Roger J Hajjar, Fadi G Akar, MOUNT SINAI SCHOOL OF MEDICINE, New York, NY

SERCA2a gene transfer to the failing heart improves its mechanical function and inhibits arrhythmic triggers caused by aberrant calcium waves. The majority of arrhythmias occur before onset of end-stage heart failure when SR function is not compromised. Examination of the electrophysiological (EP) consequences of SERCA2a gene therapy in normal hearts is imperative in determining its potential toxicity as a preventive therapy during early stages of remodeling. We hypothesized that AAV9 mediated gene transfer of SERCA2a to normal rat hearts may promote the incidence of arrhythmic triggers under conditions of intracellular calcium (Ca2+) overload. Methods: Rats underwent tail vein injection of 5x10E11vg AAV9.SERCA2a (N=8) or AAV9.GFP (N=3). 6 naïve uninjected rats served as controls (Ctl). Using high resolution optical action potential (AP) imaging, we characterized the EP substrate 6 weeks post gene transfer. Ex vivo hearts were examined during normoxic perfusion and challenge with low flow ischemia for 1h followed by reperfusion. Arrhythmia susceptibility was determined under conditions of Ca2+ overload. Results: Surprisingly, AAV9.GFP hearts exhibited markedly prolonged AP durations (APD) and slower conduction velocities (CV) compared to AAV9.SERCA2a and Ctl hearts. Challenge of AAV9.GFP but not AAV9.SERCA2a or Ctl hearts with burst pacing always resulted in VF. Remarkably, APD & CV rate relationships were identical in Ctl and AAV9.SERCA2a hearts (p=NS). Elevation of pacing rate resulted in a comparable level of AP alternans followed by loss of 1:1 capture in both groups. No evidence of triggered APs was found in AAV9.SERCA2a or Ctl hearts in response to burst stimulation. Ischemia for 1hr produced comparable changes in APD and CV. Challenge with burst stimuli during ischemia elicited arrhythmias in 2/6 Ctl and 6/8 AAV9.SERCA2a hearts (p=0.277, NS). Upon reperfusion, 1/4 & 0/2 remaining Ctl and AAV9.SERCA2a hearts exhibited VF. Conclusion: Surprisingly, AAV9.GFP but not AAV9.SERCA2a gene transfer to normal rat myocardium alters EP properties and promotes Ca2+ mediated arrhythmias. Lack of an EP phenotype of AAV9.SERCA2a suggests an upper limit of endogenous SERCA2a expression in normal rodent myocardium that is not exceeded by gene transfer.

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The Regulatory Effects Of Peroxiredoxin II On Phospholamban Phosphorylation And Cardiac Contractile Function In Vivo

Wen Zhao, Xiaojing Shi, Wenjuan Zhou, Huimin Wang, Xuepeng Geng, Shuang Zhang, Jingjing Wang, Zhengzhou Univ, Zhengzhou, China; Evangelia G. Kranias, Univ of Cincinnati Coll of Med, Cincinnati, OH; Hong-Min Liu, Zhengzhou Univ, Zhengzhou, China

Peroxiredoxin II (prxII), a cytosolic form of the anti-oxidant peroxiredoxin family, was recently found to be decreased in failing human hearts. Interestingly, in hyperdynamic hearts of two genetically modified mouse models with: a) phospholamban ablation; and b) overexpression of the active inhibitor-1 of protein phosphatase 1, the levels of this cellular peroxidase (prxII) were markedly increased. Acute overexpression of prxII by adenoviral-delivery in adult rat

cardiomyocytes (Ad-prxII) was associated with decreases in the basal rates of contraction and relaxation, as well as calcium kinetics. Accordingly, Ad-prxII-AS infected cardiomyocytes exhibited enhanced contractile parameters and Ca-kinetics. The depressed or increased contractility by Ad-prxII or Ad-prxII-AS was associated with parallel decreases or increases in phosphorylation of phospholamban (Ser16 and Thr17). To determine the in vivo effects of prxII on cardiac contractility, three transgenic lines (TG) with 2-3 fold cardiac-specific overexpression of prxII were generated and their cardiac morphologic and functional phenotypes were characterized. The TG mice exhibited no alterations in cardiac pathology or morphology up to 4 months of age. However, langendorf perfusions revealed that cardiac contractility, including the rates of contraction and relaxation (±dp/dtmax) as well as the left ventricular end systolic pressure (LVESP), were significantly depressed in TG mice (to 75, 76 and 63%, respectively), compared to WTs (100%). The depressed function was not associated with any alterations in the expression levels of key SR calcium handling proteins: SERCA2, total phospholamban, calsequestrin and ryanodine receptor. However, the levels of the phosphorylated PLN at Ser16 were found to be reduced to 50% in the TG mice, compared to WTs. These findings indicate that prxII, an anti-oxidant protein, may regulate basal cardiac contractile performance in vivo through phospholamban phosphorylation.

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Insights Into The Transcriptional Reprogramming Induced By Beta-3 Adrenergic Cardioprotection

Jaime Garcia-Prieto, David Sanz-Rosa, Fatima Sanchez-Cabo, Ana Dopazo, Valentin Fuster, Borja Ibanez, CNIC, Madrid, Spain

Background: Beta-3 adrenergic receptor (β3AR) stimulation reduces myocardial ischemia/reperfusion injury (IRI) in vivo. The molecular mechanisms that regulate this cardioprotective therapy are a matter of intense research. The aim of this study was to assess the transcriptional reprogramming underpinning cardioprotection.
Methods and Results: HL-1 cardiomyocytes were subjected to 6 hours of hypoxia followed by 18h of reoxygenation (H/R) in the presence or absence of the beta-3 adrenergic receptor agonist BRL37344 (5μM). As expected, H/R caused cardiomyocyte death, which was significantly supressed by beta-3 agonist treatment. In order to uncover the molecular basis of cytoprotection we analysed gene expression profiles in HL-1 cells subjected to hypoxia (6h) and reoxygenation (1h, 6h). Immediate reoxygentation proufoundly altered the hypoxia gene expression signature at 1h but this effect was lost upon extended reoxygenation. BRL treatment was able to reverse transcriptomic alterations associated with increased cell death, by supressing the dysregulation of cell survival pathways (possibly implicating mTORC, EGF, eNOS, JAK/Stat, and IL-10 signalling.)
Conclusion: Beta-3-adrenergic stimulation induces an adaptive transcriptional reprogramming that preserves cell viability by converging a variety of cytoprotective pathways.

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Diet-Induced Leukocyte Telomere Shortening Correlates with Extent of Atherosclerosis

GENESIO KARERE, Shifra Birnbaum, Clint Christensen, Michael Mahaney, John VandeBerg, Laura Cox, TEXAS BIOMEDICAL RESEARCH INSTITUTE, SAN ANTONIO, TX

Introduction:
Cardiovascular disease, the leading cause of death in developed countries, is commonly due to atherosclerosis. Studies have demonstrated association between leukocyte telomere shortening (LTS), extent of atherosclerotic lesions and accelerated cell senescence. Further LTS is associated with dietary intake. However, efforts to link LTS, diet and extent of lesions have been unsuccessful in humans due to difficulties controlling diet in large human population studies. To begin addressing these critical issues, we controlled dietary fat (high-fat, HF) in baboons for 2yrs - a well-developed

primate model of human atherosclerosis. This is the first study in primates showing correlation of LTS with both chronic HF diet and atherosclerotic lesions.
Hypothesis:
We hypothesized that leukocyte telomere length decreased with chronic HF diet in baboons and is correlated with extent of atherosclerotic lesions.
Methods and Results:
A cohort of pedigreed baboons (n=107; females=46, males=61) was fed a HF diet for 2yrs. Absolute leukocyte telomere lengths (LTL; kb/diploid genome) were quantified by qPCR before and after diet challenge. Total telomere length was calculated by computing the ratio of telomere quantity per single copy gene quantity (baboon LIPG). Mean LTL was significantly shorter after feeding baboons a HF diet for 2 yrs (paired t test, p=0.03). Baboons (n=232) maintained on a low fat diet for 2yrs showed no significant difference in LTL (p=0.47). These findings suggest that a HF diet accelerates LTS. Further we quantified the extent of atherosclerotic lesions in baboons after 2yr HF diet and found that LTL, adjusted for age and sex, were correlated with lesions in descending aorta (Pearson correlation, r=0.19; p=0.03). Interestingly this correlation was significant in females but not in males after adjusting for age (r=0.27, p=0.03).
Conclusions:
LTS correlates with chronic feeding with a HF diet in baboons, is significantly correlated with arterial lesions and the correlation is sex-specific. These findings suggest that LTS may be a potential biomarker of extent of atherosclerosis.

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A Deletion In The N2A Region Of Titin Carried By Muscular Dystrophy With Myositis (mdm) Mice Severely Affects Skeletal Muscle, But Not The Heart

Ida G Lunde, Inst for Experimental Medical Res, Oslo Univ Hosp, Ullevaal, Oslo, Norway; Hiroko Wakimoto, Michael A Burke, Dept of Genetics, Harvard Medical Sch, Boston, MA; Wolfgang Linke, Dept of Cardiovascular Physiology, Ruhr Univ Bochum, Bochum, Germany; Geir Christensen, Inst for Experimental Medical Res, Oslo, Norway; Jonathan G Seidman, Christine E Seidman, Dept of Genetics, Harvard Medical Sch, Boston, MA

20% of dilated cardiomyopathy patients carry mutations in the giant protein titin. Mutations are predominant in A band but also occur in I band, a domain that regulates passive tension and myocyte signaling. A recessive mouse mutation in titin I band N2A region (mdm) causes early onset muscular dystrophy with myositis and death. We assessed cardiac morphology, function, and transcriptional profiles (RNAseq) in mdm mice.
Young homozygous mdm mice (n&gt6) have reduced body weight (7gms) vs. heterozygous (20gm) or WT (17gm) littermates, with severe skeletal muscle dystrophy. Four-week old homozygous mdm mice have higher left ventricular (LV): body weight ratios. Echocardiography revealed thinner LV posterior wall and septum (LVPWd and IVSd) and normal LV diameter (LVDd); when normalized for body weight, cardiac dimensions were increased compared to WT or heterozygous mdm mice. Fractional shortening was reduced in homozygous Mdm mice (35%) vs. WT (40-41%, p&lt0.01); histology showed neither overt pathology nor fibrosis. Titin gels showed lack of difference in cardiac titin isoform pattern, consistent with RNAseq, which showed the mdm titin transcript excluded exons 107 and 108, deleting in frame 48 amino acids. 240 transcripts (0.8%) were differentially expressed (fold change &gt1.5 and &lt0.75, p&lt0.001) in homozygous vs. heterozygous mdm hearts; ANP and BNP were mildly upregulated (2- and 1.2-fold). Altered transcripts participated in extracellular and immune signaling pathways. Among titin binding partners, only calpain-3 that interacts with N2A was changed (0.6-fold), consistent with previous reports in skeletal muscle. As humans have heterozygous mutations, we stressed adult heterozygous mdm and WT mice (2 weeks of angiotensin II infusion): both had comparable hypertrophic responses (increased LVPWd and IVSd). Aged (89 week old) unstressed heterozygous mdm mice had normal cardiac dimensions and function.
The N2A region, I-band titin mdm mutation causes minimal cardiac dysfunction in mice, unlike the severe skeletal muscle phenotype.

Human I-band mutations are unlikely to cause dilated cardiomyopathy.

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Thrombospondin-1 Carries CD55 like Domains: A Novel Target for E. coli Invasion into Human Heart Cells

Tanu Rana, Pius N Nde, Stella Nowicki, Aniekanabasi N Udoko, Rajbir Singh, Candice A Johnson, Andrey Dykan, Meharry Medical Coll, Nashville, TN; Jack Lawler, Beth Israel Deaconess Medical Ctr, Boston, MA; Fernando Villata, Bogdan J Nowicki, Meharry Medical Coll, Nashville, TN

Introduction: E. coli septicemia with cardiovascular dysfunction is among most frequent causes of human mortality however, underlying molecular mechanisms remain unknown. We recently described a fatal case of uro-sepsis with E. coli, bearing invasion-ligand Dr-adhesin, resulting in myocardial necrosis. Here we investigated an unrecognized mechanism of pathogen mediated cardiac cell invasion as a contributing factor to cardiovascular pathology and patient mortality. Methods: The experiments on human cardiac fibroblasts (HCF) showed E. coli colonization and invasion. However, the primary cell receptor for Dr adhesin, CD55, was undetectable. Searching for an alternative receptor we focused on matrix protein TSP-1 which is elevated in heart during infection. We over-expressed TSP1 in HCF cells (HCF-TSP1) and challenged with Dr+ E. coli. Bacterial binding/invasion to HCF-TSP1 and wild-type (WT) HCF were compared and evaluated. To rule out the possibility of fibroblast invasion due to presence of CD55 we performed Western blotting with CD55 antibody. Analysis of TSP-1 and CD55 epitopes was done to look for bacteria binding domains. Results: A dose-dependent TSP-1-E. coli binding assay in triplicates showed bacteria-HCF specific interactions. HCF-TSP1 had 13±4 bacteria bound per cell and invasion level of 2000/well which was up to 2.5 fold higher compared to WT-HCF. Both immunostaining and immunoblotting showed presence of TSP1 and absence of CD55 in HCF-TSP1. The interaction between purified/recombinant TSP1 coated to microtiter plates showed dose-dependent attachment of E. coli which increased with TSP1 concentrations. Sequence analysis of TSP-1 and CD55-SCR-3 revealed multiple 12AA CD55-like sequences within TSP-1. CD55-like epitopes identified on TSP1 shared up to 80% identity with the CD55 functional epitopes required for complement inhibition, Dr+ E. coli binding and invasion. Conclusions: Here we identified cardiac fibroblasts as target for uro-septic E. coli invasion. We discovered TSP-1 as the receptor facilitating Dr+ E. coli invasion process and identified previously unrecognized CD55-like sequences within TSP-1 which in CD55 account for functional epitopes allowing E. coli attachment, invasion and complement inhibition.

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Genetic Dissection of Cardiac Remodeling in an Isoproterenol-induced Heart Failure Mouse Model

Jessica Wang, Christoph Rau, Milagros Romay, Rozeta Avetisyan, Yibin Wang, Jake Lusis, UCLA, Los Angeles, CA

Rationale:Family-based linkage analyses of inherited cardiomyopathies have revealed rare mutations with large effects on cardiac structure and function in dozens of genes. However, the genetic factors contributing to common, late onset forms of heart failure are largely unknown. Efforts to dissect these using genome-wide association studies in humans have been only modestly successful, largely due to environmental and genetic heterogeneity. Objective:The purpose of this study was to dissect genetic control of cardiac remodeling using an isoproterenol-induced heart failure model in mice, in which we are able to control for environmental factors in an experimental setting. Methods and Results:We characterized the changes in cardiac structure and function in response to chronic isoproterenol infusion using echocardiography in a mapping panel of 105 inbred mouse strains. We showed that cardiac structure and function, whether under normal or stress conditions, have a strong genetic component, with heritability estimates of 64% to 84%. Association analyses of cardiac

remodeling traits, corrected for population structure and multiple comparisons, revealed 3 genome-wide significant loci and 13 suggestive loci, including several loci containing previously implicated genes. Cardiac tissue gene expression profiling, expression quantitative trait loci, expression-phenotype correlation, and coding sequence variation analyses were performed to prioritize candidate gene lists and to generate hypotheses for downstream mechanistic studies.
Conclusions: Our study provided strong evidence for the contribution of common genetic variations on cardiac structure and function. The understanding of how common genetic variants contribute to cardiac remodeling will have wide spread therapeutic implications in delaying or reversing heart failure progression in human populations.

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Functional Analysis Of Microrna s In Vascular Disease

Shusheng Wang, Qinbo Zhou, Chastain Anderson, Tulane Univ, New Orleans, LA

microRNAs (miRNA, miR) are emerging as pivotal modulators of vascular development and disease. Research is my lab is focused on elucidating the functional mechanism and exploring therapeutic potential of microRNAs in vascular diseases. Our recent studies have shown that miR-23 and miR-27 in miR-23~27~24 family are required for proper angiogenesis and neovascularization in a laser-induced vascular injury model. Here we extend our study and provide evidence that miR-24 regulates actin dynamics in ECs through targeting multiple members downstream of Rho signaling, including Pak4, Limk2 and Diaph1 proteins. Consistent with the critical role for actin cytoskeleton in cell motility and proliferation, overexpression of miR-24 in ECs blocks stress fiber and lamellipodia formation, represses EC migration, proliferation and tube formation in vitro, as well as angiogenesis in an ex vivo aortic ring assay. Overexpression of miR-24 in transgenic mice represses postnatal retinal vascular development. Moreover, subretinal delivery of miR-24 mimics represses laser-induced CNV in vivo. Mechanistically, knockdown of miR-24 target protein LIMK2 or PAK4 inhibits stress fiber formation and tube formation in vitro, mimicking miR-24 overexpression phenotype in angiogenesis. Taken together, these findings demonstrate that miR-24 represses angiogenesis by simultaneously regulating multiple components in the actin cytoskeleton pathways, suggesting distinct function of miR-23~27~24 family members in angiogenesis. Manipulation of actin cytoskeleton pathways by miR-24 may represent an attractive therapeutic solution for numerous vascular diseases.

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Nppa Marks A Subset Of Embryonic Cardiomyocytes Predestined To Form Trabeculae In Zebrafish

Rima Arnaout, UCSF, San Francisco, CA; Didier Y.R. Stainier, Max Planck Heart and Lung Res Inst, Bad Nauheim, Germany; Shaun R. Coughlin, UCSF, San Francisco, CA

The development and maintenance of cardiac trabeculae is required for proper ventricular function. Aberrant trabeculation can cause heart failure, arrhythmia, and death. Yet the mechanisms controlling initiation of trabeculation are incompletely understood, partly due to a lack of markers for trabeculation during early embryogenesis. We hypothesized that natriuretic peptide A (nppa) marks trabeculae before cardiomyocytes leave the single-celled ventricular layer. While nppa has been studied in mammals, the external development of the translucent zebrafish embryo offers new insight into the earliest stages of trabeculation. We have found by in situ hybridization, and by creating a fluorescent transgenic zebrafish line, that nppa exclusively marks zebrafish cardiac trabeculae from their onset through adulthood. Live spinning disc confocal video microscopy shows that nppa:GFP-positive cells move into the trabecular layer starting at 60 hours post

fertilization (hpf). GFP-positive trabeculae are seen by 5 days post fertilization (dpf) in normal larvae. However, in cardiac troponin T2a mutants and in erbB2 mutants _ both known not to trabeculate _ nppa is still expressed, but the nppa:GFP positive cells fail to form trabecular projections. In embryos injected with a morpholino against atrial myosin heavy chain 6, where weakened atrial contraction leads to weaker blood flow without affecting the ventricle directly, GFP-positive cells form trabeculae similar to uninjected controls. Using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs), we created nppa mutants causing frameshift deletions. Mutants have poorly contractile hearts at 3 dpf and have been crossed into transgenic reporter lines in order to specifically assess trabeculation. Together, these data suggest that nppa marks a subset of embryonic cardiomyocytes destined to form trabeculae, and that nppa loss of function causes cardiac defects.

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Cyclic stretch of Embryonic Cardiomyocytes Increases Proliferation, Growth, and Expression While Repressing Tgf-β Signaling

Indroneal Banerjee, Depts of Cardiology Univ of California San Diego,, California, CA; Katrina Carrion, Depts of Pediatrics (Cardiology) Univ of California San Diego,, California, CA; Ricardo Serrano, Dept of Mechanical and Aerospace Engineering Univ of California San Diego, California, CA; Jeffrey Dyo, Depts of Pediatrics (Cardiology) Univ of California San Diego, California, CA; Sean Lund, Dept of Med Univ of California San Diego, California, CA; Erik Willems, Muscle Development and Regeneration Program, Sanford-Burnham Medical Res Inst, California, CA; Seema Aceves, 5Dept of Med Univ of California San Diego, California, CA; Rudolph Meili, Dept of Mechanical and Aerospace Engineering Univ of California San Diego,, California, CA; Mark Mercola, Muscle Development and Regeneration Program, Sanford-Burnham Medical Res Inst, California, CA; Ju Chen, Depts of Cardiology Univ of California San Diego, California, CA; Alexander Zambon, Alexander Zambon, Dept of Pharmacology Univ of California San Diego, California, CA; Gary Hardiman PhD, MUSC, Charleston, SC; Taylor A Doherty, Dept of Med Univ of California San Diego,, California, CA; Juan C. del Álamo, Dept of Mechanical and Aerospace Engineering Univ of California San Diego, California, CA; Vishal Nigam, Depts of Pediatrics (Cardiology) Univ of California San Diego, California, CA

Rationale: Perturbed biomechanical stimuli are critical for the pathogenesis of a number of congenital heart defects, including Hypoplastic Left Heart Syndrome (HLHS). While ventricular cardiomyocytes experience biomechanical stretch every heart beat, the molecular responses of embryonic cardiomyocytes to biomechanical stimuli are poorly understood. In this study, we examined how cyclic mechanical stretch modulates embryonic cardiomyocytes to improve understanding of normal and pathologic ventricular development. We hypothesize that biomechanical stimuli activates specific signaling pathways that impact proliferation, gene expression and myocyte contraction.
Objective: The objective for this study was to examine key molecular and phenotypic responses of embryonic cardiomyocytes to cyclic stretch that will provide a deeper understanding of HLHS.
Methods and Results: Embryonic mouse cardiomyocytes were exposed to cyclic stretch. Analysis of RNA-Sequencing data demonstrated that gene ontology (GO) groups associated with myofibril and cardiac development were significantly modulated. Stretch increased cardiomyocyte proliferation, size, and cardiac gene expression. Since the Tgf-β GO term was modulated by stretch, the role of Tgf-β in the cardiomyocyte response to stretch was examined. Stretched Cardiomyocytes had decreased Tgf-β expression, protein, and signaling. Functionally, Tgf-β signaling repressed cardiomyocyte proliferation, and both inotropic and chronotropic contractile function, which was assayed for confluent cell cultures by dynamic monolayer force microscopy (DMFM). Tgf-β inhibitor treatment resulted in increased cardiomyocyte size.
Conclusions: Herein, we observed that cyclic stretch promotes cardiomyocyte proliferation, growth, and gene expression. Stretch-mediated repression of Tgf-β appears to play a key role. Together these findings advance the understanding of how the

biomechanical/molecular axis modulates ventricular development.

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Myocardin-related Transcription Factors Control Epicardial EMT and Coronary Vessel Homeostasis

Michael A Trembley, Lissette S Velasquez, Corey M Hoffman, Karen L de Mesy Bentley, Eric M Small, Univ of Rochester Sch of Med and Dentistry, Rochester, NY

The epicardium is a source of multi-potent progenitor cells that envelop the heart and contribute to various cardiac lineages through the process of epithelial-to-mesenchymal transition (EMT). Although known upstream cues promote epicardial EMT via changes in gene expression and actin cytoskeletal dynamics, the link between these signals and progenitor cell motility remain unclear. Myocardin-related transcription factor (MRTF) cofactors are primarily retained in the cytoplasm through interactions with G-actin. However, upon cytoskeletal reorganization and G-actin depletion, nuclear MRTFs associate with serum response factor (SRF) to drive cellular motility and contractility gene programs reminiscent of EMT. Here, we identify the SRF/MRTF gene regulatory axis as a key mediator of epicardial EMT. We found significant enrichment of MRTF-A and -B in the epicardium prior to EMT. MRTFs were later expressed in a spatial and temporal manner concurrent with EMT and epicardial-derived cell (EPDC) differentiation. Furthermore, MRTF deletion attenuates contractile gene expression in epicardial explants and impairs migration of EPDCs into subjacent cell layers using *ex vivo* assays. Epicardial EMT and EPDCs differentiation are necessary for proper coronary vessel formation. We found that genetic ablation of MRTFs results in disrupted coronary plexus formation, endothelial cell dysfunction, and sub-epicardial hemorrhage. The vascular phenotype observed in MRTF-A/B^{epiDKO} mice results in part from the depletion (~50% reduction) of epicardial-derived coronary pericytes. These data suggest a critical role for MRTFs in coronary vessel formation by regulating epicardial EMT and mobilizing EPDCs.

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Chamber Specific Function of p38 MAP Kinases in Right Ventricle Development, Hypertrophy, and Dysfunction

Tomohiro Yokota, Vincent Ren, Yibin Wang, UCLA, Los Angeles, CA

Background:
Postnatal heart maturation is a well orchestrated process involving chamber specific changes in myocyte proliferation and growth. While the left ventricle undergoes significant growth, the right ventricle regresses relative to the left in terms of both myocyte number and size. While much attention has been focused on the left ventricular myocyte proliferation and hypertrophy, right ventricle specific changes are poorly studied. p38 MAP kinase is a well established signaling pathway for stress response and also plays an important role in cell differentiation, viability and proliferation. Its role in chamber specific postnatal heart maturation, however, has not been demonstrated.
Results and Methods:
We observed RV specific induction of myocyte apoptosis and suppression of myocyte proliferation in neonatal hearts, coinciding with a transient induction of p38 activity significantly higher in the RV relative to the LV at P3 and P7 wild-type mouse hearts. In cardiomyocyte specific p38 MAP kinase alpha&beta double KO (p38ab cdKO) mice, echocardiographic and histological analyses revealed RV specific chamber enlargement and dysfunction associated with significant postnatal lethality. Apoptotic activity in the RV was suppressed at P1 and myocyte proliferation and hypertrophy

were significantly elevated at P3 and P7 in the p38ab cdKO hearts compared to the wild-type. In contrast, no differences were observed in the LV between the p38ab cdKO and wild-type. Consequently, the surviving p38ab cdKO mice showed significant and RV specific enlargement and dysfunction, leading to eventual RV failure and pulmonary hypertension.
Conclusion: p38 a&b MAP kinases are essential to RV specific maturation in postnatal hearts. p38ab cdKO mice represent a unique RV-specific hypertrophy and heart failure model for further studies.

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Rnd3/RhoE Regulates Cardiac Ryanodine Receptor Type 2 Stability

xiangsheng yang Sr., Texas A&M Health Science Ctr, Houston, TX; Tiannan Wang, Baylor Coll of Med, Houston, TX; Xiaojing Yue, Texas A&M Health Science Ctr, Houston, TX; Xander H.T. Wehrens, Baylor Coll of Med, Houston, TX; Jiang Chang, Texas A&M Health Science Ctr, Houston, TX

Rationale: Rnd3, a small Rho GTPase, is involved in the regulation of cell actin cytoskeleton dynamics, cell migration and proliferation. The biological function of Rnd3 in the heart remains unexplored.
Objective: To define the functional role of the *Rnd3* gene in the animal heart and investigate the associated molecular mechanism.
Methods and Results: By loss-of-function approaches, we discovered a new role in which Rnd3 stabilizes the ryanodine receptor type 2 (RyR2) Ca^{2+} release channel. Genetic deletion of Rnd3 in mice resulted in embryonic lethality with heart failure and arrhythmia. Both $Rnd3^{-/-}$ embryonic and $Rnd3^{+/-}$ adult cardiomyocytes showed severe Ca^{2+} leakage. Single channel assessment showed the destabilized RyR2 channel, and this irregular spontaneous Ca^{2+} release was curtailed by protein kinase A (PKA) inhibitor treatment. Further studies found that RyR2 protein was hyperphosphorylated by PKA in the mutant heart. Remarkable increases in the PKA activity along with elevated cyclic adenosine monophosphate levels were detected *in vivo* in Rnd3-null embryos and *in vitro* in neonatal rat cardiomyocytes and non-cardiac cell lines with Rnd3 knockdown. Moreover, we found increasing β_2-adrenergic receptor (β_2AR) protein levels, but no correlated mRNA changes in both the Rnd3-null heart and non-cardiac cells with Rnd3 knockdown. Immunoprecipitation analysis demonstrated that Rnd3 and β_2AR physically interacted. Multiple post-translational modification analyses of β_2AR revealed that downregulation of Rnd3 attenuated β_2AR protein lysosomal targeting and ubiquitination, which in turn resulted in the elevation of β_2AR protein levels contributing to the activation of PKA signaling. Rnd3 deficiency had no effects on the hydroxylation- and sumoylation-mediated β_2AR protein degradation.
Conclusion: Rnd3 is a unique stabilizer of RyR2 that impacts intracellular Ca^{2+} handling in the heart.

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Retinoblastoma Gene Deletion Promotes Cardiac Dysfunction, Fibrosis and Apoptosis in Response to Pressure Overload

Paul B Ammann, Takanobu Yamamoto, Peiyong Zhai, Junichi Sadoshima, Rutgers New Jersey Medical Sch, Newark, NJ

The retinoblastoma (Rb) protein is a universal cell cycle regulator in mammals. When the Rb protein is phosphorylated by Cyclins/Cdks, it dissociates from E2F, and Rb-dependent E2F repression is subsequently inactivated. Furthermore, the Rb protein has also been implicated in the regulation of cardiac hypertrophy and apoptosis in cardiomyocytes (CMs).
To elucidate the role of Rb in response to mechanical stress, we conducted transverse aortic constriction (TAC) in cardiac-specific Rb knockout mice (cRb-KO) in vivo (C57BL/6J). Cardiac-specific deletion of Rb was achieved by crossing Rb flox/flox mice with αMHC-Cre mice. Under basal conditions, 3- to 5-month-old cRb-KO mice showed increased heart weight (HW) (left ventricular weight/ tibial length (TL): 5.93 ± 029 vs. 4.76 ± 0.14, p< 0.01), increased apoptosis as determined by TUNEL staining (0.12% vs. 0.02%, p< 0.05) and a trend towards cardiac dysfunction (-dP/dt: 4320 ± 388 vs. 5933

ABSTRACTS

± 489 mmHg/sec, $p < 0.05$) compared to control mice (Rb flox/flox).
Following 2 weeks of TAC, cRb-KO mice showed increased heart weight (HW/TL: 8.58 ± 0.35 vs. 7.50 ± 0.24, $p < 0.05$), cardiac dysfunction (ejection fraction (EF): 51.1% ± 4.0 vs. 74.3% ± 0.9, $p < 0.01$) , increased apoptosis as determined by TUNEL staining (0.48% vs. 0.05%, $p < 0.01$) and increased fibrosis as determined by Masson's Trichrome staining (1.84% vs. 1.03%, $p < 0.05$) compared to Rb flox/flox mice after TAC.
In response to 4 weeks of TAC, cRb-KO mice showed increased heart weight (HW/TL: 12.93 ± 085 vs. 9.32 ± 0.34, $p < 0.01$), lung weight (LW) (LW/TL: 18.35 ± 2.66 vs. 10.21 ± 1.93, $p < 0.01$), cardiac dysfunction (EF: 34.5% ± 8.3 vs. 64.3% ± 8.9, $p < 0.01$), increased apoptosis as determined by TUNEL staining (0,42% vs. 0,18%, $p < 0.05$) and increased fibrosis as determined by Masson's Trichrome staining (4.2 % vs. 1.1 %, $p < 0.05$) compared to Rb flox/flox mice after TAC.
Pressure gradients were similar between the cRb-KO mice submitted to 2 and 4 weeks of TAC and their respective controls.
In conclusion, our results suggest that endogenous Rb plays an important role in mediating cell survival in CMs and negatively regulates cardiac hypertrophy at baseline. Furthermore, we showed that the Rb protein is important for the maintenance of cardiac function in response to pressure overload.

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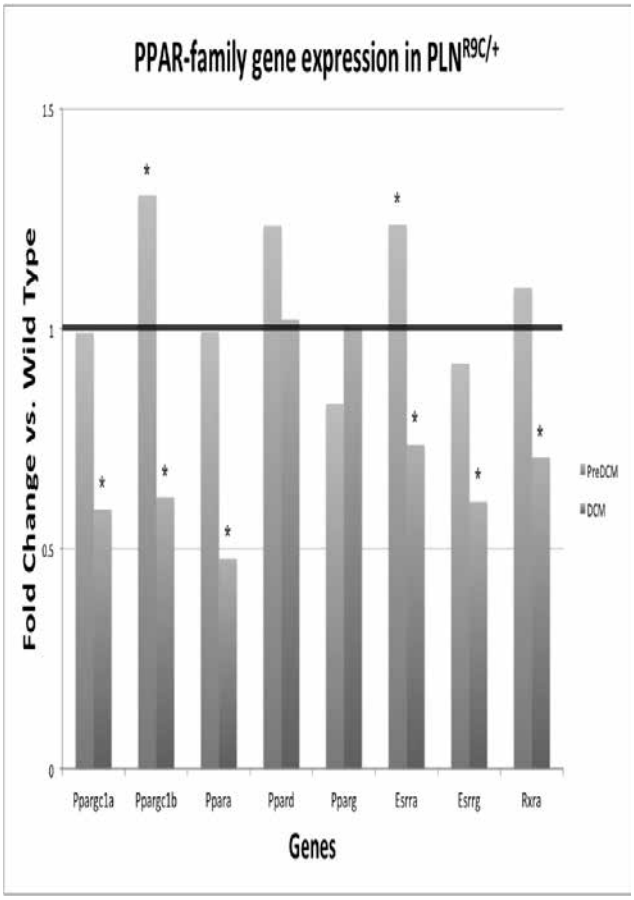
Proliferation of Cardiac Fibroblasts Defines Early Stages of Genetic Dilated Cardiomyopathy and Precedes Myocardial Metabolic Derangement

Michael A Burke, Brigham and Women's Hosp, Boston, MA; Stephen Chang, Harvard Medical Sch, Boston, MA; Danos C. Christodoulou, Harvard Medical Schoo, Boston, MA; Joshua M. Gorham, Hiroko Wakimoto, Harvard Medical Sch, Boston, MA; Christine E. Seidman, Brigham and Women's Hosp, Boston, MA; Jonathan G. Seidman, Harvard Medical Sch, Boston, MA

The complex molecular networks underpinning DCM remain poorly understood. To study distinct pathways and networks in the longitudinal development of DCM we performed RNAseq on LV tissue from mice carrying a human DCM mutation in phospholamban ($PLN^{R9C/+}$) before phenotype onset (pre-DCM), with DCM, and during overt heart failure (HF), and also on isolated myocytes and non-myocytes from DCM hearts. $PLN^{R9C/+}$ mice show progressive fibrosis (20% vs. 1% control, $p=6x10^{-33}$; n=3) associated with proliferation of cardiac non-myocytes (33% increase over control, $p=6x10^{-4}$; n=3). Consistent with this, cardiac non-myocytes have upregulated gene expression and pathways, while these are generally downregulated in myocytes. Non-myocytes were enriched in fibrosis, inflammation, and cell remodeling pathways, from pre-DCM to HF. In contrast, myocytes were enriched for metabolic pathways only with overt DCM and HF. Myocytes showed profound derangement of oxidative phosphorylation with DCM ($p=2.5x10^{-41}$; 44% (53/120) of pathway genes downregulated), suggesting mitochondrial dysfunction. Additionally, we detected probable inhibition of peroxisome proliferator-activated receptor (PPAR) signaling by diminished expression of pathway genes (Figure). DCM and hypertrophic remodeling was compared using RNAseq of a mouse model of HCM; similar patterns of fibrosis with myocyte metabolic dysregulation were evident despite unique differential gene expression patterns between models. DCM caused by $PLN^{R9C/+}$ is associated with early non-myocyte proliferation and later myocyte metabolic derangement possibly governed by altered PPAR signaling, and is common to DCM and HCM.



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Brain Derived Neurotrophic Factor Induced Upregulation Of Peroxisome Proliferator-activated Receptor Gamma Coactivator 1-alpha Signaling Prevents Hearts From Heart Failure Progression Against Pressure Overload

Ning Feng, Guangshuo Zhu, Johns Hopkins Sch of Med, Baltimore, MD; vidhya Sivakumaran, Loyola Univ, Chicago, IL; Manling Zhang, Univ of Pittsburgh, Pittsburgh, PA; Djahida Bedja, Eiki Takimoto, Nazareno Paolocci, Johns Hopkins Sch of Med, Baltimore, MD

Background: The heart is under the influence of neurotrophins (NTs) secreted from peripheral sympathetic nerves, including brain derived neurotrophic factor (BDNF). BDNF is indispensible for cardiac development and vascular wall integrity. Yet, whether BDNF signaling plays a role in governing cardiac function in response to stress is unclear. Hypothesis: BDNF signaling contributes to maintain proper cardiac structure/function in pressure overloaded mice. Results: BDNF expression is markedly down-regulated in hearts subjected to transverse aortic constriction (TAC). Cardiac specific over-expression of BDNF (BDNF-TG) or administration of a BDNF-mimetic agonist (LM22A-4) preserved cardiac function against pressure overload. In contrast, cardiac-selective deletion of the BDNF receptor, Tropomyosin related kinase receptor B (TrkB), further exacerbated heart failure. In neurons, BDNF up-regulates Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) that regulates energy metabolism and mitochondrial function/biogenesis. Oxidative stress is a major negative modulator of PGC-1a expression/activity. Exposing neonatal rat ventricular myocytes (NRVMs) to hydrogen peroxide downregulated PGC-1α, and BDNF restored it to normal levels, with a concomitant up-regulation of downstream genes involved in both mitochondrial biogenesis and oxidative stress, resulting in attenuated ROS production and increased mitochondrial biogenesis. Consistent with the cultured myocyte findings, PGC-1α and downstream genes were up-regulated in BDNF-TG mice subjected to TAC, associated with attenuated oxidative stress and improved mitochondrial biogenesis; whereas TrkB-/- mice subjected to TAC displayed further decreased PGC-1α expression with worsened oxidative stress and impaired mitochondrial biogenesis.
Conclusion: Our data show that BDNF confers protection against pressure overload via enhanced PGC-1α signaling that in turn prevents oxidative stress and improves mitochondrial biogenesis. Our data suggest BDNF/trkB is a promising new therapeutic avenue to prevent or retard heart failure.

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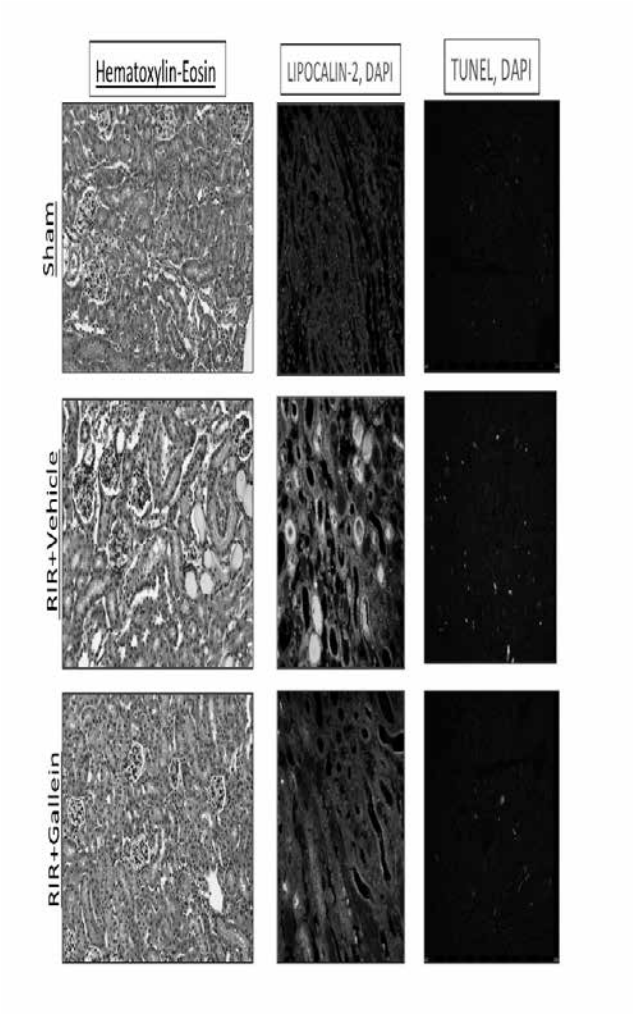
Role Of G-protein Coupled Receptor Signaling In Cardio-renal Injury

Fadia A Kamal, Joshua G Travers, Qing Ma, Prasad Devarajan, Burns C Blaxall, Cincinnati Children's Hosp, Cincinnati, OH

The kidneys play an important role in cardiovascular disease (CVD), where renal co-morbidities accompany CVD in a large proportion of patients thus complicating their treatment regimen. Moreover, the incidence of acute renal injury after cardiac surgery plays an important role in disease progression. Emerging data suggest the importance of understanding the mechanisms of cardio-renal injury and the development of novel therapies that can be safely used with cardiovascular and renal co-existing pathologies. Although the role of G-protein coupled receptors (GPCRs) in CVD has been broadly recognized, their role in renal injury remains poorly understood. We have found, in a chronic mouse model of heart failure, attenuated renal fibrosis and attenuated pathologic RAAS activation by the small molecule GPCR-Gβγ inhibitor “gallein”. To investigate the direct effects of GPCR-Gβγ inhibition on renal injury, we utilized an acute renal ischemia-reperfusion (RIR) mouse model. Gβγ inhibition by gallein pretreatment attenuated the histopathological profile of RIR, including attenuation of tubular hypertrophy, apoptosis, cast formation, and tissue Lipocalin2 expression. This was accompanied by attenuated inflammation, reflected by reduced CCL2 and ICAM1 gene expression and cellular infiltration, in addition to reduced Collagen III gene expression. These preliminary results suggest a promising protective role for Gβγ inhibition in renal injury and remodeling. Future mechanistic investigation of this possible protective effect will provide better understanding of the role of GPCR-Gβγ signaling in cardio-renal injury and remodeling and possible novel therapeutic targets.



F.A. Kamal: None. **J.G. Travers:** None. **Q. Ma:** None. **P. Devarajan:** None. **B.C. Blaxall:** None.

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Decreased Plasma Omega-3 to Omega-6 Polyunsaturated Fatty Acid Ratio Associated with Apical Ballooning Syndrome (Tako-Tsubo cardiomyopathy -TTC)

Youichi Katoh, Show Nagamine, Takeshi Wada, Hiroyuki Isogai, Dai Ozaki, Ryosuke Shimai, Midori Kakihara, Hiroshige Yuu, Ken Yokoyama, Tetsuya Oigawa, Kenji Yaginuma, Yuji Nakazato, Juntendo Univ Urayasu Hosp, Chiba, Japan

Background: Eicosapentaenoic acid (EPA) of the omega-3 polyunsaturated fatty acids (ω-3

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PUFA) family plays important roles in the prevention of cardiovascular disease, while, arachidonic acid (AA) of the ω-6 PUFA family promotes inflammatory and prothrombotic influences. Tako-Tsubo cardiomyopathy (TTC) is a heart syndrome associated with transient myocardial contractile dysfunction. Decreased endothelial function in response to acute mental stress has been reported in patients with a prior episode of TTC. However, the pathogenesis of TTC remains unclear and the relationship between TTC and EPA/AA ratio has not been elucidated.
Methods and Results: This study consisted of 10 consecutive patients with clinically diagnosed TTC. The aim of this study was to investigate the association between the plasma EPA/AA ratio and the early stage of clinically diagnosed TTC. To examine the plasma fatty acid level, blood samples were obtained from control, old myocardial infarction, and clinically diagnosed TTC patients. A clinically diagnosed TTC patients revealed a lower plasma EPA/AA ratio [Control patients, 0.58 (n=18) vs OMI patients, 0.38 (n=22) vs TTC patients, 0.12 (n=10), p=0.011]. High-sensitivity CRP levels and a low plasma EPA/AA ratio could independently predict the prevalence of TTC on multivariate logistic regression analysis [odds ratio 1.83 (95%CI 1.03-3.25), p=0.036 and odds ratio 2.05 (95%CI 1.12-3.92), p=0.02)].
Conclusion: In patients with TTC, a low plasma EPA/AA ratio was significantly associated with the early stage of clinically diagnosed TTC. The findings implicate lower EPA/AA ratio and the following endothelial dysfunction as a potential mechanism involved in the pathogenesis of this unique cardiomyopathy.

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Cardiac-Specific Gene Leucine-Rich Repeat Containing 10 (Lrrc10) is required for proper Cardiac Contractility and Responses to Biomechanical Stress

Youngsook Lee, Matthew Brody, Li Feng, Courtney Reynolds, Ravi Balijepalli, Univ of Wisconsin-Madison, Madison, WI

The molecular genetic basis for early onset dilated cardiomyopathy (DCM) is largely unknown. We have identified that Lrrc10 is a cardiac-specific gene regulated by Nkx2.5 and GATA4. Lrrc10-null (Lrrc10-/-) mice exhibit prenatal systolic dysfunction followed by early onset DCM after birth. However, mechanistic roles for LRRC10 in the regulation of cardiac pathophysiology are unknown. We hypothesize that LRRC10 expression is critical for myocyte contractility and proper responses to pressure overload. Various mouse models with pathological cardiac hypertrophy showed decreased levels of Lrrc10. To test roles of LRRC10 in response to biomechanical stress, transverse aortic constriction (TAC) was performed. Lrrc10-/- mice showed significant increases in left ventricular inner diameter dimensions and drastically reduced % fractional shortening in response to 4 weeks of TAC (13%) after 4 weeks, while no significant changes occurred in WT after TAC (26% F.S.). These data indicate that pressure overload greatly exacerbates systolic dysfunction in Lrrc10-/- vs WT hearts. Next, the role of LRRC10 in cardiac excitation contraction coupling was investigated. Myocyte contractility and calcium transients were studied using the video-edge detection system and fura 2-AM loading at basal levels and following perfusion with Isoproterenol (ISO, 10 nM) at 0.5 - 2 Hz pacing. Cell shortening was significantly reduced following perfusion with ISO in Lrrc10-/- myocytes (55% and 49% at 1 and 2 Hz, respectively) vs WT. Calcium transient analysis showed that there were no differences in peak amplitudes of [Ca2+]i between Lrrc10-/- and WT myocytes at baseline or with ISO treatment. Whole-cell voltage clamp experiments demonstrated a significant reduction in the L-type Ca2+ current (ICa,L) density in Lrrc10-/- myocytes (-2.5 ± 0.2 pA/pF) compared to WT myocytes (-6 ± 0.6 pA/pF). The inactivation of the ICa,L in Lrrc10-/- myocytes was significantly delayed. Further, co-immunoprecipitation showed that LRRC10 directly associated with Cav1.2 subunit of LTCC, which correlates well with the localization of LRRC10 to dyadic regions in adult cardiomyocytes by TEM. Therefore, LRRC10 is a novel DCM candidate gene essential for normal myocyte contractility via regulating ICa,L.

Y. Lee: None. **M. Brody:** None. **L. Feng:** None. **C. Reynolds:** None. **R. Balijepalli:** None.

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Beta3 Integrin Promotes Protein Ubiquitination and Prosurvival Signaling in Hypertrophying Myocardium

Dorea L Pleasant, Kamala P. Sundararaj, Rebecca Hartson, Sundaravadival Balasubramanian, Dhandapani Kuppuswamy, Medical Univ of South Carolina, Charleston, SC

Cardiac hypertrophy occurs in response to stress. Upon hypertrophic stimulus, β3-integrin, a cell surface receptor undergoes activation. Using β3-integrin germline knockout (β3-/-) mice, our recent work shows that β3-integrin is required for ubiquitin (Ub)-mediated prosurvival signaling during early pressure overload (PO)-induced cardiac hypertrophy. Further, attenuation of β3-integrin signaling during PO results in significant cardiomyocyte apoptosis and thus compromised ventricular function. In the present study, we ensured that the Ub that promotes prosurvival signaling in early PO is not a result of decreased proteasome function. For this, WT mice were subjected to short and long term PO by transverse aortic constriction (TAC) or Sham surgery. Greater proteasomal (chymotrypsin-like) activity was detected in TAC samples, indicating that the transient increase in protein Ub observed in PO myocardium was not due to insufficient proteasomal function but due to augmented Ub. Next, we explored whether β3-integrin activation specifically in cardiomyocytes is critical for cardiomyocyte survival, since our parallel work revealed that β3-integrin contributes to cardiac fibrosis as well. Therefore, we have generated mice with cardiomyocyte specific, conditional deletion of β3-integrin (CMΔ*itgb*3) for use in PO studies. The resulting mice contain a double transgenic system for cardiac specific expression of *Cre,* under the control of Tnnt2 promoter, and temporal manner using a Tet-On system (reverse tetracycline transactivator, rtTA). Our goal here is to utilize these mice and further elucidate the prosurvival role of β3-integrin specifically in cardiomyocytes. We are currently using CMΔ*itgb*3 and WT mice treated with or without doxycycline (DOX) and subjecting them to TAC for 72 h or 1 week. Protein and tissue samples from these mice are being analyzed for changes in β3-integrin, Ub, proteasome function and several apoptotic proteins. Ultimately, these studies seek to identify potential targets and develop novel therapeutic methods to encourage cardiomyocyte survival and prevent/delay maladaptive changes in hypertrophying myocardium.

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Paroxysmal And Sustained Atrial Fibrillation In A New Large Animal Model Of Nonischemic Heart Failure

Saad Sikanderkhel, Olawale Onibile, Gregory P Walcott, Steven M Pogwizd, Univ of Alabama at Birmigham, Birmingham, AL

Introduction: Atrial fibrillation is common in heart failure (HF). Understanding of the mechanisms of atrial fibrillation (AF) is limited by the paucity of large animal AF models, especially in the failing heart. We developed a large animal model of nonischemic heart failure (HF) in dogs by combined aortic insufficiency and aortic constriction and observed that a number of HF dogs developed paroxysmal AF on holter monitor. Here we characterize the spontaneously-occurring pAF in these HF dogs and perform electrophysiologic (EP) assessment of atrial refractoriness and AF inducibility along with echocardiographic imaging of left ventricle (LV) and left atrium (LA). Methods: HF was induced in dogs by aortic insufficiency and aortic constriction, and serial echocardiography (for LV fractional shortening (FS) and LA size) and Holter monitoring was performed. In control and HF dogs, EP study of atrial refractory period (AERP) and AF inducibility (duration and atrial cycle length (CL)) was performed. Results: By Holter monitoring, paroxysmal AF was noted in 5 dogs with episodes ranging from 15 to 94 beats long (mean of 49±27 beats, n=12). In EP studies, control dogs (N=3) exhibited AERP of 176±8 ms. Burst pacing resulted in AF of very brief duration (mean 32±24 sec) and a mean AF CL of 138±6 ms. LV FS averaged 37% and LA size averaged 4.3 cm2. HF dogs (N=5) exhibited RAERP of 150±8

(p=0.05 vs control). Two of these dogs had sustained AF with ventricular response up to 230 bpm on Holter monitor. In the other 3 HF dogs, burst pacing induced AF with a mean duration of 232±185 sec (at times with conversion to atrial flutter) and with a mean AF CL = 110±4 ms (p=0.002 vs control). Echo data showed LVFS averaged 30% and LA area of 14.9 cm2 (p=0.05 vs control). Conclusion: Thus we have developed a novel large animal model of HF that exhibits paroxysmal and sustained AF. This model will provide an opportunity for the study of underlying AF mechanisms, the progression of remodeling in HF hearts leading to AF, and the assessment of human-scale interventions to better treat and prevent this arrhythmia.

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Low-dose Interleukin-18 is Necessary to Adapt to Pressure Overload in Mice

Iwasaku Toshihiro, Hirotani Shinichi, Eguchi Akiyo, Suminatni Naoki, Okuhara Yoshitaka, Morisawa Daisuke, Sawada Hisashi, Naito Yoshiro, Masuyama Tohru, Hyogo Coll of Med, nishinomiya, Japan

Background: We previously reported that circulatory Interleukin-18 (IL-18) level elevates in heart failure (HF) patients. However, the roles of circulatory IL-18 in HF have not been fully elucidated. Continuous infusion of IL-18 induces cardiomyocyte hypertrophy and fibrosis, whereas IL-18 null mice were fragile to pressure overload. We hypothesized that systemic (circulatory) and local (cardiac) IL-18 have distinct effects on HF and cardiac remodeling. Purpose: The purpose of this study was to elucidate the role of circulatory IL-18 in adaptation to pressure overload in IL-18 null mice.
Methods: Wild type (WT) mice and IL-18 null (IL-18-/-) were subjected to transaortic constriction (TAC). Sequential serum IL-18 levels myocardial IL-18 mRNA expressions were determined by ELISA and qRT-PCR in WT mice. After two week of TAC, IL-18 null mice were administered either (IP) saline or recombinant IL-18 intraperitoneally (10ng/20g every other day). Cardiac function was assessed by transthoracic echocardiography. Two weeks after TAC, myocardial samples were obtained. Haematoxylin and eosin stained sections and Masson's trichrome staining sections were prepared.
Results: IL-18 concentration in serum and IL-18 expression in myocardial tissue increased gradually after TAC in WT mice. Forty-seven % (7/15) of TAC-operated IL-18-/- mice and 12% (2/17) of TAC-operated WT mice died of heart failure by 14days. TAC-operated IL-18-/- mice exhibited more severe left ventricular (LV) remodeling, characterized by cardiomyocyte hypertrophy, extensive interstitial fibrosis and elevation of fetal gene expressions compared with TAC-WT mice. Recombinant IL-18 given intraperitoneally improved the survival rate to 100% (10/10) following TAC operation in IL-18-/- mice. Furthermore, exogenous IL-18 administration suppressed ventricular ANP mRNA expression and myocardial cross-sectional area enlargement to non-TAC-operated level, whereas LV enlargement and contractile dysfunction were only partially suppressed in IL-18-/- mice following TAC. Conclusions: Circulatory IL-18 exerts opposing effects on cardiac hypertrophy under pressure-overload. IL-18 produced in the heart may have an effect such as preserving contractile function.

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Pulmonary Hypertension Activates Cardiac Fibroblasts To Release Paracrine Factors Regulating Cardiac Myocyte Dedifferentiation

Lori A Walker, R. Dale Brown, Kurt R. Stenmark, Peter M. Buttrick, Univ of Colorado, Aurora, CO

In virtually all models of heart failure, prognosis is ultimately determined by right ventricular (RV) function. Thus understanding the unique cellular mechanisms that contribute to RV dysfunction is of critical importance. We have approached this question by studying a large animal model that has significant resonance with human disease: exposure of the neonatal calf to hypobaric

hypoxia. This model demonstrates rapid onset of severe pulmonary hypertension (PH) and significant RV dysfunction. Strikingly, the RV of these animals shows a chamber-specific increase in markers of inflammation and local infiltration of activated pro-remodeling macrophages that are proportionate to the rise in PA pressure. In order to test the hypothesis that this inflammatory milieu directly contributes to myocyte dysfunction, we examined the effects of conditioned media (CM) from cardiac fibroblasts isolated from the PH calf on normal adult rat ventricular myocytes (ARVM) in culture. Even brief exposure (<2 days) to RV-CM results in rapid and marked dedifferentiation of ARVM to a neonatal-like phenotype that exhibits spontaneous contractile behavior. Dedifferentiated cells maintain viability for over 30 days with continued expression of cardiomyocyte proteins including TnI, sarcomeric actinin, and desmin. This response is not seen in ARVM exposed to CM from fibroblasts collected from the hypoxic LV or from the normoxic RV. We have characterized the factor(s) that are present in the RV-CM and have established that the active components are proteins with a mol wt >30 kDa and that inflammatory cytokines such as IL-18 are necessary but not sufficient to effect this phenotypic change. These data suggest that local and perhaps focal inflammation in the RV, induced by the combination of pressure overload and hypoxia, has the capacity to signal a phenotypic transformation in a population of RV cardiocytes, which likely contributes to chamber specific dysfunction. Therapies that target and interrupt this inflammatory process either by preventing in-migration of inflammatory cells or by blocking cell-cell signaling have the potential to prevent RV dysfunction.

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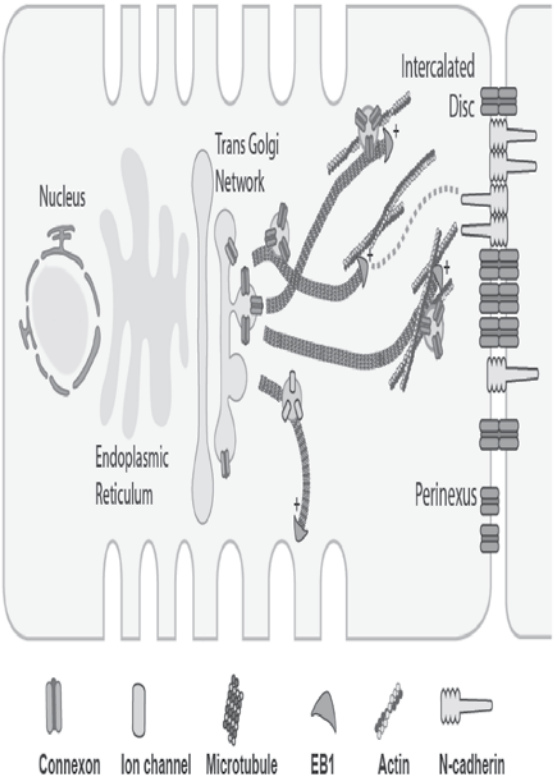
The Actin Cytoskeleton Confers Specificity of Cx43 Delivery to Intercalated Discs

Shan-Shan Zhang, Cedars-Sinai Heart Inst, Los Angeles, CA; SoonGweon Hong, Luke P. Lee, Univ of California, Berkeley, Berkely, CA; Robin M. Shaw, Cedars-Sinai Heart Inst, Los Angeles, CA

Connexin 43 (Cx43) gap junctions (GJs) electrically couple ventricular cardiomyocytes at the intercalated disc (ID), orchestrating organized organ level contraction with each heartbeat. Disease-related disruption of the Cx43 cytoskeletal trafficking machinery is associated with mislocalization of the Cx43 gap junction protein away from the ID and lethal arrhythmias. We recently found that the majority of intracellular Cx43 cargo is associated with actin, not microtubules, and is either paused or moving slowly. It is not understood why actin is involved in Cx43 trafficking. Using micropatterned HeLa cell pairs and whole-cell automated single particle tracking algorithms, we detected that distinct actin polarity exists in the cell, including highly oriented long fibers associated with fast-moving Cx43 cargo aligned toward actively forming GJ plaques. F-actin disruption with latrunculin A (LatA) leads to a loss of Cx43 cargo directionality toward the cell-cell border, as well as a marked decrease in overall microtubule length. We also found a LatA-dependent biochemical interaction between β-actin and the microtubule plus-end-binding protein EB1, which leads growing microtubules and is a necessary component of the Cx43 forward trafficking machinery. In live cell pairs, F-actin disruption resulted in a decrease in overall EB1 activity and in the number of fully extended microtubules that reach the cell-cell border. Together, these results indicate that actin contributes to the specificity of Cx43 delivery by directing EB1-based microtubule growth toward the cell-cell junction (Please refer to attached diagram).



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Deletion of Sam68 Attenuates Myocardial Hypertrophy

Junlan Zhou, Chan Boriboun, Dauren Biyashev, Gangjian Qin, Northwestern Univ, Chicago, IL

The Src-associated in mitosis 68 kDa (Sam68) belongs to the STAR (signal transducer and activator of RNA) family of RNA-binding proteins and has recently been shown to regulate angiotensin II (Ang II) signaling in vascular smooth muscle cells. In this study, we investigated the role of Sam68 in cardiac hypertrophy. Wild-type (WT) and Sam68 knockout (KO) mice were administered continuously with Ang II for 14 days via osmotic minipumps. Echocardiography analyses reveled that basal left-ventricular posterior wall thicknesses (LVPTs and LVPTd) and basal chamber sizes (LVIDs and LVIDd) are similar between the 2 groups of mice, that Ang II induced a significant elevation in LVPTs and LVPTd and reduction in LVIDs and LVIDd in both groups of mice, and that the changes of all these hypertrophic parameters were significantly attenuated in Sam68 KO than in WT mice ($p<0.05$ at day 14, n=15). The increase in the heart weight (HW) / body weight (BW) ratio was also lesser in Sam68 KO mice than in WT mice ($p<0.05$ at day 14, n=15). Then, we performed transverse aortic constriction (TAC) model. Echocardiography analyses confirmed significantly lower levels of LVPTs/d and LVIDs/d in Sam68 KO mice than in WT mice (LVPTs/d: $p<0.05$ at weeks 2 and 4, $p<0.01$ at weeks 8; LVIDs/d: $p<0.05$ at weeks 2, 4, and 8; n=12), however, left ventricular ejection fraction (LV EF) remained not significantly different at all these time points between the 2 groups. Histological analyses of fibrosis by Masson's trichrome staining demonstrated a ~ 2-fold less in collagen deposition areas in Sam68 KO than in WT mice ($p<0.05$ at weeks 8, n=12). The attenuated hypertrophic responses of Sam68 KO mice to both Ang II treatment and TAC surgery was further supported by a lower expression of fetal genes, including β-myosin heavy chain, atrial and brain natriuretic peptides in the heart of Sam68 KO mice than in WT mice (qRT-PCR). Collectively, our results suggest that Sam68 may modulate the cause of cardiac hypertrophy

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Sex Differences In Nonlinear Dynamics And Their Circadian Variation In Control Dogs And In A New Arrhythmogenic Canine Model Of Heart Failure

Yujie Zhu, Steven M Pogwizd, Univ of Alabama at Birmingham, Birmingham, AL

Introduction: Females can be more arrhythmogenic than males, and this sex difference can persist with development of chronic heart failure (CHF). The aim of this study

was to investigate sex differences in the arrhythmogenic substrate in control dogs and in a new arrhythmogenic canine model of CHF. Methods: CHF was induced in 30 dogs by aortic insufficiency and aortic constriction. Holter monitoring assessed VT and PVCs from 30 dogs, as well as traditional HRV measures and nonlinear dynamics (including correlation dimension (CD), detrended fluctuations analysis α1 (DFAα1), and Shannon entropy (SE)) at baseline, 240 days (240d) and 720 days (720d) after CHF induction. Results: At baseline, females had lower LF/HF (0.27±0.03 vs 0.33±0.02, p=0.04), CD (1.60±0.17 vs 2.21±0.15, p=0.01), DFAα1 (0.62±0.03 vs 0.72±0.03, p=0.03), and SE (2.99±0.02 vs 3.10±0.03, p=0.03 vs males). Females lacked circadian variation in LF/HF, DFAα1, and SE while males had circadian variation in all of these. Of 11 dogs with frequent runs of VT and PVCs, 95% and 91% of total VT runs and total PVCs, respectively, were in females. With CHF, all these linear and nonlinear parameters progressively declined in males and females. CHF females had less decline in LF/HF than males so that by 720 days there was no more sex difference (0.24±0.06, 0.17±0.03 in females vs 0.22±0.05, 0.18±0.01 in males at 240d, 720d). However, for nonlinear parameters of CD, DFAα1, and SE, CHF females had lower values than males (CD: 1.56±0.21, 0.99±0.32 vs 1.87±0.24, 1.50±0.34; DFAα1: 0.51±0.05, 0.43±0.04 vs 0.54±0.07, 0.48±0.04; and SE 2.93±0.08, 2.76±0.08 vs 3.01±0.11, 2.91±0.04 in females vs males at 240d, 720d). With CHF, circadian variation in CD, DFAα1, and SE were lost in both males and females.
Conclusions: There are sex differences in the arrhythmogenic substrate in control dogs and in this new arrhythmogenic canine model of moderate CHF. At baseline, females have lower sympathetic stimulation, reduced cardiac chaos, and loss of circadian variation in nonlinear dynamics. With CHF, sex differences in nonlinear dynamics persist; this reflects a loss of complexity and fractal properties that could contribute to increased arrhythmias in female CHF dogs.

Y. Zhu: None. **S.M. Pogwizd:** None.

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Sexual Dimorphisms in Cardiomyocytes Exposed to a Pathological Stimulus

Christa Blenck, Pamela Harvey, Leslie Leinwand, Univ of Colorado at Boulder, Boulder, CO

Background: Sex is an important modifier of the development of cardiovascular disease; women consistently display less hypertrophy, fewer changes in pathologic gene expression, and less cardiac dysfunction compared to men exposed to the same pathological stimuli. The signaling pathways responsible for these sexual dimorphisms, especially within the cardiomyocyte, are not well understood. Objective: To investigate sexual dimorphisms in activation of signaling pathways and expression of genes related to cardiomyocyte contractility in response to a pathological stimulus in isolated adult rat ventricular myocytes (ARVMs). Methods: ARVMs from both sexes were isolated using a Langendorff perfusion apparatus and treated with the β-adrenergic agonist isoproterenol (ISO) or vehicle (saline) for 24 hours. Activated signaling molecules were detected by performing a phospho-kinase antibody array (R&D Systems) in ARVMs isolated from two rats of each sex treated with ISO or vehicle. Total RNA was isolated from these cells, and real-time PCR was used to determine expression levels of calcium handling genes, including Cav1.2 and sodium calcium exchanger (NCX1). Results: A distinct profile of activated signaling molecules was observed between male and female ARVMs in response to ISO. Female ARVMs displayed activation of kinases such as Akt, ERK1/2, p38α, GSK3α/β and MSK1/2. Male ARVMs were characterized by an activation of signaling molecules such as PLCγ1, RSK1/2/3 and c-Jun. ARVMs of both sexes displayed activation of signaling molecules such as eNOS, STAT3 and mTOR. Because the activated signaling molecules identified with the array can modulate expression of calcium handling genes, expression of these genes was analyzed. In response to ISO treatment, female ARVMs displayed an increase in NCX1 expression normalized to vehicle (1.63 ± 0.079) as well as Cav1.2 expression (1.48 ± 0.072). However, these increases were not observed in male cells. Conclusions: Cardiomyocytes isolated from rats of different sexes display distinct responses to

the pathological stimulus ISO. Understanding the mechanisms responsible for these sexual dimorphisms can lead to the development of more effective treatment options for both men and women with cardiovascular disease.

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Crystal Structure Of Fak/mef2 Complex Reveals The Role Of Fak In The Regulation Of Mef2 Transcription Factor.

Alisson Campos Cardoso, Ana Helena Pereira, Andre Luis Ambrosio, Silvio Roberto Consonni, Sandra Martha Dias, Kleber Gomes Franchini, Brazilian Natl Lab for Biosciences, Ctr for Res in Energy and Materials, Campinas, Brazil

Members of MEF2 (Myocyte Enhancer Factor 2) family of transcription factors are major regulators of cardiac development and homeostasis. Their functions are regulated at several levels, including the association with a variety of protein partners. We have previously shown that FAK (Focal Adhesion Kinase) regulates the stretch-induced activation of MEF2 in cardiomyocytes. But, the molecular mechanisms, involved in this process, are unclear. Here, we integrated biochemical, imaging and structural analyses to characterize a novel interaction between MEF2 and FAK. An association between MEF2 and FAK was detected by co-immunoprecipitation in the extracts of stretched cardiomyocytes (10%, 60Hz, 2 hours). MEF2 and FAK staining were co-localized in the nuclei of stretched cells. Pull down assays indicated that the Focal Adhesion Targeting (FAT) domain is sufficient to confer FAK interaction with MEF2. Gene reporter assays indicated that the interaction with FAK enhances the MEF2C transcriptional activity in cultured cardiomyocytes. Also, we present a 2.9-Å X-ray crystal structure for the FAK_FAT domain bound to MEF2C (1-95), comprised by the MADS box/MEF2 domain. The structural information, when used in combination with biochemical studies, small-angle X-ray scattering (SAXS) data and reporter gene assay, lead to a mechanistic model describing how FAK binds to MEF2C and stimulates its transcription function in cardiomyocytes. We further validated this model by showing that the binding of FAK to MEF2C is essential for the hypertrophy of cardiomyocyte in response to mechanical stress. Our results present FAK as a new positive regulator of MEF2, implicated in the fine control of the signal transduction between focal adhesions and the nucleus of cardiac myocytes during mechanical stress.

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Pressure Overload-Induced Myocardial Hypertrophy Causes an Electrical Remodeling of CLC-3 Chloride Channels in Mouse Heart

Dayue D Duan, Univ of Nevada, Reno Sch of Med, Reno, NV; Ying Yu, Shanghai Jiaotong Univ Sch of Med, Shanghai, China; Guanlei Wang, Sun Yat-sen Univ, Guangzhou, NV; Lingyu L Ye, Univ of Nevada, Reno Sch of Med, Reno, NV; Yi-gang Li, Shanghai JIaotong Univ Sch of Med, Shanghai, China

Backgrand: Myocardial hypertrophy causes an increase in myocyte volume and constitutive activation of a volume-sensitive outwardly-rectifying anion channel (VSORAC). The underlying molecular mechanisms and function of VSORAC in the electrical remodeling during myocardial hypertrophy and heart failure remain undefined. We tested whether cardiac CLC-3 chloride channels play a role in the hypertrophy-induced electrophysiological remodeling. Methods and Results: The age-matched CLC-3 knockout (Clcn3-/-) mice and their wild-type (Clcn3+/+) littermates were subjected to minimally-invasive transverse aortic banding (MTAB). In 77% (44/57) left ventricular (LV) myocytes isolated from MTAB-Clcn3+/+ mice a large VSORAC current was activated under isotonic conditions. Hypotonic cell-swelling caused no changes in the VSORAC but hypertonic cell-shrinkage significantly inhibited it. This constitutively-activated VSORAC had an anion selectivity of I->Cl->Asp-, and was inhibited by tamoxifen, PKC activation and intracellular application of anti-CLC-3 antibody. In the age-matched MTAB-Clcn3-/- mice, a significantly smaller outwardly-rectifying current

was present in only 38% (36/94, P<0.05 vs MTAB-Clcn3+/+) LV myocytes. This current was neither increased by hypotonic stress nor inhibited by tamoxifen, PKC or anti-CLC-3 antibody, indicating not a VSORAC or CLC-3 current. Expression of CLC-3 protein was significantly increased in the LV tissues of MTAB-Clcn3+/+ mice but not in Sham-Clcn3+/+ and MTAB-Clcn3-/- mice. Both surface and intracardiac electrophysiological recordings revealed more atrial or ventricular arrhythmias in MTAB-Clcn3-/- mice than in MTAB- and Sham-Clcn3+/+ mice. Conclusions: Pressure-overload-induced myocardial hypertrophy causes an upregulation of CLC-3 expression and constitutive activation of CLC-3 may serve as a novel protective mechanism against the electrical remodeling during myocardial hypertrophy and heart failure.

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A Novel PKCalpha Splicing Isoform in Signaling for Cardiac Hypertrophy

CHEN GAO, UCLA, Los Angeles, CA; Jianli Gong, Susan F Steinberg, Columbia Univ, New York, NY; Yibin Wang, UCLA, Los Angeles, CA

RNA splicing is a significant post-transcriptional regulatory step to enrich the overall transcriptome complexity, yet its specific contribution to cardiac development and pathogenesis remains underexplored. Here, we performed a comprehensive deep RNA-seq analysis in murine heart to investigate the cardiac transcriptome at single exon-resolution. From the dataset, we have identified a previously un-annotated novel exon in the PKCα transcript ---PKCα-NE (Novel Exon). The novel exon encode a 16 amino acid insert adjacent to the PKCα turn motif. Interestingly, the splicing event is detected only in cardiac and skeletal muscle. Based on bioinformatics analysis, we have identified two highly conserved binding motifs for a cardiac splicing regulator_RBFox1. We further demonstrated that RBFox1 indeed functions as a positive regulator for the splicing event of this novel exon. In vitro studies showed that the NE converts PKCα from a lipid-dependent enzyme to a constitutively active kinase with high levels of autocatalytic activity and activity toward peptide substrates. The NE also increases PKCα's Km for peptide substrate. These changes in steady state kinetic parameters suggest that PKCα-NE would exert it's cellular actions most prominently when co-localized with target substrates, either on protein scaffolds or in subcellular signaling compartments. In fact, immune-precipitation for individual PKCα spicing variants followed by Mass Spectrometry exposed a specific interaction between PKCα-NE and elongation factor (eEF1A1). PKCα-WT and PKCα-NE both stimulated cardiac hypertrophy to a similar level based on myocyte size measurement, but PKCα-NE selectively increased eEF1A1 phosphorylation in association with elevated protein translational activity in cultured cardiomyocytes.
In summary, we have identified a novel cardiac specific splicing variant of PKCα that is dynamically regulated during development and heart failure. Our finding has revealed a novel signaling pathway in cardiac hypertrophy involving a novel alternative splicing event of PKCα and its specific downstream effects on protein synthesis regulation.

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Structural Characterization of Calcineurin A-Calsarcin 1 Assembly

Carlos R Koscky Paier, Alisson C Cardoso, Brazilian Natl Lab for Biosciences, Campinas, Brazil; Tatiani L Brenneli, Chemistry Inst, Univ of Campinas, Campinas, Brazil; Rodrigo V Honorato, Brazilian Natl Lab for Biosciences, Campinas, Brazil; Fábio C Gozzo, Chemistry Inst, Univ of Campinas, Campinas, Brazil; Paulo S Oliveira, Kleber G Franchini, Brazilian Natl Lab for Biosciences, Campinas, Brazil

Signaling by the calcium-dependent phosphatase calcineurin (Cn) plays key roles in regulating cardiac development, hypertrophy, and pathological remodeling. Cn binds to and is negatively regulated by calsarcins (CS), a family of muscle-specific proteins. However, the molecular mechanisms involved in the inhibition

of Cn by CS remain unclear. Understanding the architecture and structure of Cn-CS complex is critical to unravel the regulation of Cn by CS. Here we combined biochemical assays, chemical cross-linking coupled to mass spectrometry experiments (MS/MS), mutational analysis and a modeling strategy for structural characterization of CnA-CS1 assembly. The MS/MS data obtained from the cross-linked peptides of both proteins were used to guide an in silico docking of their polypeptide models. The protein complex models with the smallest estimated binding energy were clustered according to structural similarity and submitted to molecular dynamics simulation. The interacting surface of CnA was mapped in a pocket between the 1st and 3rd α-helixes and surrounding loops, while the corresponding surface of CS1 was mapped to the carboxyterminal loops within the Leu179-Phe185, Phe195-Ser199 and Thr250-Leu264 regions. Notably, the region of CnA that interacts with CS1 was found to be located in close proximity, but not coincident, to the β-sheet 14, the main binding site for the PxIxIT sequence of NFAT. Experiments performed with several CnA (FLAG-CnA) and CS1 (myc-CS1) mutants were used to validate the structural model of the CnA-CS1 assembly. The Lys40 (CnA) and Glu254 (CS1) residues were identified as critical for the complex stability. The model that emerges from this study supports the notion that CS1 interacts with an allosteric site to inhibit the activity of CnA. Alternatively, the close proximity of the CS1 to NFAT interacting site supports an interference of CS1 on the ability of CnA to bind and activate NFAT.

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Targeted Inhibition of Cardiac Ankyrin Repeat Protein Disrupts Sarcomeric ERK-GATA4 Signaling at the Titin I-band and Abrogates Agonist-Induced Cardiac Hypertrophy

Chee Lim, Lin Zhong, Manuel Chiusa, Adrian Cadar, Susan Samaras, Jeffrey M Davidson, Vanderbilt Univ, Nashville, TN

Hypertrophic cardiomyocyte growth occurs in response to various stress stimuli including biomechanical stress and neurohormonal factors. Accumulating evidence suggest that sarcomere signaling complexes play a pivotal role in the cardiomyocyte hypertrophic response by transmitting signals to the nucleus to induce gene expression. Cardiac ankyrin repeat protein (CARP, Ankrd1) is a transcriptional regulatory protein that also associates with the titin I-band spring domain, however the exact role of CARP in the heart remains to be elucidated. We report that CARP directly interacts with mitogen activated protein kinase ERK1/2 and cardiac transcription factor GATA4. Phenylephrine (PE) stimulation in cardiomyocytes induced ERK1/2 and GATA4 to transiently co-localize with sarcomeric CARP, followed by translocation of CARP and GATA4 to the nucleus. Four-and-a-half-LIM (FHL) domains proteins are part of a sarcomeric ERK2 sensory complex and knockdown of CARP by small interfering RNA (siRNA) resulted in disruption of FHL1 and FHL2. Moreover, loss of CARP attenuated PE-induced phosphorylation of ERK1/2 and GATA4, decreased GATA4 DNA binding, and prevented PE-induced cardiomyocyte growth. Mice lacking CARP have decreased FHL1 levels, and PE stimulation in wild-type mice resulted in elevated GATA4 phosphorylation and a hypertrophic response, which were completely abrogated in CARP-KO mice. We demonstrate that CARP plays an important role in PE-induced hypertrophic signaling by recruiting ERK2 and GATA4 into a titin I-band macro-molecular complex to induce GATA4 activation, followed by translocation of CARP and GATA4 to the nucleus to enhance GATA4 DNA binding and hypertrophic gene expression. Loss of CARP destabilizes FHL1 and FHL2, resulting in disruption of the PE-induced sarcomeric complex and abrogation of the cardiomyocyte hypertrophic response. These data reveal a novel role for sarcomeric titin I-band as a transcription factor activation hub that induces downstream nuclear signaling in response to agonist-induced hypertrophic stimuli.

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Small Heterodimer Partner Blocks Cardiac Hypertrophy By Interfering With GATA6 Signaling

Yoon Seok Nam, Duk-Hwa Kwon, Gwang Hyeon Eom, Hyun Kook, Chonnam Natl Univ, Gwangju, Korea, Republic of

Rationale: Small heterodimer partner (SHP; NR0B2) is an atypical orphan nuclear receptor that lacks a conventional DNA binding domain. By interacting with other transcription factors, SHP regulates diverse biological events including glucose metabolism in liver. The role of SHP in adult heart diseases has not yet been demonstrated.
Objective: We aimed to investigate the role of SHP in adult heart in association with cardiac hypertrophy.
Methods and Results: The roles of SHP in cardiac hypertrophy were tested in primary cultured cardiomyocytes and in animal models. SHP null mice showed a hypertrophic phenotype. Hypertrophic stresses repressed the expression of SHP, whereas forced expression of SHP blocked the development of cardiomyocyte hypertrophy. SHP reduced the protein amount of Gata6. By direct physical interaction with Gata6, SHP interfered with the binding of Gata6 to GATA binding elements in the promoter regions of natriuretic peptide precursor type A. Metformin, an anti-diabetic agent, induced SHP and suppressed cardiac hypertrophy. The metformin-induced anti-hypertrophic effect was attenuated either by SHP siRNA in cardiomyocytes or in SHP null mice.
Conclusions: These results establish SHP as a novel anti-hypertrophic regulator that acts by interfering with GATA6 signaling. SHP may participate in the metformin-induced anti-hypertrophic response.

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Production of Basal Lamina Collagen IV by Cardiac Fibroblasts in 3-Dimensional Cultures

Amy Bradshaw, Jessica Trombetta eSilva, Erik Eadie, Thomas K Borg, MUSC, Charleston, SC

Cardiac fibroblasts are generally considered the primary cell type that controls extracellular matrix homeostasis in the heart. Distinct changes in amounts and composition of extracellular matrix occur from birth to old age. Accordingly, age-dependent alterations in cardiac extracellular matrix are an important factor governing response to injury and pathological remodeling. Whereas cardiac myocytes are surrounded by a basal lamina, cardiac fibroblasts do not assemble a cell-adjacent basal lamina. Evidence is presented that in addition to fibrillar collagen production, cardiac fibroblasts are also the primary cell type responsible for the production of collagen IV. First, patterns and abundance of collagen IV in murine heart was established in sections from neonate, adult, and aged mice. Second, production of collagen IV by fibroblasts grown in 3-D fibrin gels was assessed by confocal microscopy and quantification by immmunoblot analysis. Finally, co-cultures of cardiac fibroblasts with myocytes were performed to show that fibroblasts are the primary cell type producing collagen IV under these conditions. The basal lamina of the myocytes plays a critical role in aligning and tethering myocytes together as well as making connections to the interstitial collagen fibers of the heart. Hence, production of collagen IV by cardiac fibroblasts would provide a mechanism by which cardiac fibroblasts assist in aligning and securing cardiac myocyte alignment and structural integrity via basal lamina production.

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Inhibition Of Matrix Metalloproteinase-13 Dependent Protease-activated Receptor-1 Activation Attenuates Fibrotic Signaling

Allison E Dixon, Univ of Cincinnati, Cincinnati, OH; Fabrice Jaffre, Harvard Univ, Boston, MA; Nigel Mackman, UNC Sch of Med, Chapel Hill, NC; Burns C Blaxall, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH

Heart failure (HF) is the leading cause of morbidity and mortality in the United States and

is characterized by progressive myocardial fibrosis, pathologic remodeling and deteriorating cardiac function. Cardiac fibroblasts (CF) are largely responsible for the secretion of ECM proteins as well as cytokines and growth factors in the heart. Upon injury or pathologic stimulation, CF transition to a myofibroblast phenotype, leading to excess production of ECM proteins and pro-inflammatory cytokines. Previous studies in our lab have indicated a role for protease-activated receptor-1 (PAR-1), the most highly expressed GPCR on CF, in pathologic cardiac remodeling. In particular, we reported the novel cleavage of PAR-1 via matrix metalloproteinase-13 (MMP-13) and cardioprotective effects of MMP-13 inhibition in an acute model of HF. Therefore, we hypothesize that MMP-13 plays an important role in cardiac remodeling through activation of PAR-1, particularly in the pathologic transition of CF to myofibroblasts.
To investigate this hypothesis, RNA was collected from hearts of mice infused with isoproterenol (ISO) for 7 days and concurrently treated with a specific MMP-13 inhibitor, WAY170523, or vehicle. To evaluate the effect of WAY170523, we used qRT-PCR to measure changes in the expression of fibrotic markers, COL1a1, COL3a1, and TGF-β. Inhibition of MMP-13 with WAY170523 attenuated expression of these genes compared to vehicle treated animals.
We previously reported that stimulation of CF and cardiomyocytes with MMP-13 induces phosphorylation of ERK1/2, a member of the MAPK family known to play a role in cardiac hypertrophy, and treatment with a direct PAR-1 antagonist decreased the activation of ERK1/2. We have found that ERK1/2 phosphorylation is directly attenuated following inhibition of MMP-13 with WAY170523.
Overall, these data suggest a role for MMP-13 dependent PAR-1 activation in pathologic myofibroblast transition and a potential therapeutic role for MMP-13 inhibition, possibly through its inhibition of ERK1/2 phosphorylation. Treatment with WAY170523 also attenuates markers of fibrosis in vivo, indicating a potential salutary role for MMP-13 inhibition in the treatment of HF.

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Regulation of Fibrotic Signaling Pathways by Desmosomal Armadillo Proteins in Cardiac Tissue

Adi D Dubash, Kathleen J Green, Northwestern Univ, Chicago, IL

The process of fibrosis, described as accumulation of myofibroblasts and excessive deposition of extracellular matrix components, is a key development in the progression of multiple different types of cardiac disease. Nevertheless, little is known about the molecular mechanisms which cause the onset of fibrosis in cardiac disease. Fibrosis is a significant component of arrhythmogenic cardiomyopathy (AC), a genetic disorder characterized by replacement of healthy cardiomyocytes (CMs) with fibrous tissue, leading to arrhythmia and in certain cases, sudden death. AC is often characterized as a “disease of the desmosome”, as mutations for all obligate desmosome proteins have been found in cases of AC, including the desmosome armadillo proteins Plakophilin-2 (PKP2) and Plakoglobin (PG). PKP2 and PG are multi-functional proteins involved in both mechanical stabilization of the cardiac area composita, as well as mediation of desmosome-related signaling pathways. We have determined that loss of PKP2 or PG in neonatal CMs causes an aberrant increase in gene expression of pro-fibrotic stimuli such as transforming growth factor beta 1 (TGF-beta1) and Interleukin-6 (IL-6). In addition, p38 MAPK, a known mediator of inflammatory fibrosis, is activated upon loss of PKP2/PG. We hypothesize that mutation or loss of PKP2 or PG cause the recruitment and activation of cardiac fibroblasts via pro-fibrotic TGF-beta and p38MAPK signaling, resulting in pathological fibrosis characteristic of AC. Indeed, conditioned media from PKP2-silenced CMs causes an increase in fibronectin gene expression by freshly isolated cardiac fibroblasts. Our future experiments will investigate whether inhibition of TGF-beta or p38MAPK signaling can alleviate fibrotic gene production. By highlighting a novel link between desmosome armadillo proteins and pro-fibrotic signaling in cardiac tissue, this study provides mechanistic insights into the pathogenesis of AC, as well as advances our knowledge of potential therapeutic targets for combating fibrosis in multiple different types of heart disease or injury.

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Elucidation Of The Fibrotic Transcription Factors Gene Network Leading To Chagas Heart Disease

Pius N. Nde, Aniekanabassi N. Udoko, Candice A. Johnson, Andrey Dykan, Girish Rachakonda, Fernando Villalta, Maria F. Lima, Siddharth Pratap, Meharry Medical Coll, Nashville, TN

Background:
Trypanosoma cruzi the causative agent of Chagas heart disease (CHD) remains incurable. The major pathology induced by the parasite is cardiac fibrosis leading to heart failure followed by death. The mechanisms of *T. cruzi* induced cardio-pathology remains largely unknown. We hypothesize that *T. cruzi* infection regulates the expression of profibrotic genes in human cardiac myocytes (HCM), tilting the heart towards a profibrotic phenotype seen in CHD patients.
Methods and Results: To elucidate the molecular mechanisms of *T. cruzi* induced cardiac fibrosis, we challenged primary HCM with *T. cruzi* for two hours and purified total RNA for microarray. We investigated changes at the whole transcriptome level on an affymetrix platform. The arrays were done in triplicates at different time points; changes in gene expression greater than 2-fold and having a Benjamini and Hochburg false discovery rate corrected p-value <0.05 were considered significant. The microarray data was validated using real-time PCR followed by PCR array and immunoblotting, to evaluate changes in the protein expression levels of fibrotic transcription factors. Protein expression levels were evaluated in triplicate and analyzed by ANOVA. The fibrotic interactome induced by *T. cruzi* in HCM was elucidated using Cytoscape. Our results indicate that exposure of HCM to *T. cruzi* upregulates the transcript levels of two transcription factors associated with fibrosis, SNAI1 (more than 2 fold up-regulated) and Early Growth Response protein 1, EGR1, (about four fold up-regulated). SNAI1 and EGR1 were increased at the protein level. Furthermore, we identified the first interactome regulating fibrosis in primary HCM induced by *T. cruzi*.
Conclusions:
This is the first report showing that *T. cruzi* upregulates the expression of profibrotic transcription factors in HCM early during the process of cellular infection and the operational fibrotic interactome. Thus, abnormal sustained expression of SNAI1 and EGR1 upregulate the expression of genes essential for conversion of HCM towards a profibrotic phenotype in CHD. Elucidation of the molecular mechanisms by which *T. cruzi* induces cardiac fibrosis will lead to the identification of new therapeutic targets for CHD.

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Loss of Sarcospan has a Deleterious Effect on Cardiac Function in Aged Mice

Michelle S. Parvatiyar, Jamie L Marshall, Maria C. Jordan, Reginald T. Nguyen, Kenneth P. Roos, Rachelle H. Crosbie-Watson, Univ of California, Los Angeles, Los Angeles, CA

Sarcospan (SSPN) has been shown to have an important role in stabilizing sarcolemmal dystrophin- and utrophin-glycoprotein adhesion complexes. Loss of sarcolemmal integrity leads to immune cell infiltration and inappropriate exchange of cellular contents with the extracellular milieu. Our laboratory has shown SSPN loss destabilizes skeletal muscle adhesion and reduces sarcolemmal dystrophin localization, whereas its complete loss due to mutation underlies development of Duchenne muscular dystrophy (DMD). Loss of dystrophin leads to cardiac dysfunction and early mortality in DMD patients. The role of SSPN in the heart is unknown. We present immunofluorescence data revealing reduction of dystrophin and the sarcoglycans with a coordinate increase of β1D integrin levels at the SSPN-null cardiac sarcolemma relative to WT. Also, SSPN loss decreases cardiac P-Akt levels, disrupting signaling promoting compensatory physiological hypertrophy. These studies suggest a fundamental role for SSPN in cardiac maintenance and function, since left ventricular

mass increases with age and upon isoproterenol administration (0.8 mg/day for two wks). Aged SSPN-null mice developed hypertrophy, evidenced as increased heart/body weight ratio and left ventricular wall dimension. The SSPN-null mice lacked the characteristic initial rise in cardiac output, left ventricular ejection fraction (LvEF %), induced by chronic β-adrenergic stimulation. Functionally, aged SSPN-null hearts had an increased E/A ratio indicating restrictive ventricular filling and decreased fractional shortening F/S (%) upon isoproterenol administration. Aged untreated SSPN-null hearts had increased fibrosis compared to aged WT controls, however isoproterenol treated SSPN-null hearts displayed exacerbated fibrotic response compared to WT. To assess whether SSPN-null hearts have altered gene expression profiles during progression of pathogenesis, qRT-PCR will be utilized to measure differences in expression of fetal gene and calcium-handling proteins. In summary, we have found that SSPN has an important role in maintaining cardiac function, its loss exacerbates the hypertrophic response and localization of stabilizing adhesion complexes at the cardiac muscle sarcolemma.

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The Role of Mixed Lineage Kinase 3 in Inflammatory Cell-Fibroblast Communication

Emily M Schulz, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH; Allison E. Dixon, Univ of Cincinnati, Cincinnati, OH; Michael S. Burhans, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH; Michelle L. Nieman, John N. Lorenz, Univ of Cincinnati, Cincinnati, OH; Val Goodfellow, Califia Biotech, San Diego, CA; Harris A. Gelbard, Stephen Dewhurst, Univ of Rochester Medical Ctr, Rochester, NY; Burns C. Blaxall, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH

Mixed lineage kinase 3 (MLK3) is a ubiquitously expressed pro-inflammatory, pro-apoptotic mitogen activated protein kinase kinase kinase (MAP3K). MLK3 is a key regulator of the p38 and c-jun terminal kinase (JNK) pathways and has been studied in cancer and neurodegenerative disease. Although the p38 and JNK pathways have been studied in the cardiac function and disease, little is known regarding the role of MLK3 in the heart. Studies in our laboratory have indicated that MLK3 RNA is highly expressed in macrophages, cardiomyocytes and cardiac fibroblasts, suggesting an important role in cardiac function. Recently published work has indicated that MLK3 plays an important role in inflammatory cell motility, leading us to hypothesize that MLK3 is involved in inflammatory cell-fibroblast communication during cardiac disease. Knockout (KO) or inhibition of MLK3 using the novel small molecule inhibitor URMC-099 does not significantly affect heart rate, mean arterial pressure, systolic pressure, minimum or maximum dp/dt compared to wild type (WT) controls as measured by invasive hemodynamics at 3 months of age. Echocardiographic analysis indicates that MLK3 KO or inhibition does not affect cardiac architecture or cardiac function, indicated by fractional shortening or ejection fraction. However, upon transaortic constriction (TAC), MLK3 KO and URMC-099 treatment results in decreases in Mac-3 positive staining at 3 and 7 days post-TAC as well as decreases in CD-45 staining 7 days post-TAC compared to WT TAC operated, vehicle treated controls, suggesting that MLK3 KO and drug treatment may attenuate the early inflammatory response after TAC. Studies examining the relationship between the MLK3 mediated inflammatory response and subsequent fibrosis and cardiac dysfunction post-TAC are currently ongoing.

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Cell-Matrix Contacts Regulate Age-Associated Cardiac Function

Ayla O Sessions, Gaurav Kaushik, Univ of California, San Diego, La Jolla, CA; Anthony Cammarato, John's Hopkins Sch of Med,

Baltimore, MD; Adam Engler, Univ of California, San Diego, La Jolla, CA

An increased deposition of ECM is observed in all advanced age heart failure patients. Therefore, it is necessary to investigate the effect of extracellular remodeling on mechanical function in genetically tractable, rapidly aging, and simple model organisms such as *Drosophila melanogaster*. The bilayered design of the Drosophila heart-tube makes it an easier model in which to study the interplay between ECM and cardiomyocytes as they regulate contraction. Here we present data from two common wildtype strains of *Drosophila* exhibiting different aging profiles in terms of cytoskeletal and ECM regulation and remodeling. Using a recently developed nanoindentation method to measure cardiomyocyte stiffness of intact *Drosophila* hearts, we have found that while *yellow-white* (*yw*) flies show midline stiffening at the intercalated discs (ICD) presenting a clear diastolic dysfunction with age, the *white-1118* (*w1118*) flies exhibit no ICD stiffening, but show an increase in thickness of the ECM layer between the ventral muscle (VM) and cardiomyocytes (CM). Paired with increased expression of ECM proteins, the *w1118 Drosophila* line may provide a good model for exploring the effect of cell-ECM contacts on regulating cardiac function with age. Knock-down of integral ECM genes LamininA and Viking (Collagen IV) result in no effect on cardiac performance in juvenile flies but causes a decrease in underlying cardiomyocyte stiffness and an increase in the contractile irregularity of heart beats. This suggests that the cell-ECM contacts in the basement membrane are intimately tied to coupling of the cardiomyocytes of the *Drosophila* heart-tube, which may have larger implications for elderly patients suffering from myocardial fibrosis and experiencing cardiomyocyte decoupling and resultant arrhythmias.

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Beta3 Integrin-mediated CTGF Expression in Cardiac Hypertrophy

Kamala P Sundararaj, Dorea L Pleasant, Sundaravadival Balasubramanian, Dhandapani Kuppuswamy, MUSC, Charleston, SC

Connective Tissue Growth Factor (CTGF/CCN2) is a fibrotic mediator overexpressed in human atherosclerotic lesions, myocardial infarction and hypertension. CTGF regulates ECM deposition, fibrosis, wound repair and angiogenesis. Our preliminary studies using wild type (WT) and global 3 integrin knockout (3-/-) mice show for the first time that pressure overload (PO) by transverse aortic constriction (TAC) induces both CTGF and 3 integrin expression in WT mice and that 3 integrin is a prerequisite for the PO-induced CTGF expression. Since both the major cells, cardiomyocyte (CM) and cardiac fibroblast (CFb) have been shown to express CTGF, exploring the primary source for the 3 integrin mediated CTGF expression is expected to identify potential targets for fibrosis. Therefore, the main focus of this proposed study is to use CM specific β3 integrin KO mice with preserved β3-integrin function in CFb as well as CFb specific β3 integrin KO mice with preserved β3-integrin function in CM and evaluate which heart cell type contributes to β3-integrin-mediated PO-induced CTGF production and ECM accumulation. Furthermore, using cells isolated from these mice, we will perform *in vitro* studie*s* to explore the mechanism by which β3-integrin mediates CTGF secretion by analyzing specific nonreceptor tyrosine kinases and transcription factors.

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Identification of Novel Cardiac Fibroblast Enriched Genes

Jessica M Swonger, Michelle D Tallquist, Univ of Hawaii at Manoa, Honolulu, HI

The leading cause of death in the United States is heart disease. While current therapies have

reduced mortality, patients surviving the initial stages of cardiac injury are left with long-term disruption of heart function including fibrosis. Pathological fibrosis in the heart is caused by excess proliferation and deposition of extracellular matrix primarily by cardiac fibroblasts (CFs). One factor required for cardiac fibroblast formation is the basic helix-loop-helix transcription factor, TCF21 (epicardin/Pod1/capsulin). Previous studies from our lab have shown that *Tcf21* null embryos lack CFs. Our current work focuses on identifying genes downstream of TCF21. Deep-sequencing identified over one hundred differentially expressed genes when comparing embryonic hearts from *Tcf21* nulls to wild types. We have verified a subset of these differentially expressed genes by qPCR and have demonstrated that these genes are also expressed in cultured primary cardiac fibroblasts. Future work will focus on determining the function of these genes during fibroblast activation and determine which of these genes are directly regulated by TCF21. The elucidation of cardiac fibroblast specific genes and their function will provide much needed information for identification of therapeutic targets aimed at cardiac fibroblast activation.

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Small Molecule Gβγ Inhibition Attenuates Cardiac Fibroblast Inflammatory and Pro-Fibrotic Signaling

Joshua G Travers, Fadia A Kamal, Michael S Burhans, Burns C Blaxall, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH

Heart failure (HF) is a devastating disease characterized by chamber remodeling, interstitial fibrosis and reduced ventricular compliance. Prolonged sympathetic overstimulation promotes excess signaling through G-protein Gβγ subunits and ultimately results in pathologic GRK2-mediated β-adrenergic receptor (β-AR) downregulation. We have recently demonstrated the therapeutic potential of the small molecule Gβγ-GRK2 inhibitor Gallein in limiting HF progression. Pathologic activation of the cardiac fibroblast (CF) induces the transition to a myofibroblast phenotype, which plays a fundamental role in myocardial fibrosis and remodeling. We hypothesized that Gβγ-GRK2 inhibition plays an important functional role in the CF to attenuate pathologic CF activation, inflammation and interstitial fibrosis. To explore the effect of Gβγ-GRK2 inhibition on inflammation and pro-fibrotic signaling, mice were subjected to 7 days of transverse aortic constriction, a pressure-overload model of HF. In addition to the attenuation in overall cardiac hypertrophy, animals treated with Gallein displayed reduced expression of pro-inflammatory cytokines, including macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1β, along with Interleukin-6, as assessed by qPCR. Gallein-treated animals also exhibited diminished pro-fibrotic signaling, as evidenced by a reduction in the expression of TGFβ, a major driver of myocardial fibrosis, and decreased expression of collagen. To recapitulate these findings in vitro, primary adult mouse ventricular fibroblasts were pathologically stimulated using Isoproterenol (ISO, β-AR agonist) or Angiotensin II and treated +/- Gallein for 24 hours. CFs treated with Gallein displayed an analogous reduction in the expression of these pro-inflammatory cytokines and collagen. In summary, these data suggest a potential therapeutic role for small molecule Gβγ-GRK2 inhibition in limiting pathologic myofibroblast activation, inflammation and interstitial fibrosis. We believe this fibroblast-targeted approach will lead to the refinement of existing targets and compounds, and possibly the generation of novel therapeutics for the treatment of HF.

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Long-term Atorvastatin, But Not Pravastatin, Treatment Leads To Repressed Mitochondrial Gene Expression And Altered Cardiac Ultrastructure

Alice Zemljic-Harpf, Univ of California, San Diego, San Diego, CA; Joseph C. Godoy, VA San Diego Healthcare Systems, San Diego, CA; Ingrid R. Niesman, Jan M. Schilling, Univ of California, San Diego, San Diego, CA; Anna Schwarz, VA San Diego Healthcare Systems, San Diego, CA; Elizabeth K. Asfaw, Erika A. Alvarez, Nancy D. Dalton, Univ of California, San Diego, San Diego, CA; Piyush M. Patel, Brian P Head, John C. Drummond, David M. Roth, VA San Diego Healthcare Systems, San Diego, CA; Georgios Kararigas, Inst of Gender

in Med and Ctr for Cardiovascular Res, Charite Univ Hosp, Berlin, Germany; Hemal H. Patel, VA San Diego Healthcare Systems, San Diego, CA

Statins reduce low-density lipoprotein cholesterol (LDL-C) and decrease cardiovascular events. Although statins are generally well tolerated, the FDA warns that statins may induce skeletal muscle side effects, cognitive changes and increased fasting glucose levels. Skeletal muscle biopsies from patients with statin myopathy have revealed defects in mitochondrial ultrastructure. Impaired mitochondrial function has been postulated as a key cause of statin-induced myopathy and hepatotoxicity. Long-term statin effects on cardiac muscle are currently unknown. Wild type mice received atorvastatin, pravastatin or vehicle daily for seven months by oral gavage. Atorvastatin and pravastatin reduced LDL-C compared to vehicle. Echocardiography at two-week intervals showed no differences in %FS, %EF, circumferential fiber shortening and ventricular wall thicknesses between atorvastatin, pravastatin and vehicle treated mice. After seven months of atorvastatin, pravastatin or vehicle administration cardiac muscles (n=21-29) were analyzed, and only atorvastatin treated hearts revealed: A) swollen and misaligned mitochondria and accumulation of protein aggregates by transmission electron microscopy (n=4, each), and B) repression of mitochondrial and endoplasmatic reticulum related genes by genome-wide expression profiling. In cultured ventricular myocytes, atorvastatin, but not pravastatin; 1) down-regulated survival pathways via inhibition of ERK1/2T202/Y204, AktSer473 and mTOR signaling (p70 S6 kinaseThy421/Ser4240 and S6 RPSER 235/236), 2) reduced protein expression of caveolin-1, dystrophin, epithermal growth factor receptor and insulin receptor β, 3) decreased RhoA activation, and 4) induced apoptosis.
LDL-C reduction by atorvastatin, but not pravastatin, was associated with a repression of mitochondrial and endoplasmic reticulum related genes, an accumulation of protein aggregates, and swollen mitochondria. This is the first report demonstrating that long-term atorvastatin treatment causes adverse effects on cardiac muscle with preserved cardiac function. Whether these changes predispose atorvastatin treated hearts to contractile dysfunction after hemodynamic stress needs further investigation.

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Obesity Causes Enhanced Sensitivity Of The Mitochondrial Permeability Transition Pore

Judith Bernal-Ramírez, Adriana Riojas-Hernández, Flor E. Morales-Marroquín, Elvía M. Domínguez-Barragán, David Rodríguez-Mier, Gerardo García-Rivas, Julio Altamirano, Noemí García, Insto Tecnológico y de Estudios Superiores de Monterrey, Monterrey, Mexico

Several mechanisms have been implicated in heart failure (HF) development due to obesity, including altered Ca2+ homeostasis and mitochondrial increased reactive oxygen species (ROS).
Besides their metabolic role, mitochondria are important cell death regulators, since their disruption induces apoptosis. The mitochondrial permeability transition pore (MPTP) formation is key in this process. Ca2+ and ROS are known inducers of MPTP, and mitochondria are the main ROS generators. However, it has not been demonstrated that MPTP formation is involved in cardiac cell death due to obesity. Therefore, the aim of this work was to determine whether Ca2+ alterations and/or MPTP opening underlie cardiac dysfunction.
We used obese Zucker fa/fa rats (32 weeks old), displaying concentric hypertrophy and cardiac dysfunction. We measured: i) Systolic and diastolic Ca2+ signaling in isolated myocytes, in basal conditions and upon β-adrenergic stimulation (β-AS), and ii) in vitro mitochondrial function: respiration, ROS production and MPTP opening.
We found that the main alteration in Ca2+ signaling in fa/fa myocytes was a decrease in SERCA Ca2+ removal capacity, since Ca2+ transient amplitude and spark frequency were unchanged. Furthermore, in fa/fa myocytes, β-AS response was preserved. On the other hand, fa/fa mitochondria respiration, in state 3

decreased, but was unchanged in state 4, when glutamate/malate were used as substrate, resulting in an small decrease in respiratory control. In addition, fa/fa mitochondria were more sensitive to MPTP opening, induced by Ca2+ and carboxyatractiloside (CAT). Moreover, fa/fa mitochondria showed increased H2O2 production, and in exposed thiol groups in the adenine nucleotide translocase, a regulatory MPTP component.
Since Ca2+ signaling is relatively normal in fa/fa cells, it does not seem to be the main contributor to the cardiac contractile dysfunction. However, given that fa/fa mitochondria showed decrease respiratory performance, were more susceptible to MPTP opening, and showed enhanced H2O2 production. We conclude that fa/fa mitochondria were more vulnerable to enhanced oxidative stress, causing MPTP opening, which could be exacerbated by SERCA slower Ca2+ removal capacity, leading to myocyte apoptosis.

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Metformin Inhibits Mitochondrial Permeability Transition Pore Opening in Human Cardiac Mitochondria

Larisa Emelyanova, Sirisha Gudlawar, Milanka Cosic, Mahek Mirza, Frahan Rizvi, Ekhson Holmuhamedov, Ctr for Integrative Res on Cardiovascular Aging (CIRCA), Aurora Univ of Wisconsin Medical Group, Aurora Health Care System, Milwaukee, WI; Francis X. Downey, Aurora Medical Group-Cardiovascular and Thoracic Surgery, Aurora St. Luke's Medical Ctr, Milwaukee, WI; A. Jamil Tajik, Aurora Cardiovascular Services, Aurora Sinai/Aurora St. Luke's Medical Ctrs, Univ of Wisconsin Sch of Med and Public Health, Milwaukee, WI; Arshad Jahangir, Ctr for Integrative Res on Cardiovascular Aging (CIRCA), Aurora Univ of Wisconsin Medical Group, Aurora Health Care System, Milwaukee, WI

Background: Type II diabetes mellitus is a major health problem contributing to increased morbidity and mortality with associated cardiovascular diseases. Metformin, an oral antidiabetic agent, has cardioprotective properties independent of their glucose lowering effect; however, mechanisms underlying cardioprotection remain poorly defined. We hypothesized that the cardioprotective effect of metformin appears to be associated with inhibition of mitochondrial permability transition pore (mPTP) that could be beneficial in diabetic hearts with attenuated endogenous cardioprotective responses.
Purpose: The aim of the study was to determine the protective effect of metformin on mPTP opening in mitochondria from human myocardium..
Methods: Mitochondria freshly isolated from the left atrial appendage of nondiabetic patients undergoing cardiac surgery were loaded with calcium- (fluo-3) and membrane potential-sensitive (Safranin-O) fluorescent dyes and challenged with sequential pulses of Ca2+ (10 μM every 3 m) in the absence and presence of different concentrations of metformin (2.5, 5, 10 mM). The sensitivity of mitochondria toward mPTP opening was assessed by abrupt release of mitochondrial Ca2+ (fluo-5N fluorescence), with simultaneous dissipation of mitochondrial membrane potential (safranin O fluorescence) and mitochondrial swelling (decrease in light scattering).
Results: Metformin caused a dose-dependent inhibition of Ca2+-induced mPTP opening with delayed mitochondrial Ca2+ release, depolarization and swelling. The tolerance of metformin-treated mitochondria to Ca2+-induced mPTP opening when compared to controls was increased from 457±71 to 600±74 nmol/mg protein at 2.5mM, 674±10 nmol/mg protein at 5 mM and 750±77 nmol/mg protein at 10 mM. Cyclosporin A (1 μM), a known mPTP inhibitor, has marginal incremental effect on metformin-induced inhibition of mPTP opening.
Conclusion: In human cardiac mitochondria, metformin inhibited calcium-overload-mediated mPTP opening that otherwise leads to mitochondrial energetic failure and cell death. Thus, metformin may help restore attenuated cardioprotection in diabetic hearts by increasing tolerance to stress-induced mPTP opening and, thus, preventing cell death.

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Dronedarone Impairs Cardiac Mitochondrial Oxidative Phosphorylation in Dose-Dependent Manner

Larisa Emelyanova, Sirisha Gudlawar, Farhan Rizvi, Ekhson Holmuhamedov, Monika Thakur, Mahek Mirza, Arshad Jahangir, Ctr for Integrative Res on Cardiovascular Aging (CIRCA), Aurora Univ of Wisconsin Medical Group, Aurora Health Care System, Milwaukee, WI

Introduction: Dronedarone (DR), a new antiarrhythmic drug, was recently shown to worsen heart failure (HF) and mortality in patients with atrial fibrillation and left ventricular dysfunction. However, the mechanism underlying the adverse effect is not known. Since, myocardium depends on mitochondrial oxidative phosphorylation (OXPHOS), we hypothesized that DR impairs mitochondrial function, which could further compropmise energetic reserves predisposing to worsening of HF and death in patients with HF. Methods: Mitochondria isolated from rat heart (2 month old, SD) were treated with DR (1, 5, 10, 20, 50 μM), and the effect on oxygen consumption rate (OCR) in State 3 (St 3, ADP stimulated), State 4 (St 4o, oligomycin) and following FCCP addition were determined using Seahorse XF24 Analyzer in the presence of glutamate/malate (complex I substrates) and succinate/rotenone (complex II substrate). Results: DR dose dependently reduced St 3 respiration both in the presence of complex I (Fig). In the presence of glutamate/malate, DR inhibited OCR by 16%, 20%, 25%, 39% and 100% at 1, 5, 10, 20, 50 μM, respectively, when compared to untreated control. At 20 μM, DR uncoupled mitochondria and increased St 4o respiration. DR at 50 μM was toxic with complete inhibition of OCR and loss of membrane potential. Similar results were observed when succinate/rotenone were used to assess complex II activity. Conclusion: DR has dose-dependent inhibitory effect on mitochondrial respiration, inhibiting OXPHOS at low concentration (1-10 μM), uncoupling at higher (20 μM) and toxic effect at 50 μM. Impairment of mitochondrial energetics could explain DR results reported in HF patients in clinical trials.

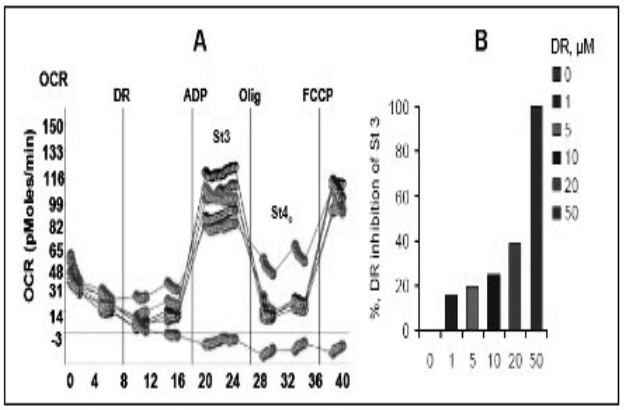

Figure. Dose-dependent effect of dronedarone (DR) on mitochondrial oxygen consumption rate (OCR) in the presence of 10 µM glutamate and 5 µM malate as substrates

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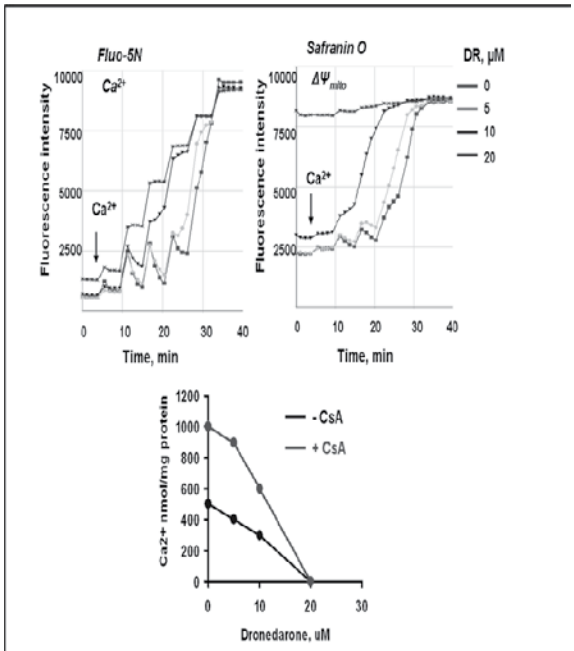
Dronedarone Alters the Sensitivity of Cardiac Mitochondria to Permeability Transition Pore Opening

Sirisha Gudlawar, Milanka Cosic, Farhan Rizvi, Ekhson Holmuhamedov, Monika Thakur, Mahek Mirza, Jamil Tajik, Francis Dawney, Arshad Jahangir, Aurora Univ of Wisconsin Medical Group, Aurora Health Care System, Milwaukee, WI

Introduction: Dronedarone (DR) is a new antiarrhythmic agent that was design to minimize side effects of Amiodarone. However, in clinical trials was shown to worsen heart failure (HF) and increased mortality. The mechanisms for these adverse effects are not known. Since mitochondria are critical for maintaining cardiac energetics and regulate, we hypothesized that DR increases sensitivity towards opening of mitochondrial permeability transition pore (mPTP) that underlies mitochondrial failure and cell death. Methods: Mitochondria were isolated from left atrial appendage tissue of patients undergoing bypass cardiac surgery (n=3). Patients did not have any history of HF or atrial fibrillation. To

induce mPTP, mitochondria were exposed with sequential additions of Ca^{2+} (Fluo-5N) in the presence of 5, 10, 20 μM of DR. The opening of mPTP was determined by monitoring abrupt release of Ca^{2+}, rapid loss of membrane potential ($\Delta\Psi$) and mitochondrial swelling. Results: DR accelerated Ca^{2+}-induced mPTP opening starting at 5 μM concentration (Fig). At concentration of 20 μM, it completely depolarized mitochondria abolishing capacity for Ca^{2+} handling and responsiveness to cyclosporine A (CsA), an inhibitor of mPTP. Conclusion: In isolated human cardiac mitochondria from patients without history of HF DR at clinically relevant concentration increased sensitivity of mitochondria to Ca^{2+} induced mPTP opening. In failing heart this can be compromised and needs to be studied. Dronedarone accelerates Ca^{2+}-induced mPTP opening in mitochondria isolated from human left atrial appendage.



Dronedarone accelerates Ca^{2+} - induced mitochondrial permeability transition pore opening in mitochondria from human left atria.

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Dissociation of Oxidative Stress from High Fatty Acid-Induced Cardiac Myocyte Death

Nicolas Gutierrez Cortes, Huiliang Zhang, Shangcheng Xu, Guohua Gong, Wang Wang, Univ of Washington, Seattle, WA

Metabolic syndrome, featured by obesity and diabetes, is an independent risk factor for cardiovascular disease. The high availability of free fatty acids characteristic of obesity has been proposed to increase fatty acid oxidation, impair mitochondrial function, stimulate reactive oxygen species (ROS) production and eventually lead to oxidative stress, which then would cause myocyte damage and heart dysfunction. However, the role of oxidative stress as the initial cause of obesity-induced heart failure is controversial. We evaluated the time-dependent effect of fatty acids in vitro in adult rat cardiac myocytes (treated with palmitate and oleate, the major saturated and unsaturated fatty acids in the plasma, respectively), and in vivo in mice fed a high-fat diet (fat calories = 60%). Palmitate and oleate dose-dependently stimulated mitochondrial respiration and superoxide flash activity, a respiration-coupled bursting ROS production event in single mitochondria. The flash frequency increased 2-fold (0.1 mM, the physiological level in plasma) and 3-fold (0.3 mM, the plasma level found in obese subjects) after only 2 hr incubation and up to 24 hr, increase that could be blocked by the CPT1 inhibitor etomoxir (100uM). Surprisingly, there was no obvious mitochondrial oxidative stress, although the high level of fatty acids caused mPTP opening and cell death at 48 hr. Further, aconitase activity was normal and NAD/NADH ratio was decreased, indicating a more reduced status rather than oxidative stress. Intriguingly, mitochondrial membrane potential was slightly lowered after only 2 hr of treatment. Finally, the high-fat diet-fed mice developed heart dysfunction after 12 weeks of treatment, but oxidative stress only appeared after 18 weeks.
In the light of these results, we hypothesize that high fatty acid supply induced multiple adaptive and mal-adaptive responses in cardiac myocytes. The increased mitochondrial respiration and bursting ROS production is offset by a mild uncoupling and increased reducing equivalents. Therefore, oxidative stress is

unlikely an initial cause of myocyte death and heart dysfunction in obesity, but rather a consequence of mitochondrial dysfunction, although the exact cause of this dysfunction remains to be elucidated.

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Cd36 Deficiency Reduces Obesity-associated Inflammation And Oxidative Stress In Heart

Tahar Hajri, Mohamed Gharib, Hackensack Univ Medical Ctr, Hackensack, NJ; Thomas V Fungwe, Howard Univ, Washington, DC

OBJECTIVE: Obesity is often associated with diabetes and cardiovascular diseases (CVD). Mounting evidence shows that diabetes is associated with structural and functional changes in the heart. CD36 protein is highly expressed in heart and regulates lipid utilization in cardiacmyocytes. In this paper, we investigated the impact of CD36 expression on obesity-associated inflammation and oxidative stress in heart. **METHODS:** Studies were conducted in control lean (WT), obese leptin deficient ($Lep^{Ob/Ob}$) and leptin deficient-CD36 null ($Lep^{Ob/Ob}$-$CD36^{-/-}$) mice. To examine obesity-associated insulin resistance, glucose uptake and insulin signaling were examined in adult mouse hearts. Presence of macrophages in heart was examined with immunohistochemisty. Oxidative stress makers and activity of anti-oxidant enzymes were measured in hearts. To evaluate substrate utilization, glucose and fatty acid oxidation was tested in primary cultures of ventricular myocytes. Finally, the activity of pro-inflammatory kinases p38 mitogen-activated protein kinases (p38-MAPK), c-Jun NH2-terminal kinases (JNK) were examined in cardiacmyocytes challenged with palmitate. **RESULTS:** In $Lep^{Ob/Ob}$, glucose uptake and oxidation in heart was lower than lean WT mice, while cardiac FA oxidation was strongly higher. Silencing CD36 in $Lep^{Ob/Ob}$ mouse markedly improved insulin sensitivity and glucose uptake in heart, but resulted in marked reduction of FA oxidation. Immunostaining of heart sections with macrophage specific antibody F4/80 showed that macrophage content was higher in myocardium of $Lep^{Ob/Ob}$ mice than $Lep^{Ob/Ob}$-$CD36^{-/-}$ mice. Moreover, oxidative stress markers, isoprostanes and reactive oxygen species, and expression of pro-inflammatory cytokines were higher in hearts of $Lep^{Ob/Ob}$ than $Lep^{Ob/Ob}$-CD36-/- mice, although the activities of anti-oxidant enzymes were comparable. Chronic overload of $Lep^{Ob/Ob}$ cardiac myocyte with palmitate strongly induced the activity of JNK and p38-MAPK, but was less effective in $Lep^{Ob/Ob}$-$CD36^{-/-}$ cardiac myocytes. **CONCLUSIONS:** These results show that CD36 deficiency induced a significant reduction of obesity-associated oxidative stress and inflammation in heart in parallel to a drop in fatty acid oxidation.

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Mitochondrial Functional Variation Contributes to Endothelial Cell Activation

David M Krzywanski, Bing Cheng, Xinggui Shen, Christopher Kevil, LSU Health Science Ctr - Shreveport, Shreveport, LA

Vascular oxidant stress contributes to endothelial dysfunction and plays a critical role in early stage cardiovascular disease (CVD) development. Changes in endothelial function due to oxidant stress may contribute to CVD initiation and progression through the development of a pro-inflammatory environment. Differences in mitochondrial function may contribute to this process and provide insight into why age of onset and clinical outcomes differ amongst individuals form distinct ethnic groups; but no reports demonstrate distinct mitochondrial functional parameters between normal cells. Consequently, we hypothesized that significant variations in normal mitochondrial function and oxidant production exist between endothelial cells from donors representing different ethnic groups. Aspects of mitochondrial oxygen utilization and oxidant production were assessed under basal and inflammatory conditions in human aortic endothelial cells (HAECs) isolated from African Americans (AA) and Caucasians (CA). Bioenergetic analysis

indicates that compared to CA, AA HAEC utilized significantly less oxygen for ATP production, possess a lower maximal respiratory capacity, and have reduced electron leak. Significant differences in mitochondrial membrane potential, decreased expression of endothelial nitric oxide synthase, and increased levels of superoxide were also observed and AA HAEC supporting a pro-inflammatory phenotype. As a marker of endothelial cell activation, AA HAEC expressed increased levels of intercellular cell adhesion molecule-1 under both basal and inflammatory conditions that could be partially mitigated but treatment with the mitochondrially targeted antioxidant MitoTEMPO. These data demonstrate that fundamental differences exist in mitochondrial oxygen utilization and oxidant production between CA and AA HAEC and that these changes may affect endothelial cell activation. These findings are consistent with the hypothesis that differences in "normal" mitochondrial function amongst ethnic groups could influence individual susceptibility by contributing to vascular inflammation, providing important insights into the mechanisms that contribute human CVD development.

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ES1 Is A Novel Mitochondrial Protein Protecting The Heart Via Direct Regulation Of Mitochondrial Energy Metabolism

Qinqiang Long, Huan Yang, Yiqun Zhou, Univ of Alabama at Birmingham, BIRMINGHAM, AL; Aibing Wang, Natl Heart, Lung, and Blood Inst, Bethesda, MD; Lan He, Yuquan Li, Teayoun Kim, Yishu Ding, Olga Zhelyabovska, Qinglin Yang, Univ of Alabama at Birmingham, BIRMINGHAM, AL

Defects in the myocardial energy metabolism have been linked to pathological cardiac hypertrophy and congestive heart failure. However, the regulation of myocardial energy metabolism remains obscure. ATP synthase is an enzyme complex in the mitochondria and plays a central role in energy metabolism. In this study, we identified ES1, a mitochondrial protein with unknown function, as a key determinant of myocardial energy metabolism via controlling ATP synthase activities. We uncovered that ES1 interacts with both α and β subunit of ATP synthase, and its expression levels in H9C2 cardiomyocytes were directly correlated to ATP synthesis and inversely to ATP hydrolysis. Cellular energetic analysis revealed that ES1 levels in H9C2 cardiomyocytes were directly correlated with mitochondrial oxidative metabolism. ATP synthase activity assays revealed increased synthesis activities and decreased hydrolysis activities on cardiac mitochondria from a mouse line with Cre-LoxP mediated, tamoxifen inducible, cardiomyocyte-restricted ES1 overexpression (TM-ES1oe) compared with mice of tamoxifen-inducible Mer-Cre-Mer (TMCM). We induced ES1 overexpression in TM-ES1oe mice (3-month-old) 7 days after transverse aortic constriction(TAC) and compared with TMCM mice with identical treatment. Echocardiography assessment revealed a substantially improved Ejection fraction (EF%) and Fractional shortening (FS%) and diminished left ventricular hypertrophy in TM-ES1oe mice compared with TMCM mice. Sections of TM-ES1oe hearts stained with Masson's Trichrome blue showed markedly decreased interstitial fibrosis compared with TMCM control. We have also generated an ES1 knockout line. ES1 knockout mice(3-month-old)showed cardiac dysfunction with decreased EF% and FS% under a basal condition. Transmission electron microscope examination revealed substantial loss of mitochondrial cristae structure on ES1 knockout hearts. These results indicate that ES1 protecting the heart by direct regulation of mitochondrial energy metabolism. ES1 may be directly involved in pathological development of cardiac hypertrophy and heart failure. We suggest that ES1 is a potential therapeutic target in treating cardiomyopathy and other heart diseases.

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ABSTRACTS

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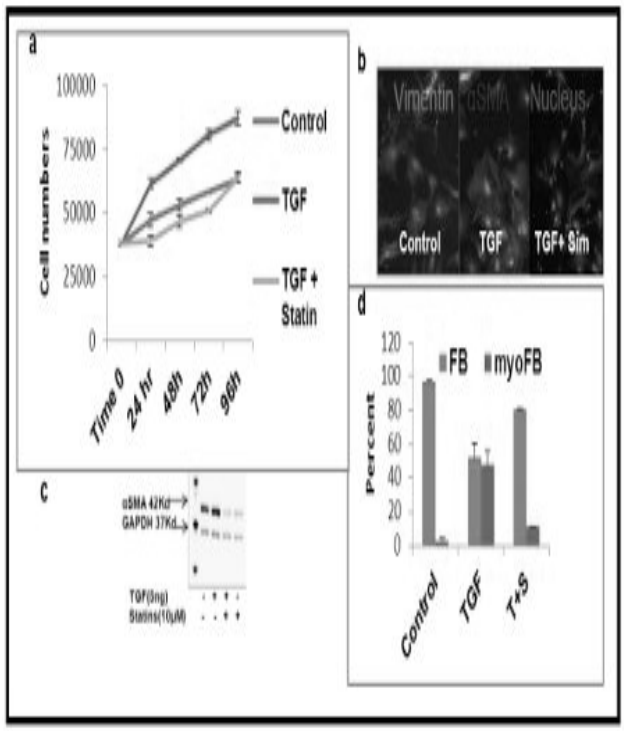
Simvastatin Inhibits TGF-β1-induced Proliferation and Activation of Cardiac Fibroblasts Through Inhibition of SMAD Pathway

Farhan Rizvi, Ramail Siddiqui, Alesandra DeFranco, Akhil Jayaprakash, Mahek Mirza, Larisa Emelyanova, Gracious Ross, Ekhson Hulmohamedov, Ctr for Integrative Res on Cardiovascular Aging (CIRCA), Aurora UW Medical Group, Milwaukee, WI; Daniel O'Hair, Aurora Medical Group-Cardiovascular and Thoracic Surgery, Aurora St. Luke's Medical Ctr, Milwaukee, WI; A. Jamil Tajik, Aurora Cardiovascular Services, Aurora Sinai/Aurora St. Luke's Medical Ctrs, Univ of Wisconsin Sch of Med and Public Health, Milwaukee, WI; Arshad Jahangir, Ctr for Integrative Res on Cardiovascular Aging (CIRCA), Aurora UW Medical Group, Milwaukee, WI

Background: HMG-CoA reductase inhibitors (statins) have been shown to reduce the incidence of atrial fibrillation (AF) and its progression but the underlying mechanisms are not fully elucidated. Since atrial fibrosis plays a major role in the development of the substrate for AF progression, we hypothesized that statins antagonize the effect of profibrotic cytokines, reducing their stimulatory effect on fibroblast proliferation, differentiation and activation. Methods: The effect of TGF-β1, a major profibrotic cytokine, on fibroblast proliferation and activation of myofibroblasts was assessed by expression of alpha smooth muscle actin (α-SMA) message (qPCR) and proteins (western and immunofluorocytochemistry) in the presence and absence of simvastatin (1-10μM). The inhibitory effect of simvastatin on SMAD 2/3 phosphorylation (western) and its nuclear translocation by TGF-β1 (5ng) was determined by immunofluorescence antibodies using fluorescent microscopy. Results: TGF-β1 treatment increased fibroblast proliferation (cell count) by 63% compared to control ($p<0.001$) at 96 hours, which was inhibited by simvastatin by 61% ($p<0.001$) (Fig a). Simvastatin also reduced TGF-β1-mediated myofibroblast differentiation (α-SMA positive) by 75% ($p=0.02$); (Fig b and c). TGF-β1 increased SMAD2/3 phosphorylation with increase in nuclear localization was inhibited by simvastatin. Conclusion: Simvastatin inhibits TGFβ-1-mediated cardiac fibroblast proliferation and myofibroblast differentiation by antagonizing SMAD phosphorylation and its translocation into the nucleus.



F. Rizvi: None. **R. Siddiqui:** None. **A. DeFranco:** None. **A. Jayaprakash:** None. **M. Mirza:** None. **L. Emelyanova:** None. **G. Ross:** None. **E. Hulmohamedov:** None. **D. O'Hair:** None. **A. Tajik:** None. **A. Jahangir:** None.

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Metallothionein Preservation Of Cardiac Akt2 Signaling By Down-regulating Trb3 Prevents Diabetic Cardiomyopathy

YI TAN, Xiaoqing Yan, Shanshan Zhou, Yong Li, Yan Li, Lu Cai, Univ of Louisville, Louisville, KY

Cardiac insulin resistance is a key pathogenic factor for diabetic cardiomyopathy, but its mechanism remains largely unclear. Here we demonstrated that diabetes significantly inhibited cardiac Akt phosphorylation from 2 weeks to 2 months in wide-type (WT) mice, but not in cardiac-specific metallothionein-transgenic (MT-TG) mice. Cardiac Akt2 expression and phosphorylation was decreased and insulin-induced cardiac Akt2 and GSK-3β

phosphorylation and glycogen synthase dephosphorylation were also decreased in WT, but not MT-TG, diabetic mice. Deletion of the Akt2 gene either in vitro H9c2 cells or in vivo significantly impaired cardiac glucose metabolic signaling. In addition, diabetes significantly increased cardiac Akt negative regulator tribbles (TRB)3 expression only in WT mice, suggesting the possible contribution of MT inhibition of diabetic up-regulation of TRB3 to Akt2 function preservation. Cardiac H9c2 cells with and without forced MT-overexpression (MT-H9c2) were treated with tert-butyl hydroperoxide (tBHP), which significantly reduced Akt2 phosphorylation in both basal and insulin-stimulating conditions only in H9c2 cells. Silencing TRB3 expression with SiRNA completely prevented tBHP's inhibition of insulin-stimulated Akt2 phosphorylation in H9c2 cells, while overexpression of TRB3 in MT-H9c2 cells completely abolished MT preservation of insulin-stimulated Akt2 phosphorylation. Forced-overexpression of TRB3 by adenovirus-mediated gene delivery in MT-TG hearts also abolished MT's preservation of cardiac insulin signaling and prevention of diabetic cardiomyopathy. These results suggest that diabetes-attenuated cardiac Akt2 function via up-regulating TRB3 plays a critical role in diabetic inhibition of insulin signaling in the heart. MT preserved cardiac Akt2-mediated insulin signaling by inhibiting TRB3, leading to the prevention of diabetic cardiomyopathy.

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Recovery of Mitochondrial Bioenergetics after Hypoxia via Regulating Pyruvate Dehydrogenase Kinase and Adenosine Monophosphate-activated Kinase

Jessica M Toli, Minzhen He, Carolyn Suzuki, Maha Abdellatif, Rutgers Univ, Newark, NJ

Mitochondrial quality control is critical for the survival of cardiac myocytes during stress. The purpose of this study was to examine the effect of metabolic substrates and regulators of metabolism on mitochondrial bioenergetics, as an indicator of mitochondrial quality, and how these factors might influence the recovery of the cell's bioenergetics after hypoxia/ischemia. By monitoring oxygen consumption rates (OCR), in real-time, in live neonatal rat myocytes and human cardiac myocyte-differentiated induced pluripotent stem cells, we found that both cell types can maintain basal OCR efficiently with any metabolic substrate; however, the neonatal cells require both glucose and fatty acid, while the human adult cells require fatty acid only, for mounting maximum reserve respiratory capacity (RRC). Our data also show that subjecting cardiac myocytes to hypoxia results in a reduction of the cells' basal OCR and oxidative phosphorylation, and exhausts the RRC, which is accompanied by an increase in pyruvate dehydrogenase kinase (Pdk) 1 and 4. Except for normalization of Pdk1 levels, there was little or no recovery of these parameters after reoxygenation. We, thus, hypothesized, that inhibition of Pdks may help recovery of the cell's bioenergetics. Indeed, our results show that by inhibiting Pdks with dichloroacetate (DCA) before or after hypoxia, the cells' bioenergetics, including OCR, oxidative phosphorylation, and RRC in neonatal myocytes, and RRC in the human myocytes fully recover within 24 h. On the other hand, activating AMP-activated kinase (AMPK) resulted in delayed (96 h) improvement of the cells' RRC that was accompanied by an increase in peroxisome proliferator-activated receptor gamma, coactivator 1α (3.5x), peroxisome proliferator-activated receptor-α (2x), and mitochondrial number (2x). These results led us to conclude that compromised mitochondrial quality can be rescued through mechanisms that regulate glucose or fatty acid oxidation by either inhibiting Pdks or activating AMPK, respectively, in rodent and human myocytes.

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Loss of beta-catenin Leads to Induction of a Chondrogenic Phenotype in Adult Mouse Heart Aortic Valves

Ming Fang, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH; Simon J. Conway, Indiana Univ, Indianapolis, IN; Katherine E. Yutzey, Cincinnati Children's Hosp Medical Ctr, Cincinnati, OH

ABSTRACTS

Objectives: Altered Wnt/beta-catenin signaling has been implicated in human heart valve disease; however, a causal relation has not been established. This study is aimed at determining the role of Wnt/beta-catenin signaling in adult valve homeostasis and disease. In endochondral bone development, Wnt/beta-catenin signaling inhibits early chondrogenesis and promotes osteogenesis. Therefore, we hypothesize that loss of Wnt/beta-catenin signaling promotes chondrogenic differentiation of valvular interstitial cells (VICs) resembling myxomatous valve disease. Methods & Results: Periostin (Postn)Cre was used for conditional loss of beta-catenin (Ctnnb1) specifically in VICs in mice. PostnCre is active in valve mesenchymal progenitors by embryonic day (E)13.5, therefore bypassing requirements for beta-catenin in endothelial-to-mesenchymal transition and endocardial cushion formation. Histology of adult heart valves was analyzed by Masson's Trichrome and Movat's Pentachrome staining, and the expression of extracellular matrix (ECM) genes was examined at both mRNA and protein level. Heart valve development appears normal at both fetal and neonatal stages with loss of beta-catenin. However proteoglycan-rich nodules form in adult aortic valves (AoVs) lacking beta-catenin at 3 months with 100% penetrance. These nodular cells are enlarged and express nuclear Sox9 and chondrogenic ECM genes, including Aggrecan, Collagen type 2 (Col1) and Col10, reminiscent of hypertrophic cartilage. Fibrillar collagens 1 and 3, as well as Versican, are excluded from the nodules, accompanied by abnormal ECM deposition throughout the AoV leaflets. Likewise, treatment of cultured porcine aortic VICs with the Wnt pathway inhibitor XAV also induces Aggrecan and Col2 gene expression in vitro. This work demonstrates that beta-catenin is required for the maintenance of normal ECM deposition and composition in adult mice, and, therefore, is indispensible for adult heart valve homeostasis. Conclusions: We have demonstrated that beta-catenin is a key factor in maintaining normal valve ECM organization in adult AoV. In the absence of beta-catenin, VICs are susceptible to chondrogenic differentiation related to myxomatous valve disease.

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Maturation Of ß-adrenergic Receptor (ß-AR) Signaling In Human Induced Pluripotent Stem Cell-derived Cardiomyocytes (hipsc-cms)

Gwanghyun Jung, HaoDi Wu, Giovanni Fajardo, Kazuki Kodo, Joseph Wu, Daniel Bernstein, Stanford, palo alto, CA

Gwanghyun Jung1, HaoDi Wu2, Giovanni Fajardo1, Kazuki Kodo2, Joseph Wu2, and Daniel Bernstein1
Stanford Cardiovascular Institute and 1Department of Pediatrics, and 2Departments of Medicine and Radiology, Stanford University School of Medicine

hiPSC-CMs are a novel model system for cardiovascular diseases, with proven utility in cardiac channelopathies, however, how accurately hiPSC-CMs recapitulate the signaling pathways regulating contractility and remodeling of adult cardiomyocytes remains to be determined. As ß-ARs are key regulators of both contractility and remodeling, we examined ß-AR signaling at different stages of hiPSC-CM maturation. hiPSCs, derived from healthy controls, were studied at 0, 14, 30, and 60d after cardiac induction under isoproterenol (ISO) stimulation ± ß1 or ß2 antagonists. Flag-tagged ß-ARs were transfected into hiPSC-CMs for trafficking studies. Compared to the ß1:ß2 ratio of 70:30 in adult ventricular cardiomyocytes, ß2-AR expression was higher in early stage (d14-30) hiPSC-CMs; ß1-ARs increased with maturation (d14-d60), reflective of the normal developmental pattern. Downstream signaling also matured from d14-60, regulating both function (PLB and TnI) and remodeling (ERK). The majority of cAMP generation and PKA activation at d30 was through ß2-ARs; by d60-90, ß1-AR signaling contributed equally. In contrast to studies in rodent cells, ß2-ARs did not show compartmentalization of PKA signaling. Between d30-60, components of the SR (RYR, PLB and CSQ) and caveolae (Cav3) matured, suggesting development of functional compartmentalization machinery. Finally, hiPSC-CMs showed ß2-AR downregulation and internalization in response to ISO, an important

response to pathologic stimulation. Thus, hiPSC-CM ß-AR signaling matures with time after cardiac induction, recapitulating many of the features of post-natal and several of adult cardiomyocytes. hiPSC-CM models of human cardiomyopathies are a reasonable platform for studying ß-AR signaling. Furthermore, the maturation of ß-AR signaling can be used as a read-out for hiPSC-CM maturity.

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Proliferation of Cardiomyocytes from a Rat Model of Heart Hypoplasia

Corin Williams, Raymond Wang, Lauren D Black III, Tufts Univ, Medford, MA

Decreased cardiomyocyte proliferation has been implicated in underdevelopment of the left ventricle in Hypoplastic Left Heart Syndrome (HLHS). However, it is not clear if cardiomyocytes in HLHS have an inherent defect that inhibits proliferation or if it is the result of abnormal external cues (eg, alterations to mechanical loading or extracellular matrix). We hypothesized that cardiomyocytes from hypoplastic hearts would be capable of proliferation if removed from the unhealthy cardiac environment. We used the rat model of congenital diaphragmatic hernia (CDH) via nitrofen treatment in the pregnant dam at E10 to induce heart hypoplasia in the developing fetuses; control dams were gavaged with olive oil only. Fetuses were harvested at E21 and the presence of CDH was determined. CDH+ hearts were smaller and abnormally shaped compared to healthy controls (*Fig. 1A*). Ki67 staining revealed that CDH+ hearts had significantly lower cell proliferation vs. healthy hearts (*Fig. 1B*). However, when cells were placed in culture, CDH+ cardiomyocytes expanded more rapidly compared to control cardiomyocytes (*Fig. 1C*). CDH+ cells also had smaller, more disorganized sarcomeres (not shown) and decreased expression of cardiac genes compared to controls (*Fig. 1D*), suggesting that CDH+ cells were less mature. Our findings suggest that cardiomyocytes from hypoplastic hearts may be arrested in an immature state but are not necessarily defective, and have potential for proliferation and growth. Strategies that aim to increase cardiomyocyte proliferation during development in HLHS may be a novel strategy to encourage left ventricular growth and lead to better outcomes in these patients.

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Exercise Protects the Heart by Preserving Mitochondrial Membrane Potential During Early Reperfusion

Rick J Alleman, Hetal D Patel, Fatiha Moukdar, David A Brown, East Carolina Univ, Greenville, NC

Exercise evokes adaptations intrinsic to the myocardium that protect against ventricular arrhythmia, yet the underlying mechanisms are not completely understood. We have previously shown that the transition to arrhythmia occurs concomitant with a collapse in mitochondrial membrane potential (ΔΨm). As our previous studies indicated that exercise preserves intracellular redox homeostasis, which directly influences mitochondrial energetics, we hypothesized that rats exposed to exercise (Ex, 10 d of treadmill running) would be protected against reperfusion arrhythmia via better maintenance of ΔΨm. To fully understand the temporal relationship between ΔΨm and cardiac electrical activity, two-photon microscopy images (using the fluorescent probe TMRM) and volume-conducted electrocardiogram were simultaneously recorded. Langendorff-perfused hearts underwent 40/30 min of ischemia/reperfusion. Exercise lowered the incidence of arrhythmia, with 3 of 8 Ex hearts experiencing tachycardia or fibrillation compared to 7 of 8 sedentary (Sed) hearts. Ex prevented the collapse of ΔΨm during the first 10 min of reperfusion (74±6.4% v 57±1.5% of baseline fluorescence intensity; $P<0.05$). To gain a more comprehensive understanding of energetics throughout the heterogeneous mitochondrial population, we then measured mean TMRM fluorescence intensity in isolated ventricular mitochondria harvested after reperfusion using flow cytometry (n=100,000 events per group). Interestingly, mean fluorescence intensity for ΔΨm was similar in Ex and Sed mitochondria (278±33 v 309 ±44 AU, respectively).

Mitochondrial respiratory control ratios were also similar in Ex and Sed (9.03±0.70 v 9.00±0.92, respectively). Taken together, the isolated mitochondrial assessment did not reflect what was observed in vivo. This suggests that either intracellular factors influenced in vivo mitochondrial energetics, or our isolated mitochondria may have been enriched with predominantly healthy mitochondria. Our studies demonstrate for the first time that exercise prevents electrical dysfunction following an ischemic insult through better preservation of mitochondrial energetics, and that this preservation is only observed in the intact organ.

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Cardiac Function Improvement in Infarcted Rats Treated with an Association of N-acetylcysteine and Deferoxamine

Michael Andrades, Hosp de Clínicas de Porto Alegre, Porto Alegre, Brazil; Amanda Phaelante, Virgílio Olsen, Univ Federal do Rio Grande do sul, Porto Alegre, Brazil; Santiago Tobar, Hosp de Clínicas de Porto Alegre, Porto Alegre, Brazil; Amanda Lopes, Nidiane Martinelli, Univ Federal do Rio Grande do sul, Porto Alegre, Brazil; Carolina Cohen, Hosp de Clínicas de Porto Alegre, Porto Alegre, Brazil; Felipe Dal-Pizzol, Univ do Extremo Sul Catarinense, Porto Alegre, Brazil; Andreia Biolo, Luis Rohde, Nadine Clausell, Univ Federal do Rio Grande do Sul, Porto Alegre, Brazil

Acute myocardial infarction (AMI) is followed by free radicals generation, which is central in cardiac remodeling. N-acetylcysteine (NAC) is an antioxidant that may have pro-oxidant activity in presence of iron ions. Thus, the association with an iron chelator might improve NAC antioxidant potential. Our aim was to test the association of NAC and an iron chelator (deferoxamine - DFX) on cardiac remodeling in a rodent model of AMI. To this end, Male Wistar rats (60 days old) were subjected to a sham surgery or subjected to myocardial infarcted by the occlusion of left ventricular descending artery. Twelve hours after, AMI was confirmed by cTnI evaluation and the animals were randomized in the following treatments: vehicle, NAC (25 mg/kg for 28 days), DFX (40 mg/kg for 7 days), or NAC plus DFX (NAC 25 mg/kg for 28 days plus DFX 40 mg/kg for 7 days). All animals were followed for 28 days. AMI induced an increase in total plasma iron levels at 7 days after the procedure and all treatments were able to decrease iron concentration. NAC/DFX was able to keep iron levels low up to 28 days. Morphofunctional assessments were made serially by echocardiography. Animals treated with NAC/DFX showed an increase in ejection fraction at 28 days when compared with vehicle group (45% ± 11% vs. 35% ± 9%,respectively; p=0.03). On histological analysis (at 28 days), NAC/DFX treated rats showed decreased oxidative stress (immunohistochemistry anti-4-hydroxynonenal, a side product of lipid peroxidation) when comparing with vehicle group (p=0.059). In conclusion, the association of NAC/DFX was able to attenuate chamber remodeling and improved left ventricle function in an animal model of AMI, whereas either NAC or DFX had no effect when given alone. The likely participation of iron and free radicals can be suggested, but further studies are necessary.

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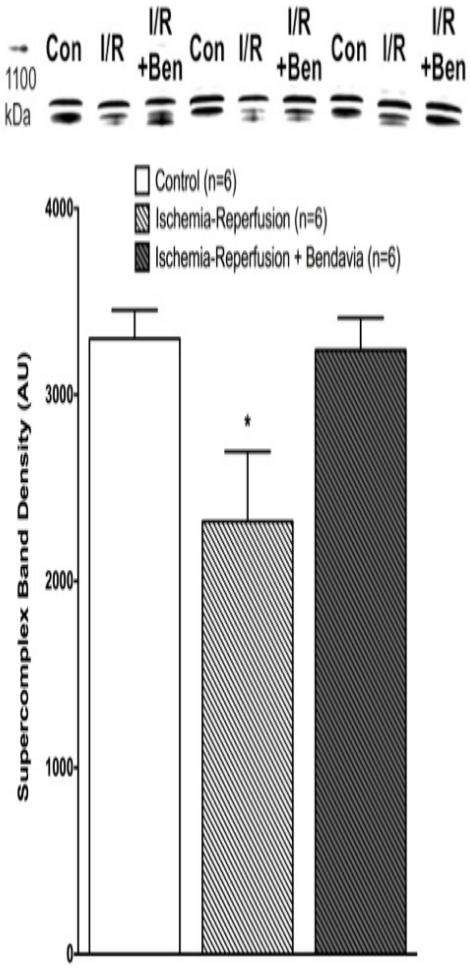
The Cardiolipin-targeting Peptide Bendavia Preserves Post-ischemic Mitochondrial Energetics by Sustaining Respiratory Supercomplexes

David A Brown, Fatiha Moukdar, Rick J Alleman, Daniel S Lark, P Darrell Neufer, Saame Raza Shaikh, East Carolina Univ, Greenville, NC

Bendavia is a mitochondria-targeting peptide currently being tested in the EMBRACE-STEMI trial for reducing injury during acute coronary syndromes. We previously showed that the cardioprotective effects of Bendavia involved improving cardiolipin-dependent mitochondrial membrane fluidity. As membrane fluidity influences the ability of proteins to assemble, we hypothesized that a consequence of augmented membrane fluidity would be higher expression of mitochondrial respiratory supercomplexes. Rat hearts (n=42) were subjected to ischemia-reperfusion (I/R) with or without 1nM Bendavia

perfusion beginning at the onset of reperfusion. Left ventricular tissue was split into one of two study arms: 1. Supercomplex expression using blue-native gel electrophoresis (BN-PAGE), or 2. High-resolution respirometry using permeabilized ventricular fibers. For BN-PAGE studies, respiratory supercomplex bands were decreased with I/R, and restored with Bendavia (see figure). High-resolution respirometry studies indicated suppressed Complex I-dependent respiration after I/R (208±19 v 42±9 pmol O2/mg*s in control v I/R, respectively; P<0.05). Complex II-dependent respiration was also lower (753±41 v 168±13 pmol/mg*s in control versus I/R; P<0.05). Perfusion with Bendavia during reperfusion significantly increased Complexes I- (100±13pmol O2/mg*s) and II-dependent (334±63 pmol O2/mg*s) respiration (P<0.05 versus untreated IR for both). Taken together, these data suggest that Bendavia's protective mechanism of action involves preserving supercomplex-dependent mitochondrial function during cardiac reperfusion.



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p55γ Suppresses VSMC Proliferation and Neointimal Formation

Chun-Mei Cao, Ning Xie, Yuan Yao, Yan Zhang, Rui-Ping Xiao, Inst of Molecular Med, Peking Univ, Beijing, China

Abnormal proliferation of vascular smooth muscle cells (VSMCs) contributes to various vascular diseases, but the factors that maintain VSMCs in a quiescent state remain poor understood. Phosphatidylinositol 3 kinases (PI3Ks) are important protein kinases that regulate vascular cell proliferation, but the biological and pathological functions of p55γ, a regulatory subunit of PI3K, and its regulation in the cardiovascular system are completely unknown. We aimed to determine the relationship between p55γ and vascular proliferation and neointimal formation. In the present study, we have demonstrated that p55γ expression is markedly downregulated in primary cultured VSMCs in response to mitogenic stimulation and in carotid arteries after balloon injury, and that overexpression p55γ profoundly inhibits mitogenic stimuli and injury induced VSMC proliferation as well as neointimal formation. p55γ overexpression inhibited, whereas knockdown of p55γ promoted PDGF-BB- and serum-induced VSMC proliferation. Importantly, *in vivo* adenoviral gene transfer of p55γ into carotid arteries attenuated, while knockdown of p55γenhanced balloon injury-induced neointimal formation. Furthermore, p55γ sequentially upregulated p53 and p21, resulting in cell-cycle arrest in S phase; knockdown of either p53 or p21 blocked p55γ-induced VSMC growth arrest. Mechanistically, p55γ interacted with and stabilized p53 protein by blocking MDM2-mediated p53 ubiquitination and degradation, subsequently activating its target gene p21. Concurrently, p55γ upregulated Bcl-xl expression, which counterbalanced p53-mediated apoptosis. These findings mark p55γ as a novel upstream regulator of the p53-p21 signaling pathway which negatively regulates

VSMC proliferation, suggesting that malfunction of p55γ may trigger vascular proliferative disorders.
* Correspondence to Chun-Mei Cao (caochunmei@pku.edu.cn) or Rui-Ping Xiao (xiaor@pku.edu.cn).

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Heterozygous Disruption of Drp1 Enhances Myocardial Injury in Response to Ischemia/Reperfusion via Inhibition of Mitophagy

Yoshiyuki Ikeda, Peiyong Zhai, Rutgers Univ New Jersey Medical Sch, Newark, NJ; Mitsuru Ohishi, Kagoshima Univ, Kagoshima, Japan; Junichi Sadoshima, Rutgers Univ New Jersey Medical Sch, Newark, NJ

Mitochondrial fission and fusion are essential for maintaining mitochondrial quality control. However, their role in stress resistance remains unknown. Since Dynamin-related protein 1 (Drp1) plays an essential role in mediating mitochondrial fission, we used cardiac-specific heterozygous Drp1 KO (Drp1-hetCKO) mice whose cardiac function is normal at 12 weeks of age. In order to evaluate the role of Drp1 in mediating stress resistance, 12-week-old Drp1-hetCKO and control (Ctr) mice were subjected to myocardial ischemia (30 min)/reperfusion (24 hours) (I/R). I/R injury increased the translocation of Drp1 from cytosol to mitochondria in Ctr mice, whereas this translocation was attenuated in Drp1-hetCKO mice.The infarct size/area at risk after I/R was significantly greater in Drp-1 hetero KO mice than in control mice (55.2 ± 3.0 vs. 40.2 ± 1.6%). Electron microscopic analyses showed that I/R significantly decreased mitochondrial mass and increased the number of autophagosomes containing mitochondria in control mice. However, these changes were significantly attenuated in Drp1-hetCKO mice, suggesting that endogenous Drp1 mediates mitochondrial fission and mitophagy after I/R (Relative mitochondrial mass, Ctr, baseline: 1.0±0.3, I/R: 0.7±0.4; Drp1-hetCKO, baseline: 1.8±0.3, I/R: 1.7±0.3). We also examined mitophagy using mitochondria-targeted Keima fluorescence. Keima has a bimodal excitation spectrum peaking at 440 and 560 nm, corresponding to the neutral and acidic pH states, respectively. The maturation of autophagosomes to autolysosomes can be monitored by measuring the fluorescence of mitochondria-targeted Keima. Fluorescent dots with high ratios of 560/440, indicating mitophagy, were significantly increased after GD in sh-scramble-transduced CMs (GD: 28.6±4.2, control 2.3±1.0 dots/cell, $p<0.01$) but not in sh-Drp1-transduced CMs (GD: 2.4±1.8, control 3.2±1.6 dots/cell, not significant), suggesting that GD stimulates mitophagy but that this is inhibited when Drp-1 is downregulated. These results suggest that endogenous Drp1 play an essential role in mediating mitochondrial fission and mitophagy, which in turn play an essential role in mediating myocardial protection in response to I/R.

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Heat Shock Proteins HSP90 and HSP70 Mediate Opioid- and GSK3β-induced Cardioprotection

Sarah K Jimenez, Bryce A Small, Stanford Univ, Stanford, CA; Anna K Hsu, Garrett J Gross, Medical Coll of Wisconsin, Milwaukee, WI; Eric R Gross, Stanford Univ, Stanford, CA

Previously, opioids were established to reduce myocardial injury in an ischemic preconditioning (IPC)-like manner, involving a central and downstream role of glycogen synthase kinase-3 beta (GSK-3β) inhibition. However, the mechanism of GSK-3β inhibition mediating cardioprotection and the protein partners

involved has not been fully elucidated. Hence, we used a non-biased sequence scan of the proteome to determine potential GSK-3β protein partners and tested whether two candidate proteins, heat shock proteins (HSP) 70 and 90, are involved in the mechanism of opioid-induced cardioprotection.
A non-biased BLAST search was performed for putative GSK-3β target substrates, based upon the sequence motif S/T-X-X-X-S/T. Approximately 700 proteins were identified to have this moiety, including many of the HSP protein class, including HSP70 and HSP90. To determine whether HSP70 or HSP90 are indeed important in opioid-induced cardioprotection, rats were subjected to an in vivo myocardial ischemia-reperfusion protocol consisting of 30 minutes of ischemia and 2 hours of reperfusion of the left anterior descending coronary artery followed by infarct size assessment. Either morphine (0.3mg/kg) or inhibition of GSK-3β using SB216763 (0.6mg/kg), reduced infarct size compared to control (42.21±1*% and 41.09±2*%, respectively versus control 60.38±1.2, *P<0.01). Inhibition of HSP70 using desoxysperguanalin (DSG), or HSP90 using radicicol (RAD), abrogated morphine-induced protection (56.09±2 and 58.64±1, respectively). Either DSG or RAD partially inhibited protection in the presence of GSK-3β (47.28±1.071 and 49.88±3.09). Our results suggest that morphine-induced cardioprotection occurs by a HSP70 and HSP90- dependent mechanism, with this HSP machinery partially required for GSK3β-inhibition-induced cardioprotection. Further understanding of this mechanism is important, considering many agents targeting HSP are currently in development as novel cancer treatments, which may have detrimental effects on the myocardial salvage mediated by opioids or by GSK3β-inhibition.

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GsMTx4-D is a Cardioprotectant against Myocardial Infarction during Ischemia and Reperfusion

Ji Li, Jinli Wang, Frederick Sachs, Thomas M. Suchyna, Univ at Buffalo-SUNY, Buffalo, NY

Rationale: GsMTx4 peptide is a selective inhibitor of cation selective mechanosensitive ion channels (MSCs) and has helped establish the role of MSCs in cardiac physiology. However, the role of MSCs in ischemic reperfusion injury was unknown. Cation imbalance appears to be a major contributor to ischemic reperfusion injury across multiple systems. MSCs may be significant contributors to the cation overload, and they are likely to be activated in reperfusion due to inhomogeneous local stresses and compromised mechanical support of the sarcolemma. Objective: The aim of this study was to determine if the D enantiomer of GsMTx4 can act as a cardioprotectant during ischemia/reperfusion in mice. Methods and Results: GsMTx4-D pharmacokinetics in the plasma and heart was monitored over 24 hrs using an LCMSMS assay. Ischemia and reperfusion in the mouse heart involved ligating a coronary artery for 20 min followed by release of the ligature. GsMTx4-D was administered by either acute intravenous injection during the ischemic event, or a day prior to the event with two days of intraperitoneal injections, once per day. Dosing of GsMTx4-D was adjusted to achieve tissue concentrations of 1-5 μM. Relative to vehicle injected animals, GsMTx4-D reduced infarct area by >40% for both acute and pretreated animals. Cardiac output was indistinguishable between sham-treated control hearts and GsMTx4-D pretreatment, and arrhythmias were also significantly reduced by intravenous injections of GsMTx4-D.
Conclusions GsMTx4-D is a potent cardioprotectant that decreases infarct area, increases cardiac output and decreases arrhythmias that are caused by ischemia and reperfusion. GsMTx4-D was not toxic and promises to be a useful therapy in reperfusion of the heart and other organs.

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ABSTRACTS

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Implication Of Mir-98 In The Cardiac Vulnerability Of Pregnancy To Ischemia Reperfusion Injury

Jingyuan Li, UCLA, Los Angeles, CA; Zoltan Pierre Arany, Beth Israel Deaconess Medical Ctr, Boston, CA; Mansoureh Eghbali, UCLA, Los Angeles, CA

We have recently reported that the late-pregnant(LP) rodent is more prone to myocardial ischemia-reperfusion (I/R) injury compared to non-pregnant(NP). However, the underlying molecular mechanisms involved in the higher susceptibility of LP to IR injury are not quite clear. The objective of this study was to investigate the role of miRNA-98(miR-98) in higher cardiac vulnerability to I/R injury in late pregnancy. NP and LP(19 days of pregnancy Sprague-Dawley rats were subjected to 45 min myocardial ischemia followed by 3 hr reperfusion in vivo (I/R group). NP and LP rats which were not subjected to I/R injury served as controls. MicroRNA microarray expression were performed. In vitro, miR-98 was knocked down or overexpressed in neonatal rat ventricular myocytes(NRVMs). Forty eight hour post-transfection, the cells were subjected to 3 hr hypoxia followed by 12 hr reoxygenation. The apoptosis was detected by TUNEL staining, and the western blot was performed to validate the computational predicted targets genes, PGC-1α and STAT3. The miRNA expression profile showed that several miRNAs were differentially expressed in LP sham compared to NP sham, particularly, miR-98 which was significantly upregulated 1.7 fold in LP sham vs NP sham. Interestingly, miR-98 was upregulated even higher (2.3 folds) in LP rats when subjected to ischemia/reperfusion injury compared to NP I/R, suggesting that both ischemia reperfusion and late pregnancy regulate miR-98. Knock down of miRNA-98 in NRVMs significantly reduced apoptosis by ~50%, while over-expression of miR98 resulted in a two fold increase in apoptosis. In-vitro overexpression of miR98-KD in NRVMS resulted in the a significant upregulation of PGC-1α(normalized to scramble control, 1.34±0.04, $P<0.05$) and STAT3(normalized to scramble control, 1.28±0.05, $P<0.05$), whereas overexpression of miR-98 resulted in downregulation of both target genes(normalized to scramble control, 0.75±0.02 in PGC-1α; 0.5±0.03 in STAT3, both $P<0.05$). In conclusion, induction of miR-98 by pregnancy possibly through downregulating PGC-1α and STAT3 may at least in part underlie the greater cardiac vulnerability to I/R injury in late pregnancy.

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Activation of ROR-α, but not ROR-β or ROR-γ, Protects against Myocardial Ischemia/Reperfusion Injury

Jun Pu, Ben He, Shanghai Renji Hosp,Sch of Med, Shanghai Jiaotong Univ, Shanghai, China; Erhe Gao, Ctr for Translational Med, Temple Univ Sch of Med,, Philadelphia, PA; Xinliang Ma, Dept of Emergency Med, Thomas Jefferson Univ, Philadelphia, PA; Yajing Wang, Dept of Emergency Med, Thomas Jefferson Univ, 1025 Walnut Street, Philadelphia, PA

Objectives: The RAR-related orphan receptors (RORs) are members of the nuclear receptor superfamily that play a pivotal role in many physiological processes, including regulation of the circadian rhythm, development, metabolism and immune function. Three different but highly homologous ROR isoforms, ROR-α, -β, and -γ, have been discovered separately. However, the functional roles of RORs in the heart have never been investigated. We investigate the role of RORs in the pathophysiology of acute myocardial ischemia/ reperfusion (MI/R) injury. **Methods and Results:**The endogenous RORα, but not RORβ or RORγ, was significantly upregulated after MI/R. Synthetic ROR agonists SR1078 and SR3335 reduced myocardial infarction and improved contractile function after MI/R. Mechanistically, ROR activation inhibited endoplasmic reticulum (ER) stress, attenuated mitochondrial impairment, reduced cardiomyocyte apoptosis, and inhibited MI/R-induced autophagy dysfunction. Moreover, ROR activation inhibited MI/R-induced oxidative stress and nitrative stress. The aforementioned cardioprotective effects of ROR agonists were impaired in the setting of cardiac-specific gene silencing of RORα, but not RORβ or RORγ subtype. In contrast, adenovirus-mediated cardiac RORα overexpression, but not RORβ or RORγ overexpression, decreased myocardial infarct size and improved cardiac function through attenuating oxidative/nitrative stress and inhibiting ER stress, mitochondrial impairment

ABSTRACTS

and autophagy dysfunction. Finally, RORα$^{sg/sg}$ mice (loss-of-function mutation in RORα), but not RORβ-null or RORγ-null mice, increased MI/R injury (greater apoptosis, larger infarct size, and poor cardiac function), exacerbated MI/R-induced oxidative/nitrative stress, and aggravated endoplasmic-reticulum stress, mitochondrial dysfunction, and autophagy dysfunction.

Conclusion: Our study provides the first direct evidence that RORα acts as a novel endogenous cardioprotective receptor against myocardial injury. RORα, but not RORβ or RORγ, is a novel cardiac protective receptor against MI/R injury, supporting for the drug development strategies specifically targeting RORα for the treatment of ischemic heart disease.

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Basis for Bicarbonate Damage in Myocardial Ischemia/Reperfusion Injury

Bruno B Queliconi, Insto de Química, USP, São Paulo, Brazil; Fernanda M Cunha, Escola de Artes, Ciências a Humanidades, USP, São Paulo, Brazil; Roberta A Gottlieb, Cedars-Sinai Medical Ctr, Los Angeles, CA; Alicia J Kowaltowski, Insto de Química, USP, São Paulo, Brazil

In the clinical setting, bicarbonate is often used to correct acidosis arising from accumulated CO2/HCO3- during ischemia. We observed that HL-1 cardiomyocytes exposed to increased [CO2/HCO3-] had more cell death after hypoxia/reoxygenation (H/R) and Langendorff-perfused rat hearts had larger infarcts after ischemia/reperfusion (I/R). In addition to buffering pH, the CO2/HCO3- pair possess an underappreciated redox activity that may contribute to injury. In order to study the effects of high CO2/HCO3- during ischemic injury independent of pH, we clamped pH using HEPES and used the mouse cardiomyocyte HL-1 cell line and isolated perfused rat hearts. HL-1 cells exposed to 10% CO2/HCO3- had no damage under basal conditions but developed exaggerated protein carbonylation and cell death after H/R. In Langendorff-perfused rat hearts, 10% CO2 was well tolerated during baseline conditions but resulted in increased protein carbonylation, cell death and larger infarcts after I/R. We hypothesized that the increased oxidative damage to proteins could be due to mitochondrial dysfunction with greater ROS production, diminished proteasomal degradation of oxidized proteins, or impaired autophagic clearance of damaged mitochondria and oxidized protein aggregates. There was no differential effect of CO2 on mitochondrial morphology or proteasomal activity in HL-1 cells. In mitochondria isolated from perfused hearts subjected to I/R under low and high CO2 conditions, there was no difference in ROS production or oxidized protein content, suggesting mitochondrial damage was not affected by CO2 level. Examination of autophagy in HL-1 cells exposed to high CO2 during H/R revealed higher LC3-II and lower p62 content. In hearts, changes in LC3-II were inconsistent; however, we detected less p62 protein, less mitochondria-associated Beclin1, and significantly more LC3 mRNA in hearts exposed to 10% CO2 during I/R. Taken together, these findings suggest that 10% CO2 affects autophagy, which could explain the accumulation of oxidatively damaged proteins. These findings point to a protective role for autophagic clearance of oxidized protein aggregates during I/R injury that may be adversely impacted by bicarbonate therapy.

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Inadequate Coupling between Ubiquitination and the Proteasome is a Major Pathogenic Factor of Myocardial Ischemia/Reperfusion Injury

Xuejun Wang, Chengjun Hu, Yihao Tian, Hongmin Wang, Xuejun Wang, Univ of South Dakota, Vermillion, SD

Targeted removal of damaged/misfolded proteins is primarily done by the ubiquitin (Ub)-proteasome system (UPS). Generally, the UPS degrades a protein molecule via two main steps: ubiquitination of the protein and subsequent degradation of the ubiquitinated protein by the proteasome. The two steps are not always directly coupled. The proteasome can directly uptake nearby polyubiquitinated proteins via its

Ub receptor subunits but extra-proteasomal Ub receptors may be required for shuttling the remote substrates to the proteasome for degradation. We have previously demonstrated that UPS function is impaired during acute myocardial ischemia/reperfusion (I/R) and this impairment plays a major role in I/R injury due to failure to timely remove damaged/misfolded proteins. The molecular underpinnings of UPS impairment in I/R injury remains poorly understood. This study shows an important role of Ubiquilin1, a *bona fide* extra-proteasomal Ub receptor, in cardiac ubiquitination-proteasome coupling during I/R. Mice with cardiomyocyte-restricted knockout of the Ubiquilin1 gene (CR-Ubqln1KO) or with Ubiquilin1 overexpression (Ubqln1OE) were subject to myocardial I/R created via left anterior descending artery ligation (for 30min) and subsequent release (for 24hr). The I/R induced decline of left ventricular maximum dP/dt, elevation of minimum dP/dt, and infarct size were significantly greater in CR-Ubqln1KO mice, and conversely, were markedly attenuated in Ubqln1OE mice, compared with their littermates with a respective control genotype. The accumulation of ubiquitinated proteins in I/R myocardium was increased by CR-Ubqln1KO and reduced by Ubqln1OE. Furthermore, in cultured cardiomyocytes, Ubqln1OE was able to enhance the degradation of a surrogate UPS substrate as well as a *bona fide* misfolded protein linked to human proteinopathy. These exciting new findings demonstrate for the first time that inadequate coupling between ubiquitination and proteasomal degradation hinders the degradation of damaged proteins in I/R myocardium and represents a major pathogenic factor to acute I/R injury. It is also suggested that facilitating the coupling is potentially a novel therapeutic strategy for reducing I/R injury.

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Involvement of Non-canonical Wnt-PKC-JNK Pathway in Angiogenesis Enhanced by MSC Overexpressing Wnt11

Meifeng Xu, Jingcai Wang, Bin Yu, Univ of Cincinnati Medical Ctr, Cincinnati, OH

Wnt11 is a key regulator of cardiac muscle cell proliferation and differentiation during heart development. Here, we hypothesized that preprogramming mesenchymal stem cells (MSC) with Wnt11 enhanced angiogenesis in ischemic myocardium. Methods and Results: An acute myocardial infarction model in SD rats was developed by ligation of the left anterior descending (LAD) coronary artery. Rat bone marrow derived MSC were transduced with Wnt11 gene (MSC-Wnt11) and transplanted into ischemic border area. The animals treated with MSC-Wnt11 showed a significantly improved cardiac function. Furthermore, fluorescent microsphere and histological studies revealed an increased blood flow and blood vessel density in MSC-Wnt11 transplanted animals. In vitro studies using conditioned medium (CdM) obtained from MSC-Wnt11 (CdM-Wnt11) showed an increased the length and number of capillary-like structure (CLS) formation and promoted migration of human umbilical vein endothelial cells (HUVECs), which was similar to that HUVECs were directly treated with recombinant Wnt11 proteins. However, these effects could be abolished by using a Wnt11 neutralizing antibody. Real-time PCR analysis indicated that the expression of novel PKCs including PKCδ, PKCε, PKCη, and PKCθ was significantly upregulated in HUVECs transduced with Wnt11 (H-Wnt11). Immunostaining and western blotting results showed that the protein levels of p-pan-PKC and p-JNK as well as VEGFA in H-Wnt11 were significantly higher than that in control HUVECs. However, no difference was detected in the expression of either p-p38 or p-ERK between H-Wnt11 and its control. Furthermore, the enhanced CLS formation and migration of HUVECs mediated by CdM-Wnt11 were partially abolished by using JNK inhibitor, SP600125 (5 μM) and PKC inhibitor, Calphostin-C (0.1 μM). Conclusion: Our results demonstrated, for the first time, that Wnt11 delivered by MSC improved cardiac function and promotes angiogenesis in ischemic myocardium, which may be associated with the activation of the non-canonical Wnt-PKC-JNK pathway.

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Glutathione-S-Transferase P Regulates Bone Marrow Derived Endothelial Progenitor Cell (EPC) Function and Neovascularization in the Infarcted Heart

Guihua Zhou, Hai Zhong, Yixin Wu, Dept of Med/Cardiovascular disease, Univ of Alabama at Birmingham, and the Birmingham VAMC, Birmingham, AL; Chiao-Wang Sun, Timothy M Townes, Dept of Biochemistry and Molecular Biology, Univ of Alabama at Birmingham, Birmingham, AL; Sumanth D Prabhu, Dept of Med/Cardiovascular disease, Univ of Alabama at Birmingham, and the Birmingham VAMC, Birmingham, AL

Glutathione-S-transferase P (GSTP) modulates proliferation of bone marrow (BM) myeloid progenitors via non-catalytic inhibition of c-Jun N-terminal kinase (JNK); however, its effects on BM-derived EPCs are unknown. We hypothesized that GSTP supports EPC mobilization while suppressing inflammatory cells, thereby promoting tissue neovascularization. We generated chimeric mice using BM-ablated wild-type (WT) mice reconstituted with either GSTP-/- (GSTP-/-c) or WT (WTc) BM. GSTP-/-c and WTc mice underwent coronary ligation or sham operation (n = 8-15/group). Compared to WTc sham, 4 w after surgery, WTc HF mice exhibited significant ($p < 0.05$): LV dilatation, dysfunction, and fibrosis; increased mortality; and augmented circulating pro-inflammatory CD11b+Ly6Chi monocytes, but comparable circulating CD34+VEGFR2+ EPCs. In contrast, compared to WTc HF, GSTP-/-c HF hearts had significant ($p < 0.01$): 1) worsening of LV dilatation (LVEDV 118 ± 30 vs 88 ± 13 μL), dysfunction (LVEF 21 ± 4 vs 32 ± 9 %), wall thinning (anterior wall thickness 0.41 ± 0.06 vs 0.49 ± 0.1 mm), and LV hypertrophy (LV/tibia length 4.9 ± 0.5 vs 4.5 ± 0.6 mg/mm); 2) increased remote zone fibrosis by trichrome staining (1.3 ± 0.2 vs 0.61 ± 0.3 %); 3) reduced capillary density (476 ± 60 vs 544 ± 37 capillary/mm2) and increased capillary area (14 ± 1.1 vs 12 ± 1.2 μm2); 4) diminished gene expression of VEGF A, VEGF B, and VEGF C; 5) and increased IL-1β, IL-6 gene expression. GSTP-/-c mice also exhibited ($p < 0.01$) increased BM JNK activation, reduced circulating EPCs (0.18±0.07 vs 0.24±0.06%), increased CD11b+Ly6Chi monocytes (2.25±0.5 vs 1.8±0.6%), but comparable anti-inflammatory CD11b+Ly6Clow monocytes at baseline as compared with WTc mice. Moreover, at 3 and 7 d after ligation, GSTP-/-c HF mice exhibited fewer circulating EPCs and anti-inflammatory monocytes, and significantly higher pro-inflammatory monocytes as compared with WTc HF mice. We conclude that GSTP promotes EPC and reparative monocyte mobilization, thereby improving angiogenic gene expression, neovascularization and LV remodeling after myocardial infarction. This suggests that GSTP polymorphisms can impact cardiac reparative capacity in humans, and that enhancing GSTP function after infarction may yield therapeutic benefit.

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Multi-Species Genome-Wide Analyses of the Specification of Individual Cardiac Cell Fates

Brian Busser, Julian Haimovich, Guokai Chen, NHLBI/NIH, Bethesda, MD; Ivan Ovcharenko, NLM/NIH, Bethesda, MD; Alan Michelson, NHLBI/NIH, Bethesda, MD

There are remarkable molecular and embryological similarities in cardiogenesis between Drosophila and vertebrates. Cells comprising the Drosophila heart can be subdivided into individual identities based on differences in morphology, function and gene expression patterns. Recent studies have shown that differential modifications of histone proteins, in vivo transcription factor (TF) binding, and the presence of particular TF binding motifs can be used as predictive signatures of the enhancers that govern cell-specific gene expression. Here we used discriminative training methods within an integrative, multi-species framework to uncover the motifs, enhancers and genes underlying cardiac cell fate decisions. As an initial step, we undertook a large-scale validation of Drosophila heart enhancers, which revealed enhancer activities in distinct subpopulations of cardiac cells. To identify related cell-specific regulatory elements, we used the validated enhancers as a training set in a machine learning approach that integrated TF motifs with ChIP data for both TF binding and histone modifications. Empirical validation of candidate

enhancers predicted by this method confirmed activity in the appropriate cardiac cells. By clustering the motifs derived from the individual cardiac classifiers, we identified and validated sequence features which discriminate specific cellular identities. Next, we asked if similar predictive signatures underlie mouse and human cardiomyocyte (CM) differentiation from embryonic stem cells (ESCs). We show that the distribution of histone marks found within differentiating human and mouse ESCs indeed predict genes potentially critical for CM differentiation, with the best predictions provided by the overlapping mouse and human candidates. We evaluated this result in a large-scale RNAi-based screen of Drosophila orthologs of the mammalian genes, which uncovered dozens of novel cardiogenic regulators whose function is being tested in differentiating human ESCs. In total, these results document the utility of computational modeling combined with empirical testing to uncover the enhancers, TF motifs and genes which characterize individual cardiac cell fates in both invertebrate and mammalian species.

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Gastrin and D1 Dopamine Receptor Interact to Induce Natriuresis and Diuresis

Yue Chen, Dept of Cardiology, Daping Hosp, The Third Military Medical Univ, Chongqing, China; Laureano D. Asico, Div of Nephrology, Dept of Med, Univ of Maryland Sch of Med, Baltimore, MD; Shuo Zheng, Dept of Cardiology, Daping Hosp, The Third Military Medical Univ, Chongqing, China; Van Anthony M. Villar, Div of Nephrology, Dept of Med, Univ of Maryland Sch of Med, Baltimore, MD; Duofen He, Lin Zhou, Chunyu Zeng, Dept of Cardiology, Daping Hosp, The Third Military Medical Univ, Chongqing, China; Pedro A. Jose, Div of Nephrology, Dept of Med, Univ of Maryland Sch of Med, Baltimore, MD

Oral NaCl produces a greater natriuresis and diuresis than the intravenous infusion of the same amount of NaCl. Gastrin is the major gastrointestinal hormone taken up by renal proximal tubule (RPT) cells. We hypothesized that renal gastrin and dopamine receptors interact to synergistically increase sodium excretion, an impaired interaction of which may be involved in the pathogenesis of hypertension. In Wistar-Kyoto (WKY) rats, infusion of gastrin induced natriuresis and diuresis, which was abrogated in the presence of a gastrin (CCK_BR; CI-988) or D_1-like receptor antagonist (SCH23390). Similarly, the natriuretic and diuretic effects of fenoldopam, a D_1-like receptor agonist, were blocked by SCH23390, as well as by CI-988. However, the natriuretic effects of gastrin and fenoldopam were not observed in spontaneously hypertensive rats (SHRs). The gastrin/D_1-like receptor interaction was also confirmed in RPT cells. In RPT cells from WKY but not SHRs, stimulation of either D_1-like or gastrin receptor inhibited Na^+-K^+-ATPase activity, an effect that was blocked in the presence of SCH23390 or CI-988. In RPT cells from WKY and SHRs, CCK_BR and D_1 receptor (D_1R) co-immunoprecipitated, which was increased after stimulation of either D_1R or CCK_BR in RPT cells from WKY rats; stimulation of one receptor increased RPT cell membrane expression of the other receptor, effects that were not observed in SHRs. These data suggest that there is a synergism between CCK_BR and D_1-like receptors to increase sodium excretion. An aberrant interaction between the renal CCK_BR and D_1-like receptors (e.g., D_1R) may play a role in the pathogenesis of hypertension.

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High-Mobility Group Protein B2 is Essential in Maintaining Normal Cardiac Gene Expression

Haodong Chen, Manuel Rosa-Garrido, Ricardo Gray, Zugen Chen, Stanley F Nelson, Univ of California, Los Angeles, Los Angeles, CA; Sarah Franklin, Univ of Utah, Salt Lake City, UT; Emma Monte, Thomas M Vondriska, Univ of California, Los Angeles, Los Angeles, CA

Heart hypertrophy is a complex disease that involves differential expression of hundreds of genes and requires highly coordinated chromatin remodeling events that must facilitate such genome-wide changes in gene expression. We examine the genome-wide distribution of a chromatin structural protein, High-Mobility Group

Protein B2 (HMGB2) in isolated cardiac myocytes with or without adrenergic receptor agonist. We report a comprehensive map of HMGB2 binding in both normal and hypertrophic cardiac myocytes, and find that HMGB2 preferentially localizes to promoters, CpG islands, enhancers and transcription factor binding sites. Moreover, we find that upon hypertrophic stimulation, HMGB2 peaks move from regulatory elements to intergenic regions. Because both HMGB2 knock-down and PHE treatment can induce hypertrophic growth, we compare gene expression dynamics between HMGB2 knock-down and PHE treatment, and find hypertrophy-related gene expression pattern upon HMGB2 knock-down is different from the one after PHE treatment. This also indicates the existence of multiple pathways that lead to hypertrophic growth. Further study reveals that HMGB2 co-localizes with many other functional elements including p300 and CTCF. Interestingly, we find that HMGB2 binding profiles are different at the binding sites of four cardiac transcription factors, MEF2A, NKX2.5, GATA4 and SRF. More specifically, HMGB2 localization is higher at MEF2A binding sites, indicating that HMGB2 may be involved in facilitating the functioning of MEF2A. Finally, we calculate the DNA bendability score based on the primary sequence of genome and correlate it with HMGB2 binding intensity. Consistent with the understanding that HMGB2 bends DNA toward DNA major groove, we find a positive correlation between HMGB2 binding and DNA bending propensity. Surprisingly, PHE treatment in NRVMs induced the relocation of HMGB2 to less bendable intergenic regions. Taken together, our data conclude that HMGB2 is required for maintaining the normal cardiac gene expression pattern, and its binding to cardiac chromatin is dynamically altered during hypertrophic growth.

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Biophysical Cues From Nano Size Pillar Affect Character Of Human Endothelial Colony Forming Cells

Long-Hui Cui, Korea Univ Anam Hosp, Seoul, Korea, Republic of; Jung-Suk Kim, Korea Univ Coll of Health Sience, Seoul, Korea, Republic of; Hyung Joon Joo, Seung-Cheol Choi, Jong-Ho Kim, Ha-Rim Seo, Chi-Yeon Park, Ji-Hyun Choi, Soon Jun Hong, Korea Univ Anam Hosp, Seoul, Korea, Republic of; Kyu-Back Lee, Korea Univ Coll of Health Science, Seoul, Korea, Republic of; Do-Sun Lim, Korea Univ Anam Hosp, Seoul, Korea, Republic of

Background: Recently, biophysical cues from nano patterned surface received extra attention. Because, numerous cells in the human body is surrounded by the nano-microenvironment. Especially for the live cells biophysical cues from nanotopography is an important factor for cell motility and pathophysiology. Human Endothelial Colony Forming Cells (hECFCs) is human peripheral blood mononuclear cells (PBMNCs) derived endothelial cell like cells which related with various disease occurrence.
Methods: To investigate the effect of biophysical cues from nano size pillar surface, we use the novel nano size pillar surface culture dish in this experiment. The diameter size of nano pillar is 120nm to 360nm and we separate the gradient topography as High (280nm-360nm), Middle (200nm-280nm) and Low (120nm-200nm) respectively. hECFCs was derived from human peripheral blood mononuclear cells (hPBMNCs) and cultured with EGM2-MV endothelial medium.
Results: Attachment of hECFCs was decreased on the High (280nm-360nm) nano size pillar area. But, proliferation and apoptosis of hECFCs on the nano size pillar surface has no significant difference with hECFCs on the flat pattern. However, single cell morphology of hECFCs on the nano size pillar surface was distinct compared with hECFCs on the flat pattern. Finally, gene expression level of ROCK, Rho and Integrin family has changed on the nano size pillar surface.
Conclusion: In this study we find that biophysical cues from nano size pillar surface can affect single cell morphology of hECFC and gene expression level. Further, through these several results we can know that ROCK family are related with biophysical cues from nanotopography and nano pillar diameter size

can affect the optimal culture condition for hECFC.

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Pulmonary Vein Thrombosis can make Pulmonary Vein Acidic States, which may be Associated with Atrial Fibrillation.

Hidekazu Takeuchi, Nagasakiken Tomie Hosp, Goto, Japan

Atrial fibrillation can cause ischemic stroke. To prevent atrial fibrillation (AF) is crucial to prevent ischemic stroke. The pulmonary vein has a myocardial layer that can generate spontaneous or triggered action potentials. The myocardial layer is extended from the left atrial myocardium. Pulmonary vein myocardium sleeve is known to be associated with generating and maintaining AF. Pulmonary vein myocardium can be classified into two types. One is short and thin myocardium sleeve, which has no potential to cause atrial fibrillation (AF). And the other is long and thick myocardium sleeve, which has potential to cause AF. The mechanisms of such myocardium sleeve changes are not understood well.
Pulmonary vein thrombosis (PVT) is believed to be rare, which was reported as a rare complication of chest surgeries such as lobectomy or lung cancers. But since 2012, I reported seven cases of PVT in elderly patients without such conditions, which suggests that PVT is not uncommon. That is a novel notion. PVT prevents arterial blood flow, which inhibits oxygen and nourishment supply and carbon dioxide excretion. Hypoxia activates hypoxia inducible factors (HIFs), and HIFs can modulate epigenetic changes, reprogramming and ES cells. Undernourishments may activate nuclear respiratory factor-1 (NRF-1) and the aryl hydrocarbon receptor (AhR). PVT can make pulmonary vein acidic states by inhibiting excretion of carbon dioxide and may modulate pulmonary vein myocardium. Under acidic states, pulmonary vein cells such as pulmonary vein myocardium cells may obtain some kinds of multipotency. After obtaining multipotency, the cells may turn into new cells to adapt changed surroundings. The changes of myocardium sleeve may be caused by acidic state conditions and HIFs, AhR and NRF-1, which seemed to modulate pulmonary vein myocardium functions. To clear these, more studies are needed.

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Characterization Of Human Plasma Proteome Dynamics Using Deuterium Oxide

Ding Wang, David A. Liem, Edward Lau, Dominic C. Ng, Brian J. Bleakley, Martin Cadeiras, Mario C. Deng, Maggie P. Lam, Peipei Ping, Univ of California, Los Angeles, Los Angeles, CA

Protein turnover half-life is a critical determinant of cardiac homeostasis and can reveal previously unknown disease mechanisms. We recently developed a theoretical method to quantify protein half-life in human using stable isotopes in deuterium oxide (D_2O). Our goal in the present study is to demonstrate the immediate clinical translation potential of the method by evaluating its safety, feasibility, efficacy, and reproducibility in 10 healthy human subjects.
The enrolled human subjects (4 females/6 males; age 22 – 51 y/o; body weight 51 – 108 kg) were labeled with a tailored protocol (UCLA IRB#12-000899), wherein each subject orally consumed weight-adjusted doses of (~45 mL each) 70% D_2O daily for 14 days to enrich body water and proteins with deuterium. Throughout labeling, the subjects maintained regular food and fluid intake, and normal daily activities. Vital signs and medical health questionnaires were taken daily till 14 days post-labeling. We followed long-term physical conditions and the physiological clearance of D_2O from body water for up to 240 days post-labeling, finding no physiological effects or signs of discomfort. To monitor label enrichment and post-labeling clearance in the subjects, we measured the D_2O level of plasma and saliva samples with GC-MS. Both body fluids were reliable sources for monitoring label enrichment kinetics, giving consistent values of individual enrichment levels (0.9–2.2%) and rates (0.15–0.40 d^{-1}) in all subjects. Post-labeling D_2O level naturally subsided with a characteristic half-life of ~7 days (0.1 d^{-1}). With LC-MS and the in-house informatics platform Proturn, we successfully characterized the turnover dynamics of >600

human plasma proteins, the largest such human dataset to-date. We detected diverse protein half-life in plasma, e.g., from albumin (18.3 d) to IGF2 (8 h). Importantly, the method can quantify protein half-life with only a single time point, suggesting it can be used to study the dynamics of one single cardiac biopsy (e.g., during heart transplant).
In summary, D_2O labeling is a safe, accessible, and effective technique for widespread clinical investigations of protein turnover dynamics. We further discuss its implications in understanding cardiac disease mechanisms.

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Haploinsufficiency Of Sam68 Attenuates Vascular Inflammation And Atherosclerosis In Apoe-deficient Mice

Shuling Han, Junlan Zhou, Gangjian Qin, Feinberg Cardiovascular Res Inst, chicago, IL

Background:Chronic inflammation in the arterial wall is a major drive of atherosclerosis and is largely mediated by the NF-kappaB signaling pathway in the vascular and pro-inflammatory cells, notably macrophages. Recently, Sam68, an adaptor protein and Src kinase substrate, has been shown to promote NF-kappaB signaling in mouse embryonic fibroblasts (MEFs). However, whether Sam68 plays a role in the development of atherosclerosis is currently unknown.Methods and Results: To evaluate the role of Sam68 in atherosclerosis, we crossed Sam68(+/-)mice (Sam68-/- mice are sterile) with ApoE(-/-)mice and obtained ApoE(-/-)Sam68(+/-)and ApoE(-/-)sam68(+/+)mice, which are both grossly normal. Fed on a regular chow, time-course analyses of atherosclerotic lesions were performed histologically throughout the aortas in these mice. ApoE(-/-)Sam68(+/-) mice displayed a significantly delayed development of atheromatous plaques as compared to ApoE(-/-)sam68(+/+)mice at 5, 7, and 10 months of age. The expression levels of pro-inflammatory factors, including TNFalpha, IL-1beta, and IL-6, in the aortas were significantly lower in ApoE(-/-)Sam68(+/-)mice than in ApoE(-/-)Sam68(+/+) mice. Consistently, in the cultured Raw264.7 macrophages, knockdown of Sam68 resulted in a significant reduction in the TNFalpha-induced expression of these pro-inflammatory genes and also in the level of nuclear phospho-p65, indicating an attenuated NF-kappaB activity. Similar results were reproduced in primary macrophages that were isolated from peritoneum or differentiated from bone-marrow mononuclear cells of Sam68(-/-) mice vs. wild-type mice. Conclusions: Our results for the first time suggest that Sam68 is a critical component of the pro-inflammatory NF-kappaB signaling pathway in macrophages and modulates the course of atherosclerosis at least in ApoE(-/-) mice. Additionally, we have performed Sam68 co-immunoprecipitation and identified by mass spectrometry a panel of new Sam68-interacting proteins. Further experiments are under way to validate those Sam68-interacting proteins that contribute to the NF-kappaB signaling pathway.

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Post-translational Modification of Histone Variants in Cardiac Hypertrophy

Mickey Miller, Aman Makaju, Li Wang, Sarah Franklin, Nora Eccles Harrison Cardiovascular Res & Training Inst, Salt Lake City, UT

While global changes in gene expression are a hallmark of cardiac hypertrophy, much less is known regarding the epigenetic factors driving these changes. Local chromatin packing and gene accessibility, which governs transcriptional status, has been correlated with specific post-translational modifications on the histone tails of nucleosomes occupying these regions. However, the specific alterations in histone post-translational modifications driving gene expression changes during cardiac hypertrophy are largely unknown. To identify myocyte specific changes in histone post-translational modifications during cardiac hypertrophy we performed label-free quantitation of nuclear proteins from isolated neonatal rat ventricular myocytes exposed to the hypertrophic agonists, phenylephrine and isoproterenol. Peptide samples were analyzed on a Thermo Orbitrap Velos Pro mass spectrometer using CID & HCD fragmentation. Differential expression analysis was performed using the Progenesis LC-MS software where modified histone peptides were

normalized against total protein expression. We observed multiple known and novel post-translational modifications on each of the four core histones, many of which changed in the setting of hypertrophy. To validate these findings in an animal model we performed the same analysis of histone post-translational modifications from cardiac tissue of mice under basal conditions or after pressure-overload induced hypertrophy. This study provides the first global characterization of myocyte specific changes in histone post-translational modifications in cardiac hypertrophy and highlight basic mechanisms of genomic reprogramming operative in disease.

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KChIP2 Mediates Ion Channel Dysregulation by Novel Transcriptional Regulation of miR-34s

Drew Nassal, Case Western Reserve Univ, Cleveland, OH; Haiyan Liu, Xiaoping Wan, Angelina Ramirez-Navarro, Eckhard Ficker, Metrohealth Medical Ctr, Cleveland, OH; Isabelle Deschenes, Case Western Reserve Univ, Cleveland, OH

Introduction: Cardiac ion channel dysregulation is a hallmark of heart failure. Consistently, the disease yields dramatic decline in Ito through loss in Kv4 and its auxiliary partner KChIP2. Notably, transcriptional changes in heart failure can be elicited through KChIP2 silencing without disease signaling, suggesting potential transcriptional capacity for KChIP2. Further, disparity between resulting transcript and protein patterns suggests a mechanism compatible with modified miRNA activity. Considering other members of the KChIP family behave as transcriptional repressors, we hypothesize that KChIP2 regulates discrete miRNAs which in turn regulate cardiac excitability. Methods and Results: A miRNA microarray was conducted on neonatal rat ventricular myocytes (NRVM) following in vitro silencing of KChIP2 by siRNA, identifying the miR-34 family as potential transcriptional targets of KChIP2. Regulation, confirmed by quantitative PCR, showed reduction in miR-34a/b/c when over-expressing KChIP2 and increase following silencing. Luciferase assays were performed on the cloned promoter for miR-34b/c which reinforced direct KChIP2 repression on the miR-34b/c promoter. Furthermore, chromatin immunoprecipitation followed by PCR identified physical interaction of KChIP2 to the promoter site. Previous studies show modified expression of KChIP2 can lead to changes in several ion channel subunits. Therefore, we investigated if this was the consequence of KChIP2 regulation via miR-34. miR-34a/b/c precursors were expressed in NRVM which reduced transcript levels of Nav1.5 and Navβ1, and reduced protein levels for Kv4.3. Reflecting these changes, peak INa was reduced following miR precursor treatment. NRVMs were exposed to 100 μM phenylephrine for 48 hrs, significantly reducing KChIP2, Nav1.5, Navβ1, and Kv4.3, while elevating miR-34b/c. Returning KChIP2 expression by adenovirus normalized these changes back towards baseline, implicating the physiologic relevance of this pathway. Conclusion: These observations describe a novel mechanism where KChIP2 regulates a host of cardiac genes through transcriptional control of miRNAs, potentially explaining electrical remodeling observed in disease states where KChIP2 is reduced.

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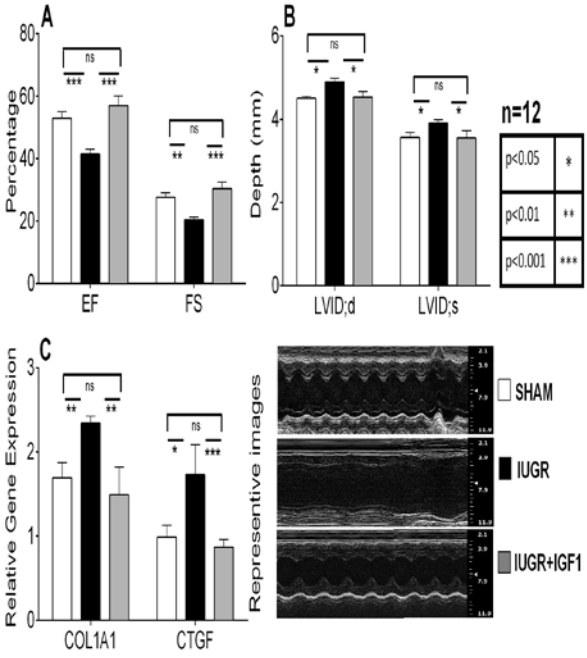
Intra-placental gene transfer of Insulin Like Growth Factor 1 Reprograms Altered Myocardial Gene Expression In Growth Restricted Mouse Model with Cardiac Dysfunction

khaled R omar, Tarek Alsaeid, Charles Klanke, Ahmed Lababidi, Mounira Habli, Cincinnati Childrens hospital medical center, cincinnati, OH

Background
Intrauterine growth restriction (IUGR), defined as birth weight <10% for gestational age, is a risk factor for adult onset cardiovascular disease. We demonstrated that Intraplacental gene transfer of adenoviral insulin growth factor1 (Ad IGF1) rescues IUGR induced cardiac dysfunction in a

surgical IUGR mouse model. The underlying molecular mechanism is unknown. We hypothesize that Ad IGF1 reprograms altered gene expression involved in collagen deposition in myocardium (Col1A1, CTGF, SRF, OPN, TGFβ)
Method
Laparotomy was performed on pregnant C57 mice at embryonic day 18 and pups were divided into 3 groups. Control: Sham operated; IUGR: by ligation of a uterine artery branch; IUGR+IGF1: injection of Ad IGF1 after ligation. Pups were delivered and followed up with cross-sectional (2D) and Doppler transthoracic echocardiography until 36 week. Hearts were collected and RNA extracted for gene expression analysis by qPCR at 36 week. Data were analyzed using ANOVA
Result
At week 36, IUGR mice showed significantly reduced EF (41 ± 6 vs 53 ± 7 vs 57 ± 9) compared to SHAM & IUGR+IGF1 (Fig A). IUGR showed significantly increased LVID;d (3.8 ± 0.3 vs 3.1± 0.49 vs 2.9 ± 0.4) as compared to SHAM & IUGR+IGF1 (Fig B). IUGR hearts demonstrated significantly increased CTGF (1.7± 0.6 vs 0.9 ± 0.3 vs 0.8 ± 0.2), and Col1A1 (2.4 ± 0.2 vs. 1.6 ± 0.4 vs 1.4 ± 0.5), as compared to SHAM & IUGR+IGF1(Fig C). No differences were detected in the other genes
Conclusion
AdIGF1 gene transfer rescues IUGR induced cardiac dysfunction and reprograms myocardial gene expression of CTGF&COL1A1. These changes may represent a potential epigenetic regulation that reprograms the fetus and attenuates the risk of adult onset CVD



K.R. omar: None. **T. Alsaeid:** None. **C. Klanke:** None. **A. Lababidi:** None. **M. Habli:** None.

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Neonatal Mouse Heart Maturation: Transcriptome-Wide Analysis Reveals Chamber-Specific Exon Enrichment of Cell Cycle Genes

Marlin Touma, Xuedong Kang, Univ of Clifornia Los Angeles, David Geffen Sch of Med, Los Angeles, CA; Jae-Hyung Lee, Sch of Dentistry, Seoul, Korea, Republic of; Xinshu Xiao, Univ of Clifornia Los Angeles, Los Angeles, CA; Yibin Wang, Depts of Anesthesiology, Univ of Clifornia Los Angeles, David Geffen Sch of Med, Los Angeles, CA

Background: During postnatal maturation of mammalian heart the neonatal cardiomyocytes undergo dramatic changes including complete maturation, proliferation arrest, and terminal exit from the cell cycle (CC). However, transcriptome-wide analysis of CC programs has not been performed in perinatal stages among different cardiac chambers. In particular, the contribution of alternative RNA splicing to the

chamber-specific CC activities is unexplored
Design/Methods: To achieve comprehensive analysis of differential expression (DE) and alternative splicing (AS) of CC-related genes in left ventricle (LV) versus right ventricle (RV) during maturation deep RNA-seq was performed on male newborn mouse LV and RV at 3 time points of perinatal transition: P0, P3 and P7. Reads were mapped to mouse Transcriptome, and to mouse Genome. Transcriptome-Wide difference in inclusion of individual exons was performed using MATS. DE genes and AS variants were defined as those with fold change ≥2, at expression level ≥3 RBKM and a false discovery rate ≤0.05. Significant gene ontology (GO) terms were determined at P-value ≤0.05. Levels of expression were validated using qRT-PCR
Results: Altogether, 2116 DE genes and 1162 AS events were observed. Among them, 109 CC-related genes were further analyzed. Distinct temporal patterns of DE and GO enrichment of CC genes in LV vs. RV during maturation were identified. Chamber -specific induction of genes involved in mitosis, karyokinesis, and cytokinesis was found at P7. RNA Splicing analysis of CC genes revealed 77 AS events. Skipping exon accounted for nearly half splicing events. Among 30 spliced exon variants, significant chamber- and temporal-specific inclusion were observed. Interestingly, the majority of AS variants exhibited opposing patterns of exon usage in RV vs. LV at p7
Conclusions: Our findings suggest novel molecular basis for chamber-specific programming of cellular proliferation and maturation in neonatal heart, including potential splicing regulation of dynamic exon enrichment of cell cycle related genes. Further functional studies to decipher putative splicing regulators of CC programming in LV vs. RV during maturation will likely lead to novel chamber-specific regenerative and therapeutic targets.

M. Touma: None. **X. Kang:** None. **J. Lee:** None. **X. Xiao:** None. **Y. Wang:** None.

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Negative Regulation Of The Fgf-16 Gene Promoter By Doxorubicin Is Associated With A Decrease In Nkx2.5 Binding

Jie Wang, Yan Jin, Peter A. Cattini, Dept of Physiology and Pathophysiology, Univ of Manitoba, Winnipeg, MB, Canada

Doxorubicin (DOX) is a widely used and effective anti-cancer drug, but it is also cardiotoxic, which can lead to heart failure, and so strategies are needed to protect the heart. Fibroblast growth factor 16 (FGF-16) is preferentially expressed and released from cardiomyocytes after birth. Evidence suggests that FGF-16 decreases the risk of heart damage and limits the negative effects of heart remodeling (hypertrophy and fibrosis) after injury in vivo. Exogenous addition of FGF-16 also increased resistance to the loss of contractility in an isolated heart model of acute DOX-induced injury. Thus, how endogenous FGF-16 production and by extension function is affected by DOX treatment is of interest. The FGF-16 gene is highly conserved between human and murine species. Alignment of sequences indicates a conserved Nkx2.5 binding site in the proximal promoter region that is associated with a previously characterized TATA box. Nkx2.5 is an important factor in vertebrate heart development and congenital disease. Furthermore, Nkx2.5 RNA levels are decreased with DOX treatment. Thus, the possibility that DOX negatively affects FGF-16, perhaps through an effect on Nkx2.5 levels or binding, was investigated. Neonatal rat cardiomyocytes were treated with DOX, and FGF-16 RNA levels decreased 75% within 6 hours as assessed by qPCR. Inhibition of transcription with actinomycinD had no effect on the DOX-induced decrease in FGF-16 RNA levels. Further support for an effect of DOX on FGF-16 transcription was obtained by transfection of cardiomyocytes with a hybrid 747 bp mouse FGF-16 promoter/luciferase reporter gene that was treated with DOX; a significant decrease in luciferase activity was observed. Electrophoretic mobility shift and chromatin immunoprecipitation assays suggest reduced Nkx2.5 protein-DNA interaction with this site after DOX treatment in vitro and in situ, respectively. These data indicate that DOX decreases FGF-16 RNA expression and this correlates with a decrease in Nkx2.5 levels and association with the proximal promoter region. A direct effect of Nkx2.5 on FGF-16 promoter activity awaits further testing. Thus, a decrease or loss of FGF-16 synthesis might contribute to the DOX-induced damage and/or response to injury.

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MiR-133 Modulates the Beta1-Adrenergic Receptor Transduction Cascade

Alessandra Castaldi, Humanitas Clinical and Res Ctr, Rozzano, Italy; Tania Zaglia, Venetian Inst of Molecular Med, Padua, Italy; Vittoria Di Mauro, Pierluigi Carullo, Giacomo Viggiani, Humanitas Clinical and Res Ctr, Rozzano, Italy; Giulia Borile, Venetian Inst of Molecular Med, Padua, Italy; Barbara Di Stefano, Univ of Palermo, Palermo, Italy; Gabriele Giacomo Schiattarella, Univ Federico II, Naples, Italy; Maria Giovanna Gualazzi, Leonardo Elia, Giuliano Giuseppe Stirparo, Humanitas Clinical and Res Ctr, Rozzano, Italy; Gianluigi Pironti, Duke Univ Medical Ctr, Durham, NC; Paolo Kunderfranco, Humanitas Clinical and Res Ctr, Rozzano, Italy; Maria Luisa Colorito, Univ of Palermo, Palermo, Italy; Giovanni Esposito, Univ Federico II, Naples, Italy; Marie-Louise Bang, Humanitas Clinical and Res Ctr, Rozzano, Italy; Marco Mongillo, Venetian Inst of Molecular Med, Padua, Italy; Gianluigi Condorelli, Daniele Catalucci, Humanitas Clinical and Res Ctr, Rozzano, Italy

Rationale: The sympathetic nervous system plays a fundamental role in the regulation of myocardial function. During chronic pressure overload, over-activation of the sympathetic nervous system induces the release of catecholamines, which activate β-adrenergic receptors (βARs) in cardiomyocytes (CMs) and lead to increased heart rate and cardiac contractility. However, chronic stimulation of βARs leads to impaired cardiac function and β-blockers are widely used as therapeutic agents for the treatment of cardiac disease. MiR-133 is highly expressed in the myocardium and is involved in controlling cardiac function through regulation of mRNA translation/stability. Objective: To determine whether miR-133 affects βAR signaling during progression to heart failure. Methods and Results: Based on bioinformatic analysis, β1AR and other components of the β1AR signal transduction cascade, including adenylate cyclase VI and the catalytic subunit of the cAMP-dependent protein kinase A (PKA), were predicted as direct targets of miR-133 and subsequently validated by experimental studies. Consistently, cAMP accumulation and activation of downstream targets were repressed by miR-133 overexpression in both neonatal and adult CMs following selective β1AR stimulation. Furthermore, gain- and loss-of-function studies of miR-133 revealed its role in counteracting the deleterious apoptotic effects caused by chronic β1AR stimulation. This was confirmed in vivo using a novel cardiacspecific TetON-miR-133 inducible transgenic mouse model (Tg133). When subjected to transaortic constriction, Tg133 mice maintained cardiac performance and showed attenuated apoptosis and reduced fibrosis compared to control mice.Conclusions: MiR-133 controls multiple components of the β1AR transduction cascade and is cardioprotective during heart failure.

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Inactivation of Cardiac Foxo1 by Insulin Signaling Is Required for Cardiac Function and Suppression of β-Myosin Heavy Chain Gene Expression

Yajuan Qi, Qinglei Zhu, Kebin Zhang, Candice Thomas, Rajesh Kumar, Kenneth M. Baker, Shaodong Guo, Texas A&M Univ Health Science Ctr, Temple, TX

Heart failure is a leading cause of morbidity and mortality in the USA and is closely associated with diabetes mellitus. The molecular link between diabetes and heart failure is incompletely understood. We recently demonstrated that insulin receptor substrate 1, 2 (IRS1, 2) are key components of insulin signaling and their dysfunction mediates insulin resistance, resulting in metabolic dysregulation and heart failure. Loss of IRS1 and IRS2 is associated with downstream Akt inactivation and in turn activation of the forkhead transcription factor Foxo1. To determine the role of Foxo1 in control of heart failure in insulin resistance and diabetes, we generated mice lacking Foxo1 gene specifically in the heart. Mice lacking both IRS1 and IRS2 in adult hearts exhibited severe heart failure, loss of mitochondria,

and a remarkable increase in the β-isoform of myosin heavy chain (β-MHC) gene expression, while deletion of cardiac Foxo1 gene largely prevented the heart failure and the loss of mitochondria, and resulted in a decrease in β-MHC expression. The effect of Foxo1 deficiency on rescuing cardiac dysfunction was also observed in db/db mice and high-fat diet (HFD) mice. Using cultures of primary ventricular cardiomyocytes, we found that Foxo1 interacts with the promoter region of β-MHC and stimulates gene expression, mediating an effect of insulin that suppresses β-MHC expression. Taken together, our study suggests that Foxo1 has important roles in promoting diabetic cardiomyopathy and controls β-MHC expression in development of cardiac dysfunction. Targeting Foxo1 and its regulation will provide novel strategies in preventing metabolic and myocardial dysfunction and influencing MHC plasticity in diabetes mellitus.

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Dysregulation of CPT2 Splicing in Diabetic Heart Disease

Elizabeth Jaworski, Curtis Nutter, Sunil Verma, Vaibhav Deshmukh, Muge Kuyumucu-Martinez, Univ of Texas Medical Branch, Galveston, TX

Diabetes mellitus is a group of metabolic diseases that are caused by elevated blood glucose levels. Individuals with diabetes have an increased risk of cardiovascular complications that include diabetic cardiomyopathy, hypertension, and coronary artery disease. Research has shown that hyperglycemia causes metabolic abnormalities in the heart such that cardiomyocytes are unable to utilize glucose for energy production due to reduced glucose intake, instead they solely depend on fatty acid oxidation for energy. Eventually, fatty acids accumulate and cause cardiac lipotoxicity, a presumed factor in the development of diabetic cardiomyopathy. Carnitine Pamitoyl Transferease 2 (CPT2) is one of the enzymes responsible for the transport of long-chain fatty acids into the mitochondria for fatty acid oxidation and energy production. CPT2 activity is elevated in diabetic hearts by mechanisms that are unclear. CPT2 is composed of 5 exons; the largest, exon 4 contains the transferase domain and is alternatively spliced in diabetes. In normal hearts, half of the CPT2 transcripts include exon 4 representing the active form of the enzyme. Through RNA sequencing analysis assay, we discovered that CPT2 is mis-spliced in diabetic hearts in a way that 70% of total CPT2 transcripts include the functional domain exon 4. The splicing change in CPT2 results in increased expression of the active CPT2 isoform in diabetic hearts. In summary, we identified a functionally important alternative splicing event in the CPT2 gene that may contribute to increased fatty acid oxidation and lipotoxicity in the diabetic heart.

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