Abstracts

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Cardiovascular Repair and Regeneration: Structural and Molecular Approaches in the Cellular Era

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Conference Program Committee
Conference Co-Chairs: Maurizio C. Capogrossi, Keiichi Fukuda, Sumanth D. Prabhu, and Mark Sussman

This annual meeting is the fourth for this premier research conference sponsored by the American Heart Association Council on Basic Cardiovascular Sciences, the world’s leading organization of cardiovascular scientists. The conference focused on how cellular-based approaches are being manipulated to enhance the repair and regeneration capabilities of the cardiovascular system with the goal of therapeutic-based interventions. The meeting featured both invited presentations and poster abstract presentations, with participants from around the world.

Abstracts for the poster presentations are provided in this special online supplement available at http://circres.ahajournals.org.
Small Molecule Targeting of Gβγ Reduces β-Adrenergic Receptor Desensitization and Normalizes Cardiac Dysfunction in an Acute Heart Failure Model
Liam M Casey, Frances Nwakama, Gabriel Vorobiof, Olga Dunavsky, Alan V Smrcova, Burns C Bixlaio; Univ of Rochester Med Ctr, Rochester, NY
Heart failure (HF) is a debilitating disease with poor prognosis, despite substantial therapeutic advances in the past two decades. Excess signaling through cardiac G-protein Gβγ subunits is an important component of HF pathophysiology. They recruit elevated levels of cytosolic G-protein coupled receptor kinase 2 (GRK, a.k.a., ORK1) to agonist-stimulated β-ARs in HF, leading to chronic β-AR desensitization and down-regulation; these events are all hallmarks of HF. Previous data has suggested that inhibiting Gβγ signaling and its interaction with GRK2 could be of therapeutic value in HF. We recently developed a novel small molecule targeting strategy to selectively inhibit Gβγ-binding interactions, and identified several Gβγ-inhibitory small molecules (Bonacci et al, Science, 2006). In particular, we identified compound M119, which blocked the interaction of purified Gβγ and GRK2 in vitro. To validate this activity in a cellular setting, we isolated adult mouse cardiomyocytes, where M119 significantly reduced β-AR-mediated membrane recruitment of GSK3β and GSK3α (PKA), thereby enhancing both adenyly cyclase activity and cardiomyocyte contractility at baseline and in response to β-AR agonist stimulation. Upon finding that the compound was biologically available following intraperitoneal injection in mice, we investigated whether the observed in vitro effects in isolated adult cardiomyocytes would translate to in vivo effects on cardiac function. Initial investigations were pursued in an acute pharmacologic HF model (30 mg/kg/day isoproterenol for 7 days). Importantly, concurrent once daily injections of M119 normalized cardiac function, morphology and GRK2 expression in this acute HF model. Collectively, our study has identified a small molecule Gβγ inhibitor capable of reducing β-AR desensitization, thereby enhancing β-AR-mediated isolated cardiomyocyte contractility and, importantly, normalizing cardiac function and morphology in an acute HF model in vivo. Our data suggest a promising therapeutic role for small molecule inhibition of Gβγ in the treatment of HF.

Encapsulation of a Small Molecule p38 Inhibitor for Cardiac Regeneration
Jay C Sy, Gokulakishnan Iyer, Milton Brown, Sergey Dikalov, Emory Univ Sch of Medicine, Atlanta, GA; Niren Murthy, Georgia Institute of Technology, Atlanta, GA; Michael E Davis; Emory Univ Sch of Medicine, Atlanta, GA
Myosin dysfunction is usually progressive and successful therapy will likely require sustained delivery. Small molecule inhibitors have great clinical potential in the treatment of cardiac disease, but their size and stability make them difficult to target to the myocardium. SB239063 has been successful in preventing cardiovascular dysfunction but treatment protocols are prolonged and may not translate to longer term benefits. Polyketals (PKSs) polymerize and encapsulate both small molecules that hydrolyze slowly at physiological pH values and degrade to non-toxic compounds. Here we show that polyketal-encapsulated SB239063 (PKSB) time-dependently inhibited TNFα-induced p38 phosphorylation in RAW macrophages. In addition, PKSB, and not empty polyketal (PK), inhibited TNFα-stimulated extracellular superoxide production as measured by accumulation of superoxide-specific product dihydroethidium, 2-hydroxyethidium (TFN 0.82 ±μM, PKSB 0.37 ±μM, p<0.05). To determine efficacy in vivo, we first established by skeletal muscle injection studies that polyketal treatment did not result in inflammatory cytokine expression or myofiber atrophy when performed in a randomized and blinded study in rats subjected to myocardial infarction. Immediately following coronary artery ligation, rats were injected with PK, PKSB, or free SB239063 intramyocardially. Three days following infarction, there was a significant reduction in p38 phosphorylation within the infarct zone of PKSB rats, with no effect of PK or SB239063. In addition, only PKSB attenuated infarct-zone superoxide production (MI 27.68± μM, PKSB 8.49 μM, p<0.05) and TNFα-production (MI 157.73 pg/ml, PKSB 103.60 pg/ml; p<0.05). In a separate double-blinded study, we examined cardiac function by MRI at 6 and 12 months. All patients showed an improvement in left ventricle EF over baseline (MI 5.1, p<0.003 at 6 months, 5.2±4.6 to 40.8±4.5, p<0.001, 12 months). Conclusion: PKSB delivered with the helical needle transcardiac catheter was safe in this small uncontrolled study in patients with chronic MI. Increased ejection fraction and other positive data trends support continued development of this therapeutic strategy in larger controlled trials.

Control of Phenotypic Plasticity of Smooth Muscle Cells by BMP Signaling Through Myocardin-Related Transection Factors
Manching Ku, Brandi N Davis, Akiko Hata, Tufts Univ Sch of Medicine, Boston, MA; Peter H Nguyen, Giorgio Lagna; Tufts-New England Med Ctr, Boston, MA
Vascular smooth muscle cells (VSMCs), unlike other muscle cells, do not terminally differentiate. In response to injury, VSMCs change phenotype, proliferate and migrate as part of the repair process. Dysregulation of this plasticity program contributes to the pathogenesis of several vascular disorders, such as atherosclerosis, restenosis and hypertension. The discovery of mutations in the gene encoding BMPRII, the type II subunit of the receptor for Bone Morphogenetic Proteins (BMPs), in idiopathic pulmonary arterial hypertension (IPAH) provided a clue that BMP signaling may affect the homoeostasis of VSMCs and their phenotype modulation. Here we report that BMP signaling potently induce SMG genes in pluripotent cells, and prevents de-differentiation of pulmonary artery smooth muscle cells (PASMCs). The BMP-induced phenotype switch requires intact RhoA/ROCK signaling, but is not blocked by inhibitors of the TGFβ and PI-3K/Akt pathways. Furthermore, nuclear localization and recruitment of the mycardin-related transcription factors (MRTF-A and MRTF-B) to a SMA-α-actin promoter was observed in response to BMP treatment in vivo. Thus, we conclude that BMP signaling modulates the phenotype of VSMCs via cross-talk with the RhoA/MRTFs pathway, and may contribute to the development of the pathological characteristics observed in patients with IPAH or other obliterative vascular diseases.

Transendocardial Autologous Bone Marrow in Chronic Myocardial Infarction Using Helical Needle Catheter: 1-Year Follow-up in an Open-Label, Nonrandomized, Single-Center Pilot Study (the TABMMT Study)
Luis de la Fuente, Salvador Med Sch Buenos Aires, Buenos Aires, Argentina; Simon H Stelter, Stanford Univ Med Ctr, Palo Alto, CA; Julio Argentieri, Eduardo Penalosa, Jorge Miano, Benjamin Koziner, Christian Bilos, Argentine Institute of Diagnosis and Treatment, Buenos Aires, Argentina; Peter A Altman; Biocardiarc Inc, South San Francisco, CA
Aims: Cell therapy has shown benefit in preclinical and clinical studies, although debate continues on the mechanism of action and the most appropriate methods for performing such therapies. We assessed the hypothesis that helical needle transcardiac delivery of autologous bone marrow (ABM) cells around regions of hypo or akinesia in chronic post-MI patients would be safe and possibly improve ejection fraction. Methods and results: 10 stable post-MI Patients with an ejection fraction (EF) <40% were enrolled. ABM cells were aspirated from the iliac crest and delivered percutaneously with a transendocardial helical needle catheter. 80x10⁶ cells were injected into 7.1±3.1 sites around the infarct to target the per-infarct zones. 2D echo left ventricle EF measurements, 24 hour Holter, and exercise tolerance testing were performed at baseline, day of procedure, 1 and 12 weeks, 6 and 12 months. There were no adverse events associated with the catheter based cell transplantation procedure. At 6 and 12 months, all patients showed an improvement in left ventricular EF over baseline (MI 5.2±4.6 to 40.8±4.5, p<0.001 at 6 months, 5.2±4.6 to 40.8±4.5, p<0.001 at 12 months). Conclusion: ABM cells delivered with the helical needle transcardiac catheter was safe in this small uncontrolled study in patients with chronic MI. Increased ejection fraction and other positive data trends support continued development of this therapeutic strategy in larger controlled trials.

GSK-3β Induces Cardiomycocyte Markers in Bone Marrow-Derived Mesenchynal Stem Cells (MSCs)
Jaeyeon Cho, Shinchi Hirotani, Junichi Sadoshima; UMDNJ, Newark, NJ
Although adult MSCs have cardiomyogenic properties, the underlying signaling mechanisms are not well understood. The Wnt pathway significantly affects cardiomyocyte differentiation from various stem cell populations, but its effects vary substantially depending upon experimental conditions. The goal of this study was to clarify the role of GSK-3β, a major inhibitory component of the Wnt pathway, in regulating cardiomyocyte differentiation from adult MSCs. Either human or mouse bone marrow-derived MSCs were treated with 5 μM of 5-aza-cytidine (5-Aza) without serum for 24hrs, which induced mRNA expression of cardiomyocyte markers, including Nkx2.5 and myosin heavy chain (α-MHC), within 3 days. 5-Aza treatment induced troponin I protein expression 5 days after treatment. We examined protein expression of GSK-3β and β-catenin, a downstream target of GSK-3β, during 5-Aza treatments. GSK-3β was increased by 5-Aza in a time-dependent manner, reaching a peak (~4 fold) on 3Day. This was paralleled by decreases in β-catenin (~50%); suggesting that the activity of GSK-3β was increased, whereas the Wnt pathway was suppressed by 5-Aza. To test the effect of GSK-3β upon cardiomyocyte differentiation, MSCs were transduced with GSK-3β-adenovirus, which induced ~15 fold expression of GSK-3β and 80–95% downregulation of β-catenin on Days 3–12. Stimulation of GSK-3β caused induction of Nkx2.5 and ANF mRNA, peaking on Days 4–5, whereas LacZ-virus did not. In order to stimulate GSK-3β by alternative methods, MSCs were isolated from conditional GSK-3β transgenic mice. Isolated MSCs were transduced with AdTA- or AdTA-adenovirus, to achieve GSK-3β expression regulated by the tetracycline (tet-Off) and -On systems, respectively. GSK-3β expression induced by the tet-Off (4.7 fold) or tet-On system (3 fold) induced mRNA expression of Nkx2.5, α-MHC and GATA4, as well as expression of protein sarcomeric α-actin and troponin I in MSCs. In contrast, MSCs treated with LacZ (10μM), an inhibitor of GSK-3β failed to induce cardiomyocyte markers such as troponin I. In summary, 5-Aza-induced increases in cardiomyocyte markers in MSCs were accompanied by activation of GSK-3β, and stimulation of GSK-3β induces expression of cardiomyocyte markers in adult MSCs.
Evidence for Paracrine Stimulation of Neovascularization by Bone Marrow–Derived Progenitor Cells: Assessment by in Vivo EPR Oximetry

Omer I Butt, Robert W Carruth, Periannan Kumarappan, Jay L Zeevi, Nicanor I Moldovan; David Heart and Lung Research Institute, Columbus, OH

Background: The activity of bone marrow progenitor cells during neovascularization is highly debated, because contradictory data in favor of direct incorporation, cell fusion or distant paracrine action are available. Therefore, a reliable and accurate testing of possibilities is highly desirable. Methods: We purified CD133- and sca-1 positive bone marrow cells by Magnetic Activated Cell Sorting (MACS), and demonstrated that they have cardiovascular (endothelial and smooth muscle cell) differentiation potential, by cultivation for 3 weeks in EGM-2 medium (Clonetics). For in vivo quantification of angiogenic activity, we developed a novel oxygen biosensor, containing an oxygen-sensitive probe detectable by Electron Paramagnetic Resonance (EPR), placed inside of nanoporous filter-limited biocompatible capsules. These devices were implanted subcutaneously in lots of six C57/B16 mice, as it follows: (i) alone; (ii) added CD133+ magnetic beads to control; (iii) added beads co-cultured with freshly isolated CD133- sca-1- bone marrow cells. Implanted-reporter pO2 was recorded non-invasively for up to 10 weeks. Tissues surrounding the implants were directly analyzed by microphotography to document the extent of neovascularization, and collected for immunohistochemistry. Results: As expected, oxygen sensors in control capsules and in capsules with autologous bone marrow cells were detected only collagen deposition, along with F4/80 macrophages and giant cells. In presence of progenitor cells, outside of gels there was an explosive development of host-derived neovascular. The gels themselves showed progressive collagenization by adipose tissue containing capillaries, apparently of host origin, in every case, after four weeks, as assessed by CD11b-positive macrophages in the vicinity. The chips supplemented with autologous as compared to control implants induced twice the oxygen concentrations (16.2 ± 0.2 mm Hg) as compared to controls (9.1 ± 0.7 mm Hg). This difference remained significantly larger (p < 0.05, n = 4) for six more weeks. Conclusion: Bone marrow progenitor cells powerfully stimulate host neovascularization, with little evidence of direct incorporation. Our approach could be used to control vascularization in ischemic hearts, as well as in the vicinity of biomaterial implants affected by fibrous encapsulation.

Phenotypic Characterization of CD133+ Cells Suggests a Potent Hemangioblast Population

Jason C Kovacic, Victor Chang Cardiac Research Institute, Sydney, NSW, Australia; David Ma, St Vincent’s Hosp, Sydney, NSW, Australia; Helen Tao, Andrea Herbert, Victor Chang Cardiac Research Institute, Sydney, NSW, Australia; John Moore, St Vincent’s Hosp, Sydney, NSW, Australia; Robert M Graham; Victor Chang Cardiac Research Institute, Sydney, NSW, Australia

Background: Although recently described, CD133+ cells may represent a potent hemangioblast population. As they may be obtained from adult humans, CD133+ cells are therefore an attractive autologous option for therapeutic angiogenesis. However, scant data exists regarding their potential contribution to neovascularization in patients with ischemic heart disease (IHD). Methods: Peripheral Blood CD133+ cells from 19 patients with stable IHD, before and after GCSF mobilization, were characterized by fluorescent-activated cell sorting (FACS). At peak GCSF mobilization, concentrated CD133+ cells were obtained using anti-CD133-conjugated magnetic beads and a CINMACS system (mean purity 59.5%). Total RNA from CD133+ cells was reverse-transcribed and quantified by real-time polymerase chain reaction (qRT-PCR) performed using primers for: α-actin, VEGF, HGF, PIGF-B, MCP-1, TNFz, PIGF. Results: FACS demonstrated expression of both hematopoietic and endothelial antigens, with high-level CD45 (100%), CD31 (>90%) and VWF (~80%); intermediate-level-1 (ICAM-1 (~30%) and E-selectin (15%); cell-specific but detectable VEGF-β-2 (2.4%), VE-Cadherin (~1%) and VCAM-1 (~1%). CD133+ cells also expressed a range of stem cell markers: CD34 (~90%), c-kit (80–95%) and MDR-1 (~10%). GCSF administration was associated with reduced CD34 (75% vs 80%, p = 0.05) and increased c-kit (85% vs 92%, p = 0.05) expression by CD133+ cells, suggestive that GSF-mobilized CD133+ cells were less differentiated (more primitive) than those obtained in the basal state. qRT-PCR indicated that the RNA expression of angiogenic cytokines by CD133+ cells was similar to VEGF, HGF,
TNF-α or less than (PSGF-B, MCP-1, PIgf; all p < 0.05) that of other relevant comparator cell populations; suggesting that CD133+ cells are unlikely to make a major contribution to angiogenesis via paracrine mechanisms. Conclusions: While expressing hematopoietic and endothelial markers, CD133+ cells from patients with stable IHD exhibit the antigenic and functional characteristics of potent progenitor cells. This phenotype supports the contention that CD133+ cells are a hemangiomab population and thus warrant further study as a therapeutic agent.

P11 Epigenetic Reprogramming During SS-Dependent Differentiation of Mouse Embryonic Stem Cells: Role of Nitric Oxide and Histone Deacetylases

Barbara Illii, Francesco Spallotta, Cnr Cardiologico Monzino, Milan, Italy; Jessica Rosati, Stefania Mattussi, Istituto Dermoapatologico dell’Imaculata, Rome, Italy; Maurizio Capogrossi, Carlo Gaetano; Istituto Dermoapatologico dell’Immaculata, Rome, Italy

Introduction: Stress shear (SS) reprograms mouse embryonic stem cells (ES) to express cardiovascular markers at least in part through the occurrence of epigenetic modifications. Nitric oxide (NO) is involved in SS signaling in vascular cells and facilitates cardiomyogenesis in ES. Here we show the results of experiments performed to assess the molecular mechanism activated by NO in the SS-dependent cardiovascular differentiation of ES. Methods and Results: Exposure of ES to the NO synthase inhibitor S-methyl-thiosourea (SMT), inhibited the SS-dependent expression of cardiovascular markers in ES, while nitric oxide donors anticipated cardiovascular differentiation in ES deprived from leukemia inhibitory factor (LIF). This result paralleled with a marked decrease in Oct4 expression, indicating a role for NO in the cardiovascular commitment of ES cells. The expression of developmentally regulated genes requires the fine tuning of the chromatin condensation/decondensation process in a temporally and spatially regulated manner. We observed NO-dependent and NO independent NO synthase expression in ES during reprogramming of ES’ cells fate. This phenomenon was transient as these molecules returned to the cytoplasm in 6 hours. Intriguingly, the direct exposure of ES to NO donors allowed the nuclear retention of these enzymes beyond the 6 hours timepoint and significantly histone H3 acetylation, indicating the presence of a prolonged histone deacetylase activity in the ES nuclei upon NO treatment. The HDAC inhibitor Trichostatin A (TSA) blocked ES differentiation towards the endothelial lineage, while class I and II HDACs specific inhibitors induced the expression of endothelial and neuronal markers respectively. Conclusions: These data suggest a direct role of NO in the regulation of class II HDACs function and in the chromatin remodelling of ES cells and may envisage new epigenetic strategies to reprogram stem cells’ fate which may be useful for cardiovascular cell-based therapies.

P12 Correlation Analysis of Endothelial Colony Forming Units and Endothelial Microparticles in Patients with Cerebrovascular Risks

Soon-tae Lee, Kon Chu, Keun-Hwa Jung, Hee-Kwon Park, Vit Nary Choe, Dong-Hyun Kim, Woo-Seok Im, Jeong-Eun Park, Jin-Hee Kim, Jae-Joon Bahn, Sang Kun Lee, Manho Kim, Jaeyu Roh; Clinical Risch Institute, Seoul National Univ Hosp, Seoul, Republic of Korea

Endothelial progenitor cell (EPC) is a surrogate biologic marker for vascular regeneration that is inversely correlated with cardiovascular risk, and endothelial microparticle (MP) is a possible indicator of vascular endothelial damage. We isolated EPC colony-forming units and endothelial microparticles (Aggrecan receptor) from each group and measured NO content in each group. We enrolled 100 patients (72% with variable cerebrovascular risks, which included patients with headache, chronic stroke, or traditional cerebrovascular risk factors. Heparinized venous blood samples (25mL) from each subject were used for the isolation of EPC. Mononuclear cells were fractionated by centrifugation on Histopaque 1077 gradients at 400 x g for 30min, resuspended using the EGM-2 BulletKit system, seeded on gelatin-coated 12-well plates, and incubated in a 5% CO2 incubator at 37°C. This method allowed colony formations of EPCs or circulating angiogenic cells (CACs), defined as a central core of rounded cells surrounded by elongated spindle-shaped cells at 7 days in culture. MP-rich plasma was prepared from citrated venous blood samples (5mL) using serial ultracentrifugation at 1500g for 15min and 3000g for 2min. Samples were frozen at -70°C till analyses. After thawing at room temperature, we counted MPs (>1 micrometer) using a Coulter Counter. EPC colony counts were correlated with endothelial MPs (p=0.039 for CD31 AnnexinV, p=0.040 for CD31 AnnexinV/CD42- Mps, p=0.129 for CD42- Mps). In particular, EPC colony count was more significantly correlated with logarithmic values of CD31 + CD42- (p=0.009), CD31 + AnnexinV + (p=0.007), and CD62E + endothelial Mps (p=0.038). In this study, we show that there exists an inverse correlation between EPC colony counts and circulating endothelial MPs, which suggests the reduced circulating endothelial regeneration pool in patients with active endothelial damage.

P13 Intn6: New Target for Angiogenesis (1) e1f3/e1f3a Specifically Targets HIV-2a for Degradation by Hypoxia- and pVHL-Independent Regulation

Li Chen, Kazuyo Uchida, Tokyo Metropolitan Institute of Med Science, Bunkyo-ku, Tokyo, Japan; Alexander Endler, Sch of Basic Medicine, Tongji Univ, Shanghai, China; Osamu Iijima, Alphagen Co, Ltd, Chiyoda-ku, Tokyo, Japan; Fukashi Shibasaki; Tokyo Metropolitan Institute of Med Science, Bunkyo-ku, Tokyo, Japan

Abstract: Hypoxia-inducible factors (HIFs) -1α and -2α are structurally similar as regards their DNA-binding and dimerization domains, but differ in their activation targets. In order to address this discrepancy, we performed yeast two-hybrid analysis and identified Intn6/e1f3a/p48 as a novel target gene product involved in HIV-2a regulation. The Intn6 gene was previously identified as a frequent integration site of the mouse mammary tumor virus (MMTV). Here, by using two-hybrid analysis, immunoprecipitation in mammalian cells and HRE-reporter assays, we report the specific interaction of HIV-2a (but not HIV-1α or Hμ-Jα) with Intn6. The results indicate that the direct interaction of Intn6 induces HIV-2a degradation. This degradation was found to be both hypoxia- and pVHL-independent. Furthermore, Intn6/Intn6a increased endogenous HIV-2a expression, and followed by induced sets of critical angiogenic factors comprising VEGF and bFGF mRNA in HeLa cells. Moreover, HIV-2a and the related factors are also expressed concomitantly in human endothelial cells. We proved that Intn6/Intn6a induced mRNA of HIV-2a, bFGF, IL6, and IL8 in HUVEC cells, and these induction triggered to mediate the cord of formation on matrigels. These results indicate that Intn6 is a novel and critical determinant of HIV-2a-dependent angiogenesis not only in HeLa cells, but also in human endothelial cells. Thus, Intn6/Intn6a transfer may be an effective therapeutic strategy in pathological conditions such as heart and brain ischemia, and obstructive vessel diseases.

P14 AMP-Kinase Activates Ubiquitin Ligases in Cardiomyocytes

Kedryn K Baskin, Peter Razeghi, Heinrich Taegtmeyer, Univ of Texas Health Science Ctrr, Houston, TX

Left ventricular hypertrophy (LVH) is an independent risk factor for cardiovascular morbidity and mortality. Studies have shown that regression of LVH improves cardiac function. Conventional strategies used to reverse cardiac hypertrophy currently focus on decreasing pro-hypertrophic signaling. However, this approach has often been unsuccessful because of the vast redundancy within the pro-hypertrophic signaling network. Cardiac size, whether hypertrophic or atrophic, is determined by the balance of protein synthesis and degradation. Here we propose a new approach to reverse cardiac hypertrophy through the activation of pro-atrophic signaling pathways. Studies in skeletal muscle have already shown that the Ubiquitin Proteosome Pathway (UPP) is the major signaling pathway responsible for skeletal muscle atrophy. The activation of the two muscle-specific ubiquitin ligases, Muscle and atrophy F-box protein (Mafbx/Atrogin-1) and Muscle Ring Finger 1 (MuRF-1), increase protein degradation in vivo and in vitro. The UPP breaks down proteins in an ATP-dependent manner. Because the central determinant of the levels of ATP in the cell is 5’ Adenosine Monophosphate Kinase (AMPK), we propose that AMPK plays a role in the regulation of Mafbx and MuRF-1, thereby reversing cardiac hypertrophy through the UPP. In order to investigate the role of AMPK in the activation of Mafbx and MuRF-1 in vitro and in vivo, we used two model systems: neonatal rat ventricular myocytes (NRVM) and Mafbx and MuRF-1 knockout mice. In both models, AMPK was activated by treatment with the anti-diabetic drug Metformin (1,1-dimethyl biguanide HCl) or AICAR (5’-phosphoribosyl-5-aminimidazole-4-carboxamide). Our data show that Metformin and AICAR both increase transcript levels of Mafbx/Atrogin 1 and MuRF-1 in vitro. We conclude that AMP kinase potentially activates the UPP in cardiomyocytes. One of the consequences may be enhanced availability of amino acids for energy provision.

P15 Functioning Engineered Cardiac Tissue from Skeletal Muscle–Derived Stem Cells

Kelly Clause, Univ of Pittsburgh, Pittsburgh, PA; Joseph P Timney, Children’s Hosp of Pittsburgh, Pittsburgh, PA; Li J Liu, Burhan Gharibeh, Kazuro L Fujimoto, William R Wagner, John C Ralphie, Bradley B Keller, Johnny Huard, Kimimasa Tobita; Children’s Hosp of Pittsburgh, Pittsburgh, PA

The limited capacity of the injured myocardium to reactivate cardiomyocyte regeneration is a major barrier to the restoration of cardiac function. Numerous types of stem cells are under investigation as alternative cell sources for functioning cardiomyocyte replacement. Here, we describe our initial success in differentiating functioning cardiomyocytes from rat skeletal muscle derived stem cells (MDSCs) using MDSC aggregation and three-dimensional engineered cardiac tissue (ECT) culture techniques. MDSCs were isolated via the preplate technique from neonate Lewis rat hind-leg muscles. The freshly isolated MDSCs were expanded and a 45-day/Scal1/ Mafbx/Atrogin-1) ECT culture was used to test our hypothesis that MDSCs, when cultured in ECT, differentiated into a contracting muscle phenotype, and this differentiation was characterized by the expression of cardiac specific genes including Nkx-2.5, α- and β-cardiac myosin heavy chains, cardiac α-actin, and connexin-43. Confocal microscopy identified elongated cells aligned to the ECT longitudinal axis and these elongated cells also expressed concomitantly in human endothelial cells. We proved that Intn6/Intn6a induced mRNA of HIV-2a, bFGF, IL6, and IL8 in HUVEC cells, and these induction triggered to mediate the cord of formation on matrigels. These results indicate that Intn6 is a novel and critical determinant of HIV-2a-dependent angiogenesis not only in HeLa cells, but also in human endothelial cells. Thus, Intn6/Intn6a transfer may be an effective therapeutic strategy in pathological conditions such as heart and brain ischemia, and obstructive vessel diseases.
P16 Implant and Cellular Therapy: A New Option of Treatment for Patients with Chronic Coronary Disease with Intractable Angina or Cardiac Failure Without Possibility of Surgical Revascularization

Roberto J. Fernandez Vina, Sr, Fernandez Vina Foundation and Maimonides Univ, Buenos Aires, Argentina; Liliana Carnozzi, Fernandez Vina Foundation, San Nicolas, Argentina; Andres Pinto; Fernandez Vina Foundation and San Nicolas Clinic, Argentina; Francisco Vrsalovic, Fernandez Vina Foundation and San Nicolas Clinic Argentina, San Nicolas, Argentina; Oberdan Andrin, Fernandez Vina Foundation and Maimonides Univ Buenos Aires, San Nicolas, Argentina; Federico Fernandez Vina, Fernandez Vina Foundation and San Nicolas Clinic, Argentina; Liliana Carnozzi, Fernandez Vina Foundation, San Nicolas, Argentina; Andres Pinto; Fernandez Vina Foundation and San Nicolas Clinic Argentina, San Nicolas, Argentina

Objectives: Adult stem cells derived from bone marrow are being used in the treatment of chronic myocardial ischemia and myocardial infarction. Method 69 patients with refractory angina or cardiac failure that had no possibility of surgical revascularization were included. The age of patients (66 men and 3 women) was more than 55 years old. Results: Echocardiograms showed extensive myocardial dysfunction with diastolic failure; in 18 patients anterior and left nectrosis, and in 43 multiple ischemic areas. Ventriculography revealed increment of EDVolume and ESystole Volume, with FE oscillating between 22% and 30%. The Cell Implant was made by Retrograde Injection through the Sinus Coronary Vein. The average of cells implanted (CD34+ and CD38-) was of 2.2 x 10^6. Follow up: After a period of one month a progressive increase of sectors contractility was observed in the echocardiograms. After 120 days it was observed that FE had improved between 38% and 43%. Scintigraphy revealed improvement of the perfusion in the 53 patients in the perinecrotic and diffuse ischemic areas, and 48 patients were subjected to Ventriculo-grapathy after 90 to 120 days and it was observed that the FE improved up to 38%. Conclusions: The cellular implants have demonstrated improvement of the function, survival, surgery, in areas with severe dysfunction, and a FE increase, observed by echocardiograms, and Ventriculography. 78% of the patients, showed an imro-vement of the contractility of periphery of scars. It was not observed any progression of coronary occlusive disease after a period of 2 years. 43 patients have achieved 3 year of evolution and they are asymptomatic or in functional class I.

P17 Cardiac Progenitors During Zebrasih Heart Regeneration

Kazu Kikuchi, Kenneth D Poss; Duke Univ Med Cntr, Durham, NC

Recent studies have identified populations of cardiac stem cells, or cardiac progenitors (CPs), in the adult mammalian heart. Some CPs are reported to have the potential to restore muscle function when transplanted into infarcted hearts. Although these progenitors show little or no natural regenerative ability, the observations indicate that the mammalian heart has the potential for myocardial regeneration through CPs. In contrast with mammals, teleost zebrafish naturally regenerate cardiac muscle. In a recently published study, we found evidence that undifferentiated progenitor cells in the zebrafish by gene expression analyses, and generate transgenic reporter lines to visualize the activity of CPs during regeneration. In addition, we will examine the differentiation potential of candidate CPs in vivo during regeneration using genetic fate mapping. This study will provide unique insights into vertebrate CPs and their participation in natural regeneration. Results from this study may have implications for establishment of therapeutic regenerative myocardia treatments.

P18 Cardiomyocyte Grafts Contract, Alter the Mechanical Properties of Surviving Host Myocardium, and Improve Cardiac Function in Infarcted Rat Hearts

Frederick S Korte, Alicia Moreno-Gonzalez, Charles E Murray, Richard Regnier; Univ of Washington, Seattle, WA

Cell transplantation studies on infarcted myocardium have shown improved cardiac function. However, little is known about the underlying cellular mechanisms that lead to these improvements. The goal of this study was to determine if neonatal rat cardiomyocytes transplanted into one week old infarct region of adult rat hearts: 1) contract, 2) alter the passive mechanical properties of infarcted tissue, 3) lead to changes in the mechanical properties of remote host myocardium far from the infarct, and 4) improve global myocardial function. Thin tissue strips were dissected from infarcted hearts and chemically demembranated before active and passive mechanical properties were measured (grafted cells identified by fluorescent label CM-Dil). Nine weeks after transplantation, cardiomyocyte grafts produced Ca2+-dependent force, while non-treated infarcted tissue did not produce active force. The Ca2+sensitivity of force was increased in cardiomyocyte grafts (pCa50 = 5.67 ± 0.07, n = 6) and remote myocardium from cell-treated hearts (pCa50 = 5.64 ± 0.07, n = 4) as compared to control myocardial strips (pCa50 = 5.41 ± 0.03, n = 14) (P < 0.05). The maximal rate of force development (a0) for cardiomyocyte grafts (7.8 ± 0.8 s⁻¹, n = 8) was similar to that of neonatal cardiomyocyte grafts (7.7 ± 0.8 s⁻¹, n = 3), but much higher than that of control myocardium than all other groups. Passive stiffness was increased in scar tissue by an order of magnitude compared to adult control or remote myocardium. Interestingly, passive stiffness in cardiomyocyte grafts was similar to control strips, but the collagen content of cardiomyocyte grafts was similar to that of scar strips. Finally, fractional shortening as assessed by echocardiogram was significantly increased in cell-treated infarcted hearts (25.4 ± 1.9, n = 6) as compared to non-treated infarcted hearts (17.3 ± 0.8, n = 6). These studies demonstrate that cardiomyocyte grafts have the potential to remuscularize the injured heart, may contribute positively to heart function by remodeling or restructuring infarcted tissue, and may indirectly affect the mechanical properties of remote myocardium. Supported by HL63487 to F.S. Korte, AHA 0510170Z to A. Moreno-Gonzalez, AHA 0140040N and HL61683 to M. Regnier and HL64387 to C. E. Murray.

P19 Identification of Myocardial and Vascular Precursor Cells in Human and Mouse Epidemic

Federica Limana, Cntr Cardiologico Monzino, Rome, Italy; Antonella Zacheo, Istituto Dermatopo dell’Immacolata, Rome, Italy; David Mocini, Ospedale San Filippo Neri, Rome, Italy; Giovanni Borsellino, Fondazione Santa Lucia, Rome, Italy; Roberta De Mori, Antoiena Mangoni, Istituto Dermatopo dell’Immacolata, Rome, Italy; Massimo Santini, Massimo Stabano, Alberto Costantino, Ospedale San Filippo Neri, Rome, Italy; Giulio Pompili, Antonia Germansi, Cntr Cardiologico Monzino, Rome, Italy; Maurizio Capogrossi; Istituto Dermatopo dell’Immacolata, Rome, Italy

Background: During development, the epicardium is the source of multipotent mesenchymal cells which give rise to coronary vessels, including endothelial cells, smooth muscle cells and, possibly, cardiomyocytes. Recent studies have identified populations of cardiac stem cells, or cardiac progenitors (CPs), in the murine model of myocardial infarction (MI), epicardial cells transduced with a lentiviral vector which retains the ability to give origin to myocardial precursors and vascular cells. These factors which are released in the pericardial fluid after infarction. In agreement with this hypothesis, when MI was induced maintaining the pericardial cavity intact, a significant number of CPs in the pericardium were mobilized upon injury to regenerate lost tissues. To test this hypothesis, we will identify candidate CPs in the zebrafish by gene expression analyses, and generate transgenic reporter lines to visualize the activity of CPs during regeneration. In addition, we will examine the differentiation potential of candidate CPs in vivo during regeneration using genetic fate mapping. This study will provide unique insights into vertebrate CPs and their participation in natural regeneration. Results from this study may have implications for establishment of therapeutic regenerative myocardia therapies.

P20 Efficacy of Intramyocardial Autologous Angiogenic Cell Precursors Injection for Ischemic Cardiomyopathy

Kitiban V Arom, Permyos Ruengsakulrach, Vibul Jotisakulratana; Bangkok Heart Hosp, Bangkok, Thailand

Introduction: The objective is to determine efficacy of intramyocardial angiogenic cell precursors (ACPs) injection in ischemic cardiomyopathy (ICM) methods. Twenty-three patients (cell group) underwent intramyocardial ACPs injection. Twenty three case-matched patients from heart failure database treated by medical means were served as controls. There was no statistically significant different between the cell and the control groups in relation to preoperative left ventricular ejection fraction (LVEF) and co-morbidities. In the cell group, mean age was 61.0 ± 10.5 years. Mean LVEF was 25.8 ± 7.5%. NYHA Class was 3.0 ± 0.9. Thirteen underwent cell injection alone and 10 underwent combination Off-pump CABG (OPCAB) and cell injection. ACPs expressed CD34, CD133, KDR, Tie-2, CD144, von Willebrand factor, C031™®, concomitant binding of Ulex-Lectin and uptake of acetylated low density lipoprotein (Ac-LDL), secreted interleukin-8, vascular endothelial growth factor and angiogenin were injected into the non-viable myocardium and hypokinetic segments in the cell group. The quality of life evaluated by Short Form 36 demonstrated improving of physical function, role-physical, role-emotion, general health, and vitality domains in cell group at 6 months' follow up. LVEF changed significantly in 82.6% of patients (19/23). The LVEF improved from 25.8 ± 7.5% to 32.2 ± 10.9% (P < 0.001) at 145 ± 7 to 106.5 days in the cell group. There was no significant difference in changes of LVEF in the cell injection alone and the combination OPCAB with cell injection (6.5 ± 5.9 vs. 7.2 ± 8.8 point %, P = 0.8). The change in the LVEF did not differ between the cell group and controls (6.8 ± 7.1 vs. 0.8 ± 15.1 point %). Conclusions: Intramyocardial ACPs injection is safe in the ICM patients. The NYHA class and quality of life were improved. The LVEF was significantly improved in the cell group even though the change of LVEF was not significant different from the controls. Large-scale placebo-controlled studies are in progress.
Early Elevation of Matrix Gla Protein After Bare and Drug-Eluting Stent Implantation in Patients With Stable Angina

Konstantina Bouki, Panagiotis Vavoulis, Dionisos Chagopoulos, Konstantinos Paravolakis, Dimitris Elefopoulos, Evangelia Kapsali, Anastasia Perpina, Thomas Apostolou; General Hosp of Nikaia, Piraeus, Greece

BACKGROUND: Matrix γ- Carboxyglutamic Acid -protein (MGP) is a major vascular calcification inhibitory factor that is strongly expressed in human calcified atherosclerotic plaques. A down regulation expression of MGP confer an increased risk of plaque calcification and myocardial infarction. Experimental data demonstrated that the endothelium when exposed to oxidative, or inflammatory stimuli regulates the process of calcification by enhancing the expression of MGP. In this study, we investigated the response of the endothelium, regarding serum levels of MGP, after Percutaneous Coronary Intervention (PCI). METHODS: Serum MGP levels were measured (by ELISA), in peripheral, pre-PCI (MGP A), 4–6 hours (MGP B) and 24 hours post-PCI (MGP C) and 30 days later (MGP D), in 85 patients (pts), (male 71, age 62±10 years) with stable angina pectoris. RESULTS. PCI was performed in 111 vessels using drug-eluting (DES, n=53) or bare metal (BS, n=32) stents. The serum MGP concentrations increased significantly shortly after PCI, returned to baseline after 24 h and remained unchanged at 30 days later (see Graph). This increase of MGP levels was suppressed in pts with: multifocal coronary artery disease (CAD), (p=0.04), multiple treated vessels (p=0.004), longer than 18mm implanted stents (p<0.001). Changes in MGP levels did not effect, left ventricular weight, cardiac myocyte size, and number of nuclei per cardiomyocyte were determined in hearts from rats that had an AV fistula or sham surgery. Evaluation of isolated, unstressed myocytes (fistula versus sham) at 3, 5, 7 and 14 days post-surgery revealed that male rats experienced a significant decrease in cardiomyocyte length (117.6 ± 4.5 μm vs. 93.8 ± 2.3 μm) as well as an increase in the percentage of mononucleated cardiomyocytes (11% vs. 18%) at 5 days post-fistula. This decrease was transient, with restoration of normal average lengths by 14 days post-fistula. In contrast, females had progressive increases in cardiomyocyte length (sham 95.6 ± 6.7 μm; 7-days fistula 108.1 ± 3.6 μm; 14-days fistula 113 ± 9.9 μm) and the percentage of mononucleated cardiomyocytes decreased (sham 10.5 ± 3.4%; 7-days fistula 5.4 ± 0.4%; 14-days fistula 3.25 ± 1%). These findings, together with negative staining for proliferating cell nuclear antigen (PCNA) in cardiomyocytes isolated from male rats at 5 days post-fistula, suggest cytoskinesis is occurring in male hearts. Concurrent with the divergent remodeling of the cardiomyocyte, tumor necrosis factor-α was increased in the myocardium of male hearts, together with degradation of myocardial collagen and impaired cardiomyocyte adhesion to the extracellular matrix. These changes were not present in female hearts. Therefore we conclude that TNF-α, which is known to activate matrix metalloproteinases, is a key factor contributing to the gender differences seen in cardiomyocyte hypertrophy and myocardial remodeling.

Gender Differences in Cardiac Remodeling and Inflammatory Cytokine Expression Induced by Volume Overload in Rats

Yan Du, David B Murray, Jason D Gardner, Joseph S Janicki, Gregory L Brewer; Univ of South Carolina, Columbia, SC

In previous studies we have described gender differences in myocardial remodeling (i.e., eccentric versus concentric) induced in response to an infrarenal aorticaval (AV) fistula. Accordingly, this study sought to determine if this gender dimorphism was the result of differences in cardiomyocyte remodeling. To this end, ventricular weight, cardiac myocyte size, and number of nuclei per cardiomyocyte were determined in hearts from rats that had an AV fistula or sham surgery. Evaluation of isolated, unstressed myocytes (fistula versus sham) at 3, 5, 7 and 14 days post-surgery revealed that male rats experienced a significant decrease in cardiomyocyte length (117.6 ± 4.5 μm vs. 93.8 ± 2.3 μm) as well as an increase in the percentage of mononucleated cardiomyocytes (11% vs. 18%) at 5 days post-fistula. This decrease was transient, with restoration of normal average lengths by 14 days post-fistula.

Interaction of Angiotensin II Receptor Blocker Losartan with Uric Acid

Seok-Min Kang, Woochul Chang, Soyeon Lim, Namsik Chung, Ki-Chul Hwang; Yonsei Univ, Seoul, Republic of Korea

Background: Serum uric acid (SUA) is currently recognized as a risk factor for cardiovascular diseases including hypertension and heart failure. It has been demonstrated that an angiotensin II receptor blocker (ARB), losartan, decreases SUA level resulting from its inhibitory action on the renal uric acid transporter (URAT1), whereas other ARBs, candesartan and valsartan, have no lowering effect. Recent studies show that uric acid causes vascular smooth muscle cell (VSMC) proliferation by entering cells via a functional URAT1. In this study, we examined the interaction of ARBs with URAT1 in the VSMCs. Methods and Results: Following 6 hours incubation of uric acid, the expression of URAT mRNA in rat aortic VSMCs was increased dose-dependently with a peak at concentration (80 μg/ml) of uric acid. Losartan inhibited uric acid-stimulated expression of URAT at the concentration of 100 μM, whereas candesartan and valsartan were not inhibitory for the expression of URAT. Uric acid significantly activated p38 and extracellular signal-regulated kinases (ERKs) as well as expression of cyclo-oxygenase-2 (COX-2) mRNA and monocyte chemoattractant protein-1 (MCP-1) production. Losartan dramatically inhibited p38, ERKs activation, COX-2 and MCP-1 production in uric acid-stimulated rat aortic VSMCs. Conclusion: Our data indicate that losartan may have an inhibitory effect on the expression of URAT in uric acid-stimulated rat aortic VSMCs. Therefore, losartan would have an anti-inflammatory and antiproliferative activity by modulation of entering cells of uric acid via URAT.

Cardiac Cells with Stem Cell Characteristics Can Be Isolated from Patients with Various Heart Diseases

Israel M Barbash, Ayelet Itzhaki, Neufeld Cardiac Research Institute, Ramat Gan, Israel; Efrat Rakanian, Nofrat Mishali, Bashier Shonk Yosef, Shabs Med Ctr, Ramat Gan, Israel; Jonathan Leor; Neufeld Cardiac Resch Institute, Ramat Gan, Israel

INTRODUCTION: Cardiac progenitor cells (CPC) have been isolated mostly from murine hearts and were shown to have stem cells characteristics. However, characterization and functional assessment of human derived CPCs is scarce. We aimed to develop efficient method for isolating CPC and to characterize them with respect to cell markers and differentiation capabilities. METHODS and RESULTS: Myocardial samples (septum, left and right atrial appendage) were donated during all types of heart surgery and percutaneous RV septum biopsies. Tissues were enzymatically dissociated, and the CPC created typical clones, possessed self-renewal capacity and expressed stem cell markers including C-KIT, CD133, MDR1, and DATA 4. CPC were successfully isolated from patients aged 0–81 years, diabetics (24%), hypertensive (36%) and from patients with coronary artery disease (45%) and impaired LV function (42%). CPC were injected into athymic nude rat myocardium to examine myogenic differentiation. One week after injection, some cells still expressed stem cell markers while others expressed specific human cardiac marker, such as human cardiac troponin I and human cardiac α-actin (green) with early sarcomere formation (Figure, arrow), indicating that some of the implanted human cells developed into early cardiomyocytes in vivo. CONCLUSIONS: Our preliminary findings suggest that adult human hearts retain unique cell population with stem cell markers and myogenic potential. We successfully isolated and expanded these cells from hearts of patients with heart disease and showed their regenerative potential.

Use of a Novel Technology to Identify N-Linked Glycoproteins on the Cell Surface of Cardiac Progenitor Cells: Discovering Markers of Differentiation

Rebekah L Gundry, Steven T Elliott, Johns Hopkins Univ Sch of Medicine, Baltimore, MD; Bernd Wollschled, Damaris Bausch-Fluck, Swiss Federal Institute of Technology, Zurich, Switzerland; Kenneth R Boehler, National Institute on Aging, National Institutes of Health, Baltimore, MD; Jennifer E Van Eyk, Johns Hopkins Univ Sch of Medicine, Baltimore, MD

To enhance discovery of specific biomarkers of differentiation, a new, directed proteomic approach is being employed to identify cell surface N-linked glycoproteins present at discrete stages during in vitro differentiation of stem cell-derived cardiac and skeletal mesodermal lineages. In this initial study, the cell surface glycoprotein capture (CSGC) method was used to covalently label the extracellular domain of glycoproteins present on intact C2C12 cells (murine myogenic, skeletal lineage) with a biotin tag. Following enzymatic digestion, all biotin-labeled glycopolypeptides were captured using streptavidin beads and the N-linked glycopeptides, specifically, were subsequently released using N-Glycosidase F. The resulting peptides were analyzed using high mass accuracy MS to identify the proteins and confirm the site of glycosylation. Further analyses verified the presence of a known or predicted transmembrane domain, the presence of the conserved N-linked glycosylation sequence motif (NXS/T) at each glycosylation site, and finally that the glycosylation sites mapped to the extracellular domain. The two biological replicates had greater than 80% specificity for the N-linked cell surface glycoproteins (specificity is defined as number of N-linked glycopeptides captured / total number of peptides captured) and 67% non-redundant cell surface N-linked glycoproteins have been identified. Examples include cell surface receptors (macrophage mannose receptor, basic fibroblast growth factor receptor 1, ephrin type A and B receptors), cell adhesion proteins (N-cadherin, N-cadherin, and nectin 1, cadherin family proteins) and N-linked glycoproteins. One particularly interesting protein found by the current study is N-cadherin. As the mRNA expression of N-cadherin is known to be elevated in cardiac tissue when compared to other cell types, it could potentially be specific for early detection of the cardiac lineage. The expression of N-cadherin on C2C12 cells has been validated by immunohistochemistry, thus confirming the CSGC results. It is predicted that this targeted proteomics approach will be useful for identifying cell surface markers that could be used for monitoring differentiation and selecting pure cell populations.
Transcriptional Variability in Stem Cell Populations Isolated from Adult Mouse Myocardium
Claudia O Rodrigo, Tina A Shehadeh, Karel Caliero, Nanette H Bishopric, Univ of Miami Miller Sch of Medicine, Miami, FL

Although the mammalian heart has low regenerative potential, a number of research groups have reported isolation of cardiac stem/progenitor cells from adult myocardium based on the expression of surface markers. One interesting aspect of these studies is the number of different surface marker patterns used to identify these cell populations. It is not known whether these differences reflect the presence of multiple stem cell types, or distinct stages of development of a single pluripotent progenitor. We have isolated a clonogenic cell population from adult mouse myocardium that is c-kit+ , sca-1+ . Different cell morphologies were observed within the initial isolates and have been described by other groups. Individual clones were therefore isolated for the purposes of analyzing differences in gene expression. We have now isolated 65 clones from whom 42 have been maintained in culture for at least 20 –30 passages. We have selected 6 clones based on their different morphology for transcriptional analysis using a focused stem cell array. About 4% of the transcripts (37 in 94) related to stem cell maintenance and differentiation were highly abundant (Cts > 39) in all 6 clones. Using unsupervised clustering based on Euclidean Distance measure, we used the transcriptional profiles to present the 6 clones in a hierarchical clustering image. This algorithm was able to group clones based on similarity in expression profile. We have calculated absolute transcript levels from Cts > 35. The most abundant shared transcripts included those involved in cell proliferation; c-myc, Ccn1 and p300. Clone 42 was the most divergent and has a unique star-like morphology compared to the others. The most divergently expressed genes among the different clones were Cxcl12, involved in chemotraction, the growth factor FGF1, the homeobox transcription factor Msx1 and the lineage markers MyoD1 and Flk-1/KDR. The latter have now been identified as a marker for a multipotent cardiac progenitor cell in the early embryo. Our results suggest that clonal differences might be an important factor to be taken into account during stem cell therapy design.

The Hematopoietic Actions of G-CSF Improve Cardiac Function and Repair After Myocardial Ischemic Injury
Alex Bobik, Baker Heart Rsch Institute, Melbourne, VIC, Australia; David Curtis, Royal Melbourne Hosp, Melbourne, VIC, Australia; Peter Kanellakis; Baker Heart Rsch Institute, Melbourne, VIC, Australia

Granulocyte-colony stimulating factor (G-CSF) administered after myocardial infarction (AMI) improves heart function and repair. Although initial studies suggested that improved function/repair were due to differentiation of hematopoietic cells into cardiomyocytes and blood vessels, more recent data indicate that the hematopoietic cells remain in the hematopoietic phenotype and that direct effects of G-CSF on cardiomyocytes may be responsible for its beneficial effects. We tested this hypothesis using G-CSF receptor deficient mice in a model of AMI. Wild type mice were reconstituted with either wild type bone marrow cells (control) or G-CSF receptor deficient bone marrow (G-CSFR BM-/-). Injury was induced by occlusion of the coronary artery, followed by treatment with saline or G-CSF for 5 days. Twenty eight days later cardiac function was assessed after placement of a Miller catheter into the left ventricle as was cardiac repair. Effects on cardiac repair were assessed immunohistochemically, measuring Cxcl12 – vessels and small alpha-actin+ and GATA-4+ cardiomyocytes in the infarct zone. The G-CSF control mice had 20 to 50% better cardiac function (P<0.05, LVEDP and LVEF) compared to saline-treated control mice (P<0.05); G-CSF also increased vessels in the infarct zone (1.9-fold, P<0.05) and immature cardiomyocyte (alpha-actin+, GATA-4+/+ numbers in the infarct zone (1.8-fold, P<0.05). The G-CSF did not increase the expression of collagen I, FN1 or EDA-FN in the 3.3D cell line. Focal adhesion marker, -SMA was confirmed by Western blotting and immunocytochemistry. Notch1 is activated in border zone cardiomyocytes coincident with phosphorylated Akt, and infarcted hearts receiving adenoviral NICD exhibit better function after six weeks than vehicle and EGFP virus injected controls, implicating Notch signaling in a cardioprotective role following cardiac injury. Conclusion: Notch activation in cardiomyocytes is mediated through c-Met and Akt survival signaling pathways, and Notch signaling in turn enhances Akt activity. This suggests a positive survival feedback mechanism between Notch and Akt signaling in adult myocardium following injury.

Notch and P13K Signaling in Cardiac Myocytes in Vitro and in Vivo
Natalie Gude, Gregory N Emmanuel, John Muraski, Mark A Sussman; SDSU Rsch Foundation, San Diego, CA

Introduction: The Notch network regulates multiple cellular processes, including cell fate determination, development, differentiation, proliferation, apoptosis and regeneration. Notch receptor activation generates the Notch Intracellular Domain (NICD), which translocates to the nucleus and turns on target genes, including Hes1. Notch activity influences HGF/c-Met receptor and PI3K/Akt signaling cascades. Delineating connections within the Notch/c-Met/Akt signaling axis is essential in understanding the role of Notch signaling in cardiovascular diseases. The aim of this study was to determine whether Notch signaling is critical to understanding myocardial cell survival during ischemia-reperfusion injury. Hypothesis: We will show that Notch signaling is critical to understanding myocardial cell survival during ischemia-reperfusion injury.

Method: Notch signaling was assessed in myocytes isolated from neonatal rat hearts. Notch signaling was mediated by adenoviral NICD. NICD was also administered in vivo in a rat model of myocardial infarction. NICD was administered either alone or in combination with HGF and/or Akt kinase inhibitors.

Results: NICD treatment significantly improved infarct size and cardiac function. NICD treatment increased Akt phosphorylation and prevented protein expression of Notch signaling in a dose dependent manner. NICD treatment significantly increased Akt phosphorylation. NICD treatment significantly increased Akt phosphorylation in a dose dependent manner. NICD treatment increased Akt phosphorylation and prevented protein expression of Notch signaling in a dose dependent manner. NICD treatment significantly increased Akt phosphorylation. NICD treatment significantly increased Akt phosphorylation in a dose dependent manner. NICD treatment increased Akt phosphorylation and prevented protein expression of Notch signaling in a dose dependent manner. NICD treatment significantly increased Akt phosphorylation.
Soluble Natriuretic Peptide Receptor-Related Fragment (sNRF) Inhibits NP Action and Is Increasing in the Failing Heart

Scott Kuhn, Nathan Airhart, Ronald Wells, Charles T Roberts, Michael Silberbach; Oregon Health Sciences Univ, Portland, OR

In heart failure, the beneficial effects of natriuretic peptides (NPs) are blunted, in part, due to NP receptor (NPRA) unresponsiveness caused by an unknown mechanism. We initially identified sNRF in a yeast 2 hybrid screen of a human heart library. The sNRF cDNA transcript encodes the C terminus of NPRA (NPRA820–1061), its 5' end extends 215 nt into intron 15 of the NPRA gene, followed by exons 16–22 of the fully spliced NPRA mRNA sequence. Another group subsequently reported (GenBank BX329044) a similar cDNA derived from placenta. In primary cultures of neonatal rat cardiac fibroblasts (CF), sNRF expression blocks NP’s inhibitory effects on α-smooth muscle actin expression, a key marker of CF differentiation. In other experiments sNRF appears to potentiate TGF-β action independently of NPs. To determine if sNRF mRNA is differentially expressed in human heart disease, total RNA was extracted from LVs of 13 hearts explanted from patients undergoing cardiac transplantation and was compared to normal LV (n = 4). Quantitative real-time RT-PCR using primers corresponding to the intron 15 sequence and the 3' end of NPRA exon 17 revealed sNRF mRNA expression in the failing hearts was higher than any of the normal hearts. Relative sNRF expression was 26-fold higher compared to the mean control value (range 2.8- to 91-fold, p < 0.0001). In the same experiment, we determined the levels of full-length NPRA mRNA using a TaqMan probe that spans exons 7 and 8 of the full-length NPRA transcript that are upstream of the presumed sNRF transcription initiation start site and, as such, would not recognize sNRF. There was no difference between the expression levels of full-length NPRA in the normal versus diseased hearts (0.8-fold compared to the mean control value, range 0.4- to 2.2-fold) indicating that regulation of sNRF from its putative promoter in intron 15 is independent of NPRA expression initiated at the NPRA promoter flanking exon 1. We conclude that sNRF expression inhibits critical NPNF functions and may have NP-independent effects. sNRF mRNA is increased in the clinical setting of heart failure. Whether sNRF inhibition reverses NP resistance or blocks TGF-β effects in heart failure remains to be determined.

Electro-Gene Transfer in the Heart

Bryan A Boone, William G Marshall, Jr, Sylvia I Gogarte, Jose D Burgos, Margaret K Baldwin, Michele L Danielson, Mary J Larson, Dianne R Carotto, Yolimar Cruz, Kenneth E Ugen, Univ of South Florida College of Medicine, Tampa, FL; Richard Heller, Univ of South Florida College of Medicine, Tampa, FL; Richard Heller, Univ of South Florida College of Medicine, Tampa, FL

This study evaluated gene delivery to the heart using electroporation to deliver plasmids to cardiac myocytes in situ to increase levels of gene expression. Thirteen pigs (neutered males, 25 - 30 kg) underwent median sternotomy with general endotracheal anesthesia. Four asynchronous electroporation parameters (field strength and pulse width) were evaluated to determine which would yield the highest levels of expression using injected luciferase plasmid in the left ventricular anterior wall, each followed by electroporation. One site of plasmid delivery without electroporation was used as control. After 48 hours, 4 animals underwent injection site excision with a 6mm punch. The level of luciferase expression was quantified using commercial firefly luciferase (Sigma, St Louis, MO) with levels expressed as pg/mg of tissue. Electroporation parameters that yielded the highest levels of luciferase expression (100 V/cm field strength / 250 ms pulse width) were used to deliver VEGF plasmid after injection into the anterior left ventricular wall. Five animals were maintained for 48 hours and four animals for 7 days. An ELISA kit was used for detection of VEGF. Myocardial luciferase expression at injection sites was markedly increased (25X) with electroporation versus control (Table 1). Myocardial VEGF expression delivered with electroporation at injection sites was also greatly enhanced (4–5X) versus plasmid injection alone (Table 2) after 48 hours, but levels dropped to background levels within 7 days. No serum elevation of VEGF was noted. Gene delivery to the in situ heart using electroporation has been accomplished and leads to increased expression.

Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>pg Luciferase / mg tissue</th>
<th>S.D.</th>
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<tbody>
<tr>
<td>P-E</td>
<td>431.47</td>
<td>247.84</td>
</tr>
<tr>
<td>P-E</td>
<td>(200 V/cm, 150 ms)</td>
<td>2,138.78</td>
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<tr>
<td>P-E</td>
<td>(100 V/cm, 150 ms)</td>
<td>2,729.76</td>
</tr>
<tr>
<td>P-E</td>
<td>(100 V/cm, 250 ms)</td>
<td>9,103.76</td>
</tr>
<tr>
<td>P-E</td>
<td>(100 V/cm, 20 ms)</td>
<td>3,190.32</td>
</tr>
<tr>
<td>P-E</td>
<td>(100 V/cm, 250 ms)</td>
<td>7,364.55</td>
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Cardiac Proteasome Complexity: Subpopulations with Distinct Molecular Compositions

Oliver Drews, UCLA, Los Angeles, CA; Robert Wildgruber, BD GmbH, Munich, Germany; Chenggong Zong, UCLA, Los Angeles, CA; Ulle Sukup, BD GmbH, Munich, Germany; Glen Young, UCLA, Los Angeles, CA; Nissim Mikael, BD GmbH, Munich, Germany; Aldrin V Gomes, Ping Peipei; UCLA, Los Angeles, CA

The ubiquitin-proteasome system plays a key role in protein degradation in mammalian cells. At the proteasome core, the subunits form a complex of three catalytic and three regulatory complexes. This complex of multi-protein complexes. Using these, 20S complexes were separated into subpopulations with distinct isoelectric points (pI; Mol Cell Proteomics. 2006; 5: S22). The separated complexes retained their structural integrity and proteolytic activities. To provide insights in the functionality of cardiac 20S proteasomes and protein proteolysis in the context of heart failure, we used 20S subpopulations isolated from failing heart or normal heart. In this presentation we will present evidence that cardiac 20S subpopulations potentially play different roles in cellular events similar to the immunoproteasome in the immune response. In cardiovascular systems, they might be involved in modulating cardiac function. Our studies provide the foundation to analyze proteasome subpopulations in the heart.

Distinct Functional Complexes of 20S Proteasomes in Murine Heart and Liver

Aldrin V Gomes, Glen Young, Oliver Drews, Xiaohai Li, Chenggong Zong, Peipei Ping; UCLA, Los Angeles, CA

The proteasome system represents a key component in the intracellular protein degradation machinery. Despite advancements in proteasome research, which suggests its involvement in over 20 diseases, mechanisms pertaining to its molecular regulation remain poorly understood. An organole targeted proteomic effort was undertaken to comprehensively characterize highly purified heart and liver 20S proteasomes. Blue-native gel electrophoresis combined with LC/MS/MS was used to delineate the molecular composition of this organellar. Heterogeneity was observed with respect to the components of the three inducible β subunits in the 20S proteasomes: the β2i expression in the purified 20S complexes was found to be 40% higher in the liver compared to the heart by immunoblotting. Corroborating this finding was the large difference in the average peak area of the two most intense peaks from the heart β5 mass spectra (47%) compared that to the liver 20S (100%). Immunoblotting showed that the liver 20S proteasomes contained higher relative amounts of phosphorylated Ser, Thr and Tyr residues than the heart 20S. A difference in proteasome function of these two organs was also observed. The chymotrypsin like proteolytic activity of the cardiac 20S was significantly greater than that of liver 20S; whereas the caspase like proteolytic activity of the liver 20S was greater than that of cardiac 20S. The 20S proteasomes from both heart and liver contained at least 10 different associating partners. One binding partner, protein phosphatase 1 (PP1), which was associated with both heart and liver 20S complexes, showed distinct functional roles in the 20S complexes from the different tissues. Addition of PP1 to cardiac 20S significantly enhanced the β5i and confer altered substrate specificities and turnover. Immunodetection of these subunits in proteasome complexes with distinct pIs demonstrated variations in assembly. Subunit β1i was 47% higher expressed in complexes with pI 5.21 than in those with pI 5.28. Since β2i was significantly lower in complexes with pI 5.21, the analyzed complexes present at least in part hitherto unreported intermediate proteasomes in cardiac tissue. Complexes with different pIs exhibited distinct proteolytic activities as well. Proteasome subpopulations potentially play different roles in cellular events similar to the immunoproteasome in the immune response. In cardiovascular systems, they might be involved in modulating cardiac function. Our studies provide the foundation to analyze proteasome subpopulations in the heart.

Increased Expression of Heat Shock Protein 25 in Doxorubicin-Treated Failing Hearts

Kaushik Vedam, Yashinori Nishijima, Mahmood Khan, Periannan Kuppusamy, Jay L Zweier, Govindasamy Ilangovan; Ohio State Univ, Columbus, OH

Doxorubicin (DOX) is known to cause cardiotoxicity in cancer patients, who are treated with this drug or its cocktails, for various malignancies. Recent studies have shown that loss of cardiac oxidative stress-sensitive proteasome subunits potentially play different roles in cardiotoxicity. Small heat shock proteins are induced in response to oxidative stress. Thus we have developed a hypothesis that over expression of small Hsps may serve as marker of oxidative stress in DOX-treated failing hearts. In the present work we investigate the level of Hsp25 and its phosphorylated isoforms in DOX-treated failing hearts. Mice (n = 10) were injected (IP) with 6mg/kg body weight for 8 weeks. Control groups were treated with equal volume of saline for the same period of time. The hemodynamic and contractility of the heart in these mice were followed by echocardiography and electrocardiogram. At the eighth week, development of heart failure was confirmed by reduced ejection fraction and contractility by echocardiography. ECG also showed S-T segment elongation consistently. After confirming the heart failure, the animals were sacrificed and their hearts isolated. The isolated hearts were

Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>pg VEGF / sample</th>
<th>S.D.</th>
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<tr>
<td>P-E</td>
<td>117.96</td>
<td>66.30</td>
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<tr>
<td>P-E</td>
<td>(1000 V/cm, 250 ms) [2 days]</td>
<td>494.90</td>
</tr>
<tr>
<td>P-E</td>
<td>(100 V/cm, 250 ms) [7 days]</td>
<td>2,085.50</td>
</tr>
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then minced to obtain the heart lysates. We determined the level of total, s-15, and s-85 phosphorylated Hsp25 in control and DOX treated group. In DOX treated group, we found three times increased expression of Hsp25 as well as the phosphorylated isoforms. The Western blot showed increased number of spots in DOX treated cases. Three major spots were noticed in control, while 6 different spots were found in DOX treated. In addition to this, we carried out Mcl-Me analysis of these proteins and confirmed that they indeed correspond to the Hsp25 proteins. We conclude from these studies that, in doxorubicin treated failing hearts, there is increased expression of Hsp25 due to oxidative stress.

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**Gradual Reoxygenation After Prolonged Myocardial Ischemia Improves Cardiac Function and Reduces Oxidative Stress in a Porcine Model: An Optimizing Postconditioning Strategy**

Ulf Abdel-Rahman, Peter Rietsteke, Soheyl Bejati, Klaus Zwicker, Stefan Kerscher, Karima Tizi, Ulrich Brandt, Anton Moritz; JW Goethe-Univ, Frankfurt, Germany

**Objective:** Hyperoxic reoxygenation of the ischemic myocardium after aortic declamping may aggravate myocardial reperfusion injury in cardiac surgery. The study assessed a new gradual reoxygenation protocol for postconditioning and its effects on cardiac function and oxidative stress.

**Methods:** Twenty-one adult pigs were put on cardiopulmonary bypass (CPB) with prolonged aortic cross-clamping (60min). After aortic declamping, pigs underwent hyperoxic reoxygenation (PaO2 250–350 mmHg) for the initial 10min of reperfusion in group I (n = 10). In group II (n = 11), gradual reoxygenation started for two minutes at low (paO2 40–50 mmHg), and continued for another 8 minutes at a higher oxygen levels (paO2 50–90 mmHg). Animals were weaned from CPB after further 20min of normoxygenic reperfusion. Cardiac function was measured by conductance catheter technique during 60min post CPB. Oxidative stress was assessed by detection of oxygen radicals using electron paramagnetic resonance (EPR) spectroscopy as well as by neutrophil oxidative burst assays within the coronary sinus blood (CS) after aortic declamping.

**Results:** End-systolic pressure volume relationship (ESPVR) and peak left ventricular pressure rise (dP/dtmax) were significantly less decreased in group II after CPB end. Correspondingly, early oxygen radical production and oxidative burst assays in CS were significantly reduced in group II compared to group I during and after reperfusion. Conclusion: Gradual reoxygenation in the initial reperfusion period leads to significantly less reduced cardiac function and oxidative stress after prolonged myocardial ischemia, and serves to optimize postconditioning strategies.

**P38**

**The Myocardial Protective Effect of Pioglitazone Is eNOS- and iNOS-Independent**

Yochai Birnbaum, Yumi Ye, Yu Lin, Ming-He Huang, Charles V Lui, Reggio J Perez-Polo; Univ of Texas Med Branch, Galveston, TX

**Background:** Endothelial nitric oxide synthase (eNOS) activation with subsequent subsidence NO3 (iNOS) and cyclooxygenase-2 (COX2) activation are essential for mediating the myocardial infarct size (IS) limiting effects of statins. In the rat, the peroxisome proliferator-activated receptor γ (PPARγ) agonist pioglitazone (PIO) limits IS and increases myocardial eNOS- and iNOS- levels without activating eNOS and iNOS. The protective effect is blocked by COX2 inhibitor. Hypothesis: PIO limits IS in the eNOS−/− and iNOS−/− mice. **Methods:** Male C57BL/6 wild-type (WT), eNOS−/− and iNOS−/− mice received PIO 10 mg/kg/IP or water (P) on days for 3 days. Mice underwent 30min coronary artery occlusion and 4h of reperfusion, or hearts were harvested and subjected to ELISA. Results: PIO reduced IS in the WT, as well as in the eNOS−/− and iNOS−/− mice (Figure). Myocardial eNOS- and iNOS- levels, were increased by PIO in the WT (40.2±0.8 vs. 26.0±0.7 pg/mg; **p < 0.001**), eNOS−/− (41.8±0.5 vs. 23.4±0.4 pg/mg; **p < 0.001**), and iNOS−/− mice (40.6±0.8 vs. 24.5±0.4 pg/mg; **p < 0.001**). Conclusions: The myocardial protective effect of PIO is eNOS and iNOS independent. As eNOS activity decreases with age, diabetes and advanced atherosclerosis, this effect may be relevant in the clinical setting.

**P39**

**Snf1-Related Kinase (SNRK) Is Upregulated in Ischemic Tissue, and Its Overexpression Increases Cell Death**

Michael A Burke, Hossein Ardehali; Northwestern Univ, Chicago, IL

Snf1 related kinase (SNRK) is a novel kinase homologous to the AMP-activated protein kinases (AMPK) of mammals and the Snf1 kinase of yeast. While these proteins serve to regulate intracellular metabolism during low energy states, the primary function of SNRK has not been delineated. SNRK is expressed in heart and vascular tissue and is upregulated during apoptosis in neuronal cells. However, it is not clear whether its upregulation promotes or inhibits cell death. The objective of this study was to determine the effects of ischemia on SNRK levels and whether SNRK upregulation affects cell death in vitro. Dog hearts were subjected to 5 hours of low-flow ischemia followed by reperfusion of heart tissue and Western blot analysis. The level of SNRK was increased in response to the low flow ischemia. Furthermore, in explanted heart samples for end stage cardiomyopathy, the levels of SNRK was significantly increased. To better understand the role of SNRK in cell death, we then overexpressed green fluorescent protein (GFP) tagged SNRK in HEK293 cells in the presence and absence of hydrogen peroxide (H2O2). Cells were then treated with tetramethylrhodamine ethyl ester (TMTRE), a marker of mitochondrial membrane potential and thus cell viability followed by flow cytometry or trypan blue. Overexpression of SNRK induced cell death compared to GFP transfected cells in the absence or presence of H2O2 (p < 0.001). Western blot analysis of GFP and SNRK showed that overexpression resulted in agrgresome formation in some cells. The significance of agrgresome formation is not clear, but was unexpected given the relative normal hydrophobicity of the SNRK protein. These results suggest that SNRK plays a role in cell death and is upregulated in ischemia and cardiomyopathy. Thus, SNRK represents a potential therapeutic target to abrogate the cell death that accompanies ischemic heart disease.

**P40**

**Inhibition of Ischemic Cardiomyocyte Apoptosis Through Targeted Ablation of Bnip3 Restraints Postinfarction Remodeling**

Faisal Syed, Univ of Cincinnati, Cincinnati, OH; Maike Krenz, Janka Wasapanura, Cincinnati Children’s Hosp, Cincinnati, OH; Xiaoping Ren, W K Jones, Univ of Cincinnati, Cincinnati, OH; Jeffrey Robbins, Cincinnati Children’s Hosp, Cincinnati, OH; Gerald Dom, Abhinav Dwan; Univ of Cincinnati, Cincinnati, OH

Cardiomyocyte apoptosis contributes to early infarct expansion and late global ventricular dysfunction after myocardial infarction. Bnip3, a proapoptotic BH3-only Bcl2 family protein, is transcriptionally upregulated in cardiomyocytes by hypoxia. We hypothesized that Bnip3 mediated cardiomyocyte apoptosis contributes to post-infarction LV remodeling. We used gene targeting and conditional overexpression to evaluate the effects of Bnip3 on in vivo cardiomyocyte apoptosis, and determine the consequences of ablatuing murine Bnip3(SK0) on cardiomyocyte death, infarct size, and ventricular remodeling after surgical ischemia-reperfusion (IR) injury induced by reversible LAD artery occlusion for 60min. Bnip3(SK0) did not affect hearts of unreated mice. After IR, Bnip3(SK0) mice had no differences in mortality, early infarct size (at 24h by gadoxinolin enhanced MRI) or late infarct size (at 3 weeks by MRI and pathology) as compared to wild type (WT). Two days after IR, apoptosis was significantly diminished in Bnip3(SK0) peri-infarct myocardium (6.7±0.8% vs 11.5±1.3% in WT, p < 0.011) and remote myocardium (3.7±0.7% vs 6.0±0.4% in WT, p < 0.046). Three weeks after IR, Bnip3(SK0) mice exhibited preserved global left ventricular systolic performance as compared with systolic dysfunction in WT (change in LVEF from 24 hours to 3 days: 11±8% vs -20±7% in WT, n=6–9, P < 0.015) and significantly diminished left ventricular diastolic volume: 46±11% vs 108±17% in WT, P < 0.007; and LV end-systolic volume: 35±20% vs 181±41% in WT, P < 0.005). Also, the myocardial scar was significantly thicker in Bnip3(SK0) (0.91±0.12mm vs 0.61±0.35mm in WT, P < 0.047). These observations suggest myocardial salvage by apoptosis inhibition following IR injury in Bnip3(SK0) mice. Forced cardiac expression of Bnip3 increased cardiomyocyte apoptosis in unsterred mice (1.21±0.14% vs 0.17±0.02% in controls; n=4, P < 0.029), causing progressive left ventricular dilatation and diminished systolic function that recapitulates Bnip3-mediated post-IR remodeling. In conclusion, post-ischemic cardiomyocyte apoptosis mediated by Bnip3 is a major determinant of ventricular remodeling in the infarct heart.

**P41**

**Glucose Phosphorylation and Mitochondrial Binding Are Required for the Protective Effects of Hexokinase I and II**

Lin Sun, Shetha Shukair, Tejaswitha J Naik, Farzad Moazed, Hossein Ardehali; Northwestern Univ, Chicago, IL

Alterations in glucose metabolism have been demonstrated in diverse disorders, ranging from heart disease to cancer. The first step in glucose metabolism is carried out by the hexokinase (HK) family of enzymes. Overexpression of HKI and HKII in tissue culture protects against oxygen-induced cell death. The protective effects of these enzymes are thought to be due to either an increase in glucose phosphorylation or closure of the mitochondrial permeability transition pore (mPTP) as a result of HK binding to the voltage dependent anion channel (VDAC) on the mitochondria. VDAC is believed to form part of mPTP, opening of which leads to cell death. In order to determine the relative contribution of mitochondrial binding and glucose phosphorylating activities of HKs to their overall protective effects, we expressed full length HKI and HKII, their truncated proteins lacking the mitochondrial binding domains, and catalytically inactive proteins in tissue culture. Overexpression of full length proteins resulted in complete protection against cell death, decreased levels of reactive oxygen species, and inhibition of mitochondrial permeability transition in response to H2O2. However, the truncated and mutant proteins only exerted partial effects. Similar results were obtained in primary neonatal rat cardiomyocytes. To explore the role of VDAC in the protective effects of HKs, we then measured the phosphorylation level of this protein in cells overexpressing HKs. Overexpression of FL-HKI and FL-HKII resulted in a 5–10 fold increase in VDAC phosphorylation, while the mutant and truncated proteins resulted in a smaller increase. The mechanism for VDAC phosphorylation appears to be through PKCγ, as inhibitors of this kinase led to a reversal of this process. These results suggest that both glucose phosphorylation and inhibition of mPTP contribute to the protective effects of HKI and HKII. Furthermore, overexpression of HKI and HKII lead to VDAC phosphorylation in a PKCγ dependent pathway. These findings bear implications of HK.
overexpression and binding to the mitochondria as a potential clinical treatment strategy for various forms of human disease.

**ErbB2 Blockade and Downregulation Lead to Cardiomyocyte Cell Death Through Distinct Pathways**

Leo I Gordon, Amareaswara Singh, Sheila Prachand, Lloyd Lieberman, Lin Sun, Michael A. Buro, Tejasmita J Nadimpalli, C. Benson Pratt, Cleveland Clinic, Cleveland, OH; Hossein Ardeshil; Northwestern Univ, Chicago, IL

The tyrosine kinase receptor erbB2 (or Her2 in humans) is a member of the epidermal growth factor (EGF) family. It is highly expressed in many cancer types, and its overexpression is correlated with a poor prognosis in breast and ovarian cancer. Treatment with Herceptin (a monoclonal antibody against the extracellular domain of erbB2) has resulted in a significant improvement in survival of breast cancer patients overexpressing erbB2. However, this agent also causes cardiomyopathy and exacerbates anthracycline-induced cardiotoxicity. Treatment of isolated cardiomyocytes with anti-erbB2 induces apoptosis through modulation of Bcl-xl and -xS. In this report, we studied the mechanism for the deleterious effects of erbB2 antibody (Ab) or erbB2 protein downregulation in cardiomyocytes. We hypothesized that the deleterious effects of erbB2 Ab in cardiomyocytes are mediated through an increase in the levels of reactive oxygen species (ROS). Treatment of neonatal rat cardiomyocytes with erbB2 Ab resulted in a dose dependent increase in ROS production and cell death compared to control IgG treated cells. Reduction of erbB2 protein using RNA interference led to an increase in cell death but did not alter the levels of ROS. Furthermore, dog hearts subjected to ischemia displayed lower levels of erbB2 protein, suggesting a role for this protein in ischemic injury of the hearts. Similar results were obtained in explanted hearts of patients with ischemic cardiomyopathy. Finally, we also showed that erbB2 signaling in cardiomyocytes is through a PKCε-dependent pathway. These results provide evidence for a role for erbB2 in cardiomyocytes at baseline and ROS induction as a possible mechanism of the deleterious effects of Herceptin on the heart. Thus, therapies targeting oxidative stress may have beneficial effects in cancer patients receiving this form of therapy.

**Cardiac-Specific Deletion of Protein Phosphatase 1 β Leads to Reduced Myocardial Function**

Manunik Auger-Messier, Allen J York, Cincinnati Children’s Hosp Med Ctr, Cincinnati, OH; Angus C Naim, The Rockefeller Univ, New York, NY; Jeffrey D Molkentin; Cincinnati Children’s Hosp Med Ctr, Cincinnati, OH

Protein phosphatase 1 (PP1) plays a fundamental role in the control of cardiac function by regulating critical intracellular calcium handling proteins. For example, cardiac-specific overexpression of PP1 catalytic subunit (PP1C) in mice produces a phenotype of hypertrophy with suppressed contractility and an increase in cardiac ejection fraction. In this study, we validated PP1β targeted deletion using a cardiac-specific Cre transgenic mouse (MerCreMer) and a tamoxifen-inducible Cre transgenic mouse (Ai14). In both MerCreMer and Ai14 mice, the PP1β protein was successfully deleted from the heart. However, while PP1β deletion in MerCreMer mice did not affect basal cardiac function, PP1β deletion in Ai14 mice led to decreased contractility and increased diastolic filling fractions. These results demonstrate that PP1β is necessary for normal cardiac function, and that PP1β deficiency in certain cardiac tissues may lead to adverse cardiac remodeling.

**A Cyclin D2-Rb Pathway Regulates Cardiac Myocyte Size and RNA Polymerase III After Biomechanical Stress in Adult Myocardium**

E Angulo, A Garcia, Univ of California, Los Angeles, Los Angeles, CA; M Sano, Baylor College of Medicine, Houston, TX; S S Chan, Univ of California, Los Angeles, Los Angeles, CA; S J Goodfellow, Univ of Glasgow, Glasgow, United Kingdom; H J Xu, M Anderson Cancer Ctr, Houston, TX; M C Jordan, K P Ross, Univ of California, Los Angeles, Los Angeles, CA; R J White, Univ of Glasgow, Glasgow, United Kingdom; M B Schneider, Baylor College of Medicine, Houston, TX; W R MacLeilian; Univ of California, Los Angeles, Los Angeles, CA

Normally, cell cycle progression is tightly coupled to the accumulation of cell mass (cell growth). The molecular mechanisms that regulate hypertrophic growth and the mechanisms whereby proliferation and cell growth are coupled are poorly understood. We have identified Cyclin D2 (CycD2), a G1 cyclin implicated in mediating S phase entry, as a potential regulator whereby proliferation and cell growth are coupled are poorly understood. We have identified Cyclin D2 (CycD2), a G1 cyclin implicated in mediating S phase entry, as a potential regulator whereby proliferation and cell growth are coupled.

**Cyclooxygenase-2 by Atorvastatin Leads to Reduced Expression of Proinflammatory Mediators and Attenuation of Hypertrophic Growth**

Angus C Nairn, The Rockefeller Univ, New York, NY; Jeffery D Molkentin; Cincinnati Children’s Hosp Med Ctr, Cincinnati, OH

Hypertrophic growth in response to transverse aortic constriction (TAC) was attenuated in CycD2-/- mice compared to wildtype mice (5.45 ± 0.29 mg/g CycD2-/- versus 8.41 ± 0.14 mg/g CycD2+/+; P < 0.05). Blocking the increase in CycD2 with siRNA in response to hypertrophic agonists prevented phosphorylation and inactivation of CycD2-target Rb in vitro. When mice deficient for Rb (Crbl-/-) were subjected to TAC, hypertrophic growth was potentiated (5.23 ± 0.3 mg/g Crbl-/- versus 6.44 ± 0.11 mg/g Crbl+/+; P < 0.05). Hypertrophic growth requires protein synthesis and transcription of RNA genes by RNA polymerase III with increased sensitivity to hypertrophic signals. This load-induced increase in RNA pol III activity is augmented in Rb-deficient hearts, consistent with the established role of Rb as a direct repressor of the B-H1 subunit of RNA pol III-specific transcription factor B (TFIB). We performed co-immunoprecipitation studies, which showed that Rb binds to B-H1 in basal conditions but this association is disrupted in response to a hypertrophic stimulus. These investigations identify an essential role for the CycD2-Rb pathway as a governor of cardiac myocyte enlargement in response to biomechanical stress and, more fundamentally, as a regulator of the load-induced activation of RNA pol III.

**The Induction of Cyclooxygenase-2 by Pioglitazone Is eNOS- and iNOS-Independent, Whereas Both Are Essential for the Induction of Cyclooxygenase-2 by Atorvastatin**

Yochai Birnbaum, Yumei Ye, Yu Lin, Ming-He Huang, Charles Y Lui, Regino J Perez-Polo; Univ of Texas Med Branch, Galveston, TX

Background: Three-day treatment with oral atorvastatin (ATV) increases the expression and activity of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) in the adult rat heart in vivo. Although ATV increases COX2 expression in the eNOS” mouse, this COX2 is inactive, because it is not S-nitrosylated. On the other hand, ATV fails to increase COX2 expression and activity in the iNOS” mouse. In the rat,...
pioglitazone (PIO) activates COX2 without upregulating eNOS and iNOS. We studied whether PIO upregulates COX2 expression and activity in eNOS- and iNOS-/- mice. Methods: WT, eNOS- and iNOS-/- mice received PIO (10 mg/kg/d) or water alone by oral gavage for 3 days. On the fourth day hearts were harvested and assessed for eNOS, iNOS, and COX mRNA levels (r-PCR) and COX2 activity (ELISA). Results: PIO caused a small increase in eNOS mRNA in the WT and iNOS-/-, but not in the eNOS-/- group of contrast, while the hypothesis that iNOS mRNA decreases by PIO in the WT and iNOS-/- mice. COX2 mRNA levels were significantly increased by PIO in the WT, as well as the eNOS- and iNOS-/- mice. Similarly, COX2 activity was increased by PIO in the WT (13.8±0.33 vs. 3.36±0.12 ml/min/mg; p<0.001), eNOS1 (6.05±0.21 vs. 3.24±0.10 ml/min/mg; p<0.001) and iNOS1 (2.08±0.32 vs. 1.54±0.17 ml/min/mg; p<0.001) mice. PIO caused a small, yet significant decrease in COX1 activity in all three groups of mice. Conclusions: In contrast to the effects of statins, PIO upregulates COX2 expression and activity in an eNOS and iNOS independent mechanism.

A Novel Regulatory Pathway of Bone Morphogenetic Protein Smad-Dependent Signaling by BMP Receptor II and Tribbles3

Mun Chen Chan, Peter Nguyen, Brandi Davis, Sackler Sch of BioMed Sciences, Boston, MA; Keyong Du, Gioro Lagna, New England Med Ctr, Boston, MA; Alkito Hata; Sackler Sch of BioMed Sciences, Boston, MA

Bone Morphogenetic Proteins (BMPs) signaling is crucial in regulating a myriad of biological processes, including cell growth, differentiation, and apoptosis. BMP signal binds the BMP receptor complex, which is made up of two different transmembrane serine/threonine kinases, BMPRI and BMPRII. BMPRII is unique compared to other Type II receptors of the TGFβ superfamily as it contains a long (500 amino acid) tail domain. Little is known about the biological role of this evolutionarily conserved domain, but mutations in this tail domain is linked to Idiopathic Pulmonary Arteriole Hypertension (IPAH). IPAH is characterized by thickening of peripheral arteries caused by uncontrolled division and possible dedifferentiation of vascular Smooth Muscle cells (vSMCs). Here we report that Tribbles-like protein 3(Tbr3) is a binding partner of BMPRIi tail domain (BMPRII_TD). Upon BMP stimulation, Tbr3 associates with BMPRII_TD and facilitate the ubiquitination of Smad2 by Smurf1. Smurf1 is a E3 ubiquitin ligase which targets both BMPRI and Smad1/5, and leads to repression of Smad dependent signaling. Therefore, the degradation of Smurf1 by Tbr3 leads to a stabilization of Smad1/5 targets, and ultimately potentiation of BMP-Smad-dependent signaling. In isolated vSMCs, downregulation of Tbr3 inhibits BMP-mediated cellular responses. This leads to an increase in both proliferation and de-differentiation, both characteristic pathological features of pulmonary arteries from IPAH patients. This work therefore identifies Tbr3 as a critical component in a novel mechanism of regulation of the BMP pathway by BMPRII. Furthermore, this work allows for a better understanding of the molecular mechanism behind PHH, an important step in developing novel strategies of treating this disease.

Regulators of G-Protein Signaling 5 Proteins May Upregulate Expression of Voltage-Gated K+ Channels in Response to Angiotensin II, Therby Inhibiting Angiotensin II-Mediated Arterial Contraction

Ick-Mo Chung, Sch of Medicine, Ewha Womans Univ, Seoul, Republic of Korea; Lawrence D Adams, Jagadambika J Gunaje, Jun Xue, Kanchan Chitaley, Louis F Santana, Stephen M Adams; Jagadambika J Gunaje, Jun Xue, Kanchan Chitaley, Louis F Santana, Stephen M Adams; Jagadambika J Gunaje, Jun Xue, Kanchan Chitaley, Louis F Santana, Stephen M Adams

Regulators of G protein signaling (RGS) proteins inhibits signal transduction of G protein-coupled receptors by increasing GTPase activity. We previously observed differential expression pattern of the G4 substantially in various G-protein coupled receptors: Sprague-Dawley (SD) rats. And, consistent with this observation, angiotensin II (Ang II)-induced contractility was significantly less in the abdominal aorta (AA), expressing RGS 5 transcript 10 folds as much as thoracic aorta (TA), compared with TA. Ang II is known to inhibit voltage gated K+ channels (K+) in vascular smooth muscle cells (SMCs), leading to depolarization and contractions of SMCs. We tested the hypothesis that RGS 5 may regulate K+ thereby regulating Ang II-mediated force generation. Expressions of three K+ (K1, K2, 1.5, and K2.1) transcripts were measured by real-time reverse transcripción Q-PCR in arterial SMCs from SD rats. Expressions of K1 1.5 and K2 1.2 mRNA, normalized to GAPDH, were greater in AA SMCs versus TA_SMCs 2.5 fold for K1 and 5.6 fold for K2.1. Cultured SMCs from SD rats were transfected with pRRES2-EGFP vector expressing either 1) human RGS 5/EGFP or 2) EGFP only, as a negative control, using Human Ad5MSC Nucleofector kit. Successful transfection of human RGS 5 was verified by Western blot assay. Then transfected SMCs were treated with Ang II (100 nM) for various time period (0–24hrs) after serum starvation. Expressions of all K1.2, K1.5, and K2.1 mRNA tend to be increased by Ang II treatment (1–24 hrs), compared with baseline (0 hr), in human RGS 5/EGFP transfected SMCs. SMCs transfected with human RGS 5/EGFP and Ang II-treated for 24 hrs had an increased expressions of transcripts of all K1.2, K1.5, and K2.1 mRNA, compared with SMCs transfected with EGFP only and treated with Ang II for 24 hr. In conclusion, RGS 5 may upregulate transcription expression of K+ in arterial SMCs in response to Ang II, thereby inhibiting Ang II-mediated vascular contraction.

Targeting Focal Adhesion Kinase with siRNA Prevents and Regresses Load-Induced Cardiac Hypertrophy in Mice

Carolina F Clemente, Thais F Tornatore, Thais H Theisen, José Robert M Sousa, Kiebler Franchini; State Univ of Campinas, Campinas, Brazil

Hypertrophy is a critical event in the onset of failure in chronically overloaded hearts. FAK (Focal Adhesion Kinase) has attracted particular attention as a mediator of load and agonists-induced hypertrophic growth of left ventricle (LV). We demonstrated here that myocardial FAK expression and activity increase in the pathological LV hypertrophy. A novel finding was the activity coincident with the LV chamber dilatation and impaired function. To elucidate the role of FAK in the pathophysiology of LV hypertrophy and failure induced by chronic pressure overload we used specific small interference RNA (siRNA). Myocardial FAK expression was reduced to ~20% at one and to ~50% at seven and fifteen days after seven and fifteen days after severe myocardial infarction (MIF) targeted to FAK. Transient FAK silencing prevented as well as reversed the hypertrophy and the fibrosis of chronically overloaded LV, while preserving the chamber function, despite the persistent pressure overload. However, FAK depletion did not influence basal cardiac function or structure in mice that underwent short heart failure. A novel finding was the activity coincident with the LV chamber dilatation and impaired function. To elucidate the role of FAK in the pathophysiology of LV hypertrophy and failure induced by chronic pressure overload we used specific small interference RNA (siRNA). Myocardial FAK expression was reduced to ~20% at one and to ~50% at seven and fifteen days after severe myocardial infarction (MIF) targeted to FAK. Transient FAK silencing prevented as well as reversed the hypertrophy and the fibrosis of chronically overloaded LV, while preserving the chamber function, despite the persistent pressure overload. However, FAK depletion did not influence basal cardiac function or structure in mice that underwent short heart failure. A novel finding was the activity coincident with the LV chamber dilatation and impaired function. To elucidate the role of FAK in the pathophysiology of LV hypertrophy and failure induced by chronic pressure overload we used specific small interference RNA (siRNA). Myocardial FAK expression was reduced to ~20% at one and to ~50% at seven and fifteen days after severe myocardial infarction (MIF) targeted to FAK. Transient FAK silencing prevented as well as reversed the hypertrophy and the fibrosis of chronically overloaded LV, while preserving the chamber function, despite the persistent pressure overload. However, FAK depletion did not influence basal cardiac function or structure in mice that underwent short heart failure.
vivo as a CK2 inhibitor activates TFE-1 dependent promoters. They propose that the TEF-1:CK2 association may regulate transcription by three mechanisms: (1) CK2 phosphorylation directly regulates MCTA-DNA binding by TEF-1; (2) TEF-1 can recruit CK2beta and associated proteins to DNA, regulating the activity of nearby transcription factors or chromatin structure; and (3) TEF-1 can recruit the CK2 holoenzyme to muscle promoters, where it can regulate the activity of other transcription factors that are CK2 targets, such as MEF2C.

Unfolded Protein Response and Hypoxia-Inducible Factor 1α Stability in Primary Culture of Rat Adult Cardiomyocytes
Ekaterina Fomicheva, Terri G Edwards, Joseph M Metzger; University of Michigan, Ann Arbor, MI
It was recently published that activation of the Unfolded Protein Response (UPR) occurs in infarcted mouse hearts and hypoxic cardiac myocytes. Interestingly, it was also noticed that a stabilization of Hypoxia Inducible Factor 1 alpha (HIF-1α) occurs under the same conditions. We hypothesized that UPR might exert an effect on HIF-1α levels in adult rat cardiac myocytes. Methods: We developed a double viral system (DVS) as a tool for tracking HIF-1α abundance in myocytes. The DVS consisted of a sensor and an effector virus: the sensor virus has 6xHIS6E in the promoter and an oxygen dependent domain (ODD) in fusion with the yeast GAL4-p65 protein and activated by the presence of HIF-1α. The effector consisted of six GAL4 binding sites upstream of a minimal viral promoter, and is able to activate signal by the binding of the GAL4-ODD-p65 protein to activate the reporter luciferase (Luc) gene. Results: We transduced freshly isolated myocytes with the sensor 6xHRE-Mp-GAL4-p65 and the 6xHUSB effector virus. Virus encoding Renilla luc was used as an internal control and CMV-Luc virus was used as a control for adenoviral efficiency. HIF-1α was induced by exposure to a gas mixture (0.5% O2, 5% CO2, 95% N2) compared to normoxia (95% air and 5% O2). Under normoxic conditions, two different treatments were applied after 24 hours following viral transduction; calcium ionophore A23817 was added to the medium to induce UPR and DMOG was used to inhibit prolyl hydroxylase activity. After the next 24 hours, myocytes were lysed and Dual Luc assay was performed (firefly Luc activity was normalized to Renilla Luc). Dual Luc assays (n=8–9) displayed the highest amplification of expression for group under hypoxia 1.18x101±1.2x102 RLU vs normaloxygen 1.81±0.2 RLU (P<0.0001). An elevated level of expression was observed in myocytes treated with A23817 3.38x103±8.6x102 and with DMOG 3.80x103±3.3x102 RLU compared to myocytes kept in normoxia 1.81±0.2 RLU (P<0.0001). The expression in myocytes transduced with CMV-Luc 9.31x101±1.6x100 RLU was also elevated compared to normoxia 1.81±0.2 RLU (P<0.0001). Conclusions: Our data showed UPR has an effect on HIF-1α abundance in adult rat myocytes. These results indicate that UPR and HIF-1α may contribute to preserving the myocardium during hypoxic stress.

Gender-Specific Role of Transcription Factor NF-κB in Left Ventricular Remodeling on Pressure Overload
Christina Gehre[, Laura Zelaraya, Claudia Noack, Anke Renger, Rainer Dietz, Claus Scheidereit, Martin W Bergmann; Max-Delbrueck-Cntr, Berlin, Germany
We have shown that in vivo cardiac specific inhibition of nuclear factor k B (NFκB) leads to an attenuation of other transcription factors that are CK2 targets, such as MEF2C.

Effects of Graded Levels of Thyroid Activity on Cardiac Function and Arteriolar Density in Rats
Yingheng Liu, Rebecca A Redetzk, Suleman Said, Cardiovascular Inst, Sioux Falls, SD; Gabriella M de Escobar, Instituto de Investigaciones Biomedicas Alberto Sols, Madrid, Spain; A Martin Gerdes, Cardiovascular Inst, Sioux Falls, SD
The link between thyroid dysfunction and cardiovascular diseases has been recognized for more than a hundred years. While overt hypothyroidism leads to impaired cardiac function and possibly heart failure, the cardiovascular consequences of borderline low thyroid function are not clear. Establishment of a suitable animal model would be helpful. In this study, we characterized a rat model to study the relationship between cardiovascular function and graded levels of thyroid activity. We used rats with surgical thyroidecemy and subcutaneous implantation of various T4 doses (100μg, 30μg, 10μg, 3μg, 1.5μg, 0μg) and 65μg/100g/d) for three weeks. In terminal experiments, cardiac function was evaluated by echocardiography and hemodynamics. Myocardial arteriolar density was also quantified morphometrically. Thyroid hormone levels in serum and heart tissue were determined by RIA assay and cardiac histology alone, and/or low thyroid conditions. Results: We suggest that gender-specific functional changes may be helpful in decisions regarding treatment of borderline thyroid conditions.

Activation/Phosphorylation of Protective Protein Kinase PKCε Attenuates the Degradation of This Kinase: A Novel Mechanism for Cardioprotection
Aladin V Gomes, Ming Lu, Chengong Zong, Beniam Berhan, Dawn Pantaleon, Xing Qiao, Guangbin Wu, Wang, Zhen Li, Peijei Ping; UCLA, Los Angeles, CA
Recent investigations suggest an important role of new protein synthesis in the manifestation of a cardioprotective phenotype. However, very little is known regarding whether protein degradation plays a role in cardioprotection, and if so, what specific mechanisms are involved in this process. To address this issue, we conducted experiments to comprehensively examine the role of the ubiquitin-proteasome system in modulating degradation of a previously well-characterized cardioprotective protein, protein kinase Cε (PKCε). The role of PKCε degradation was found to be tightly coupled with its ubiquitination and was significantly inhibited by three structurally independent inhibitors of the proteasome system, epoxomicin, clasto-lactacystin β-lactone and MG132. Selective activation of PKCε in cardiac cells enhanced PKCε phosphorylation and reduced PKCε protein degradation, demonstrating that phosphorylation/activation of PKCε renders this kinase less susceptible to degradation. This effect of phosphorylation on proteasome dependent degradation was also confirmed by phosphorylation site mutagenesis studies on PKCε. The half-life of PKCε T566E and PKCε T566A (a mutation which prevents phosphorylation of this residue) in Cos7 cells was determined by pulse-chase experiments to be ~11 and ~8 hours respectively. Furthermore, cardiac tissues from PKCε transgenic mice exhibiting a cardioprotective phenotype showed increased PKCε activity, elevated PKCε phosphorylation, decreased PKCε ubiquitination, and reduced PKCε degradation compared with those in wild type control mice. Taken together, these data demonstrate (i) that the ubiquitin-proteasome system modulates PKCε degradation in cardiac cells and (ii) that activation/phosphorylation of PKCε in cardioprotection attenuates the degradation of this kinase, suggesting a possible critical role of the ubiquitin-proteasome system in sustained activation of protective proteins and thereby cardioprotection.

Exclusive Signaling of α1-Adrenergic Receptors via 1 Splice Variant of Phospholipase Cβ1 (b) in Rat Cardiomyocytes
David R Grubb, Huy Huyh, Oliver Vasilevski, Elizabeth A Woodcock; Baker Heart Rech Institute, Melbourne, Australia
Activation of Gq in cardiomyocytes can lead to cardiomyocytes protection, hypertrophy or apoptosis, depending on the cell type and the signaling pathway. In this study we investigated the role of PLCβ1 splice variants in cardiomyocytes protection. PLCβ1 splice variants in cardiomyocytes include PLCβ1a and PLCβ1b, which differ only in their extreme C-terminus. PLCβ1a has a C-terminal PDZ interacting domain, and PLCβ1b has a C-terminal proline rich sequence, suggesting the possibility of differential localisation and thus different function. We addressed the question of the role of PLCβ1 splice variants in cardiomyocytes protection using a rat model of myocardial infarction. We found that both PLCβ1a and PLCβ1b containing a nuclear localisation sequence, and localizing to nuclei in non-excising cells, was not observed in NRVM. GPP-PLCβ1a was also excluded from the nucleus, and localised to the cytosol. An Western blotting showed that PLCβ1b was pre-coupled with Gαq, neither PLCβ1a nor PLCβ1b was found to be pre-coupled with Gq, demonstrating an unexpected level of specificity in downstream Gα signaling. As RNAi was not successful in knocking down PLCβ1 expression, myristilated-peptides correponding to the C-terminal regions of PLCβ1a and PLCβ1b were used. When added to NRVM,
the PLCβ1β C-terminal peptide eliminated the PLC response to α1-AR agonists, and changed the rapid dissociation of PLCβ1β from the sarcolemma into the cytoplasm. The PLCβ1β C-terminal peptide did not affect PLCβ1α or PLCβ3. The PLCβ1α C-terminal peptide had no effect on the α1-AR agonist response or PLCβ1β, PLCβ1α or PLCβ3 intracellular localisation. This implies that a sarcolemmal protein specifically binds the unique C-terminal region of PLCβ1β. Such a protein could target PLCβ1β to the sarcolemma and to the caveolae where it associates with Gβγ5. This provides the potential to selectively inhibit PLC activation via Gq in heart by inhibiting the sarcolemmal targeting of PLCβ1β.

SERCA2α Overexpression Induces eNOS Activation in Human Coronary Artery Endothelial Cells

Lahouaria Hadri, Djamel Leboche, Yoshiaki Kawase, Mount Sinai Sch of Medicine, NYU, New York, NY; Richard Pelosi, Targeted Genomics, Seattle, WA; Krizstina Zsebo, Celladon, San Diego, CA; Anne-Marie Lompre, CHU Pitié-Salpêtrière, INSERM U821, Paris, France; Roger J Hajar; Mount Sinai Sch of Medicine, NYU, New York, NY

Endothelial dysfunction, characterized mainly by an impairment of nitric oxide (NO)-dependent vasodilatation, plays an important role in the pathophysiology of coronary artery disease. Recently, we have shown that adenoviral overexpression of SERCA2α increases coronary flow in a rat model of diabetic cardiomyopathy. However, the importance of SERCA2α therapy and the mechanisms by which it affects the regulation of vascular tone and NO production in endothelial cells is currently unknown. The aim of this study is to investigate the role of SERCA2α gene transfer in the regulation of eNOS expression and activity, NO production and proliferation of cultured human coronary artery endothelial cells (HCAEC). To confer long-term stable expression adeno-associated-virus (AAV-1) vector was used. HCAEC were infected with AAV1- β-1-galactosidase (as a control) or AAV1-human SERCA2α for 7 days. The eNOS expression was then analyzed by RT-PCR and Western-blot and activation was monitored in HCAEC-infected cells by measuring eNOS phosphorylation at Ser 1177. The proliferation of HCAEC was performed with colorimetric immunoassay for the quantification of cell proliferation, based on measurement of BrdU incorporation during DNA synthesis. Control non-infected cells or cells infected with AAV1- β-1-galactosidase expressed low levels of eNOS, however, infection with AAV1-human SERCA2α exhibited a significant increase in eNOS protein and mRNA levels. Immunoblot analysis also showed that stimulation with histamine (1μM, 10 min) resulted in a robust increase in phosphorylated eNOS at Ser 1177 with AAV1-human SERCA2α compared to control cells. eNOS-Ser 1177 phosphorylation is calcium dependent. HCAEC, infected with AAV1-SERCA2α for 7 days, and then infected with an adenovirus overexpressing parvalbumin, a protein that buffers intracellular calcium ions, for 24 hours, partially decreased eNOS phosphorylation. In addition, overexpression of SERCA2α did not inhibit HACEC proliferation compared to the control cells. Since SERCA2α overexpression leads to an increase in the reticulum endoplasmic calcium load, our data suggest that SERCA2α may be involved in the modulation of eNOS expression and activity through regulation of temporal kinetics of Ca2+ transient.

The Calcium Binding Protein Calmyrin Modifies the Myocardial Stress Response in Vivo

Joerg Heineke, Jian Xu, Michelle Sargent, Allen York, Jeffery D Molkentin; Children's Hosp Med Ctr, Cincinnati, OH

We identified the gene calmyrin as being upregulated in cardiomyocytes undergoing hypertrophy. Calmyrin is a 22kDa Ca2+-inmyosin switch protein, which changes conformation and translocates to the membrane upon Ca2+ binding. Calmyrin is highly expressed in the neonatal mouse heart, and in the adult myocardium its expression and localization to the sarcolemma are upregulated by pressure overload stimulation. However, the functional role of calmyrin in the heart is completely unknown. Here, we generated cardiomyocyte-specific transgenic mice that utilize an inducible tetracycline-regulated α-Myc heavy chain promoter system to determine the gain-of-function effects of calmyrin in the heart. We obtained 4 independent lines of calmyrin inducible transgenic mice (Tg). We focused our analysis on lines 26.6 and 27.5, which did not exhibit any overt phenotypes. Adult onset calmyrin overexpression did not affect cardiac structure or function at baseline. Interestingly, however, during pressure overload the transgenic mice developed an exaggerated hypertrophic response (HW/BW: 27.5, which had a robust myocardial overexpression of calmyrin, was protected against ischemia/reperfusion injury and had a significantly smaller infarct (0.16 vs. controls 6.6 0.16 p = 0.001). Furthermore, the TG1a C-terminal peptide had no effect on the α1-AR agonist response or PLCβ1β, PLCβ1α or PLCβ3 intracellular localisation. This implies that a sarcolemmal protein specifically binds the unique C-terminal region of PLCβ1β. Such a protein could target PLCβ1β to the sarcolemma and to the caveolae where it associates with Gβγ5. This provides the potential to selectively inhibit PLC activation via Gq in heart by inhibiting the sarcolemmal targeting of PLCβ1β.

GATA4 Prevents Death in Cardiac Myocytes Lacking α1-Adrenergic Receptors but Is Not Required for α1A-Mediated Survival Signaling

Yuan Huang, Sanford Rech/USD, Sioux Falls, SD; Megan Egetheng, Sanford Sch of Medicine/USD, Vermillion, SD; Casey D Wright, Chastity L Merkwan, Quan-hai Chen, Nicole L Baye, Qiangrong Liang, Timothy D O’Connell; Sanford Rech/USD, Sioux Falls, SD

Recently, we demonstrated a direct protective effect of the α1A-adrenergic receptor subtype (α1A-AR) in cardiac myocytes and defined an α1A-ERK signaling pathway that is required for myocyte survival signaling. Previous studies suggested that the cardiac-specific transcription factor GATA4 is activated by ERK and mediates survival signaling in cardiac myocytes. Here, we examined GATA4 as a downstream regulator of α1A-ERK survival signaling in cardiac myocytes. As with our previous studies, we used cultured α1BKO cardiac myocytes, which lack α1-ARs and are susceptible to cell death induced by a variety of stimuli, including norepinephrine (NE), doxorubicin and H2O2. In the current study, myocyte death was induced by 1 μM (NE) and measured by annexin V/propidium iodide staining. Our results show that overexpression of GATA4 is sufficient to protect α1BKO cardiac myocytes from NE-induced cell death (control, 6.1 ± 0.4, NE, 17.2 ± 0.6%, GATA4 control, 7.8 ± 0.5; GATA4 NE, 11.0 ± 0.4%, n = 4–5, P < 0.05). However, in α1BKO myocytes expressing the α1A-subtype, phenylephrine (PE, α1 agonist) did not increase the phosphorylation of GATA4, although PE increased ERK phosphorylation and a constitutively active MEK-1 (upstream activator of ERK) increased GATA4 phosphorylation. Furthermore, aortic constriction increased GATA4 phosphorylation, which express the α1A-subtype and are protected from NE-induced cell death relative to α1BKO myocytes, we found that siRNA mediated knockdown of GATA4 did not reverse the protective effects of the α1A-subtype in response NE (control siRNA, 6.6 ± 1.2, NE, 8.2 ± 1.6%, G4 siRNA control, 6.9 ± 0.7; NE, 5.6 ± 0.2%, n = 2). The failure of the α1A-subtype to phosphorylate GATA4, the increased phosphorylation of GATA4 following aortic constriction in α1BKO mice, and the failure of GATA4 knockdown to reverse α1A-mediated survival signaling altogether indicate that GATA4 is not required for α1A-mediated survival signaling. In summary, these results demonstrate that whereas overexpression of GATA4 is sufficient to rescue α1BKO myocytes from NE-induced cell death, GATA4 is not required for α1A-mediated survival signaling in cardiac myocytes.

Regulation of Akt/PKB Activity by P21-Activated Kinase in Cardiomyocytes

Satoru Kobayashi, Kai Mao, Sanford Rech/USD, Sioux Falls, SD; Zahara Jaffer, Fox Chase Cancer Cntr, Philadelphia, PA; Zhiwei Huang, Troy Lackey, Sanford Rech/USD, Sioux Falls, SD; Jonathan Chemoff, Fox Chase Cancer Cntr, Philadelphia, PA; Qiangrong Liang; Sanford Rech/USD, Sioux Falls, SD

Akt/PKB is a critical regulator of cardiac function and morphology, and its activity is governed by dual phosphorylation at active loop (Thr308) by phosphoinositide-dependent protein kinase-1 (PDK1) and at carboxyl-terminal hydrophobic motif (Ser473) by a putative PDK2. P21-activated kinase-1 (Pak1) is a serine/threonine protein kinase implicated in cardiac hypertrophy, and was shown previously to activate Akt through an undefined mechanism. Here we report Pak1 as a potential PDK2 that is essential for Akt activity in cardiomyocytes. Both Pak1 and Akt can be activated by multiple hypertrophic stimuli or growth factors in a phosphatidylinositol-3-kinase (PI3K)-dependent manner. Pak1 overexpression induces Akt phosphorylation at both Ser473 and Thr308 in cultured cardiomyocytes and in transgenic heart. Pak1 and Akt can be activated by multiple hypertrophic stimuli or growth factors in a phosphatidylinositol-3-kinase (PI3K)-dependent manner. Pak1 overexpression induces Akt phosphorylation at both Ser473 and Thr308 in cultured cardiomyocytes and in transgenic heart. Conversely, silencing or inactivating Pak1 gene diminishes Akt phosphorylation in cultured cardiomyocytes and in gene targeted mouse heart. Purified Pak1 can directly phosphorylate Akt at Ser473 but not at Thr308 (Figure), suggesting that Pak1 may be a relevant PDK2 responsible for Akt Ser473 phosphorylation in cardiomyocytes. Our results connect two important regulators of cellular physiological functions and provide a potential mechanism for Pak1 signaling in cardiomyocytes.

Plant Estrogens in the Rodent Diet Negatively Impact Genetic Heart Disease in Males

John P Khoshla, Elizabeth D Luczk, Tom H Cheung, Univ of Colorado at Boulder, Boulder, CO; Brian L Stauffer, Univ of Colorado Health Sciences Cntr, Denver, CO; Leslie A Leinwand; Univ of Colorado at Boulder, Boulder, CO

The perception that soy food products and dietary supplements will lead to healthy hearts has been recently challenged by our demonstration that cardiac disease in male mice with hypertrophic cardiomyopathy (HCM) is worsened by the traditional soy-based rodent chow.
compared to a milk-based diet. We provide evidence here that the dietary phytoestrogens, genistein and daidzein (in the soy-based chow) are sufficient to cause the poor phenotypes seen in soy-fed HCM male mice including ventricular dysfunction, myocardial fibrosis, induction of beta-myosin heavy chain, and activation of caspase-3. We show that soy and phytoestrogens differentially activate genes in males compared to females. We also show that diet has the greatest impact on the genetic landscape of the healthy heart, more so than gender or HCM alone, and that this dietary effect is magnified by disease.

Different Routes of Stem Cell Delivery: Which One Is the Preferred Way?
Hussein Rayatzadeh, Mohammad H Mandegar; Dept of Cardiovascular Surgery, Shariati Hosp, Tehran Univ of Medical Sciences, Tehran, Iran (Islamic Republic of)

Background: Cardiovascular repair and tissue engineering has emerged as one way for treatment of end-stage heart failure. However, to date it is not clearly defined which one is the preferred route for stem cell delivery. Methods: We briefly reviewed the trials that had applied different routes for the stem cell (SC) delivery into the injured cardiac muscle. Results: There are different routes for SC delivery into cardiac muscle including intravenous, transcoronary, transcendocardial, and transepigastric injections. The best for administering SCs highly depends on the patients’ revascularization plan; whether they are going to undergo PTCA, CABG and even medical therapy alone. Additionally it is necessary to notify that beside some advantages all the routes have their specific limitations. For instance, perfect transcendocardial injection is impossible unless the NOGA mapping is available; consequently in centers that are not equipped with this technology, like many third-world countries, it is limited to utilize. Also, larger cells like mesenchymal SCs and skeletal myoblasts are not suitable candidate for intravenous injection because a large number of them are trapped into capillary systems. Conclusion: Transcendocardial injection of SCs is the most common route to date. Trials applying intracoronary injection of SCs, during CABG are rare and include low number of patients. However, it seems that as a result of the better access to most of infracted regions and direct vision of the surgeon, it is suitable for patients undergoing CABG. Taken together, it seems that a large-scale study is inevitable to compare different routes of SC delivery and discover the best.

Synthetic Small Molecules that Initiate Cardiac Lineage Commitment and Enhance the Cardiogenic Potential of Human Peripheral Blood Stem/Progenitor Cells
Hesham Sadek, Jenny Hishe, Daniel Garry, Eric Olson, Jay W Schneider; UTSW, Dallas, TX

New strategies are needed to enhance the effectiveness of hematopoietic stem/progenitor cells (HSCs) in the repair of damaged myocardium, and to discover the molecular and cellular mechanisms underlying cardiac regeneration. We have taken a small molecule approach by screening a synthetic organic chemical library for pharmacologic activators of Notch2, a signature gene of cardiac fate, using a luciferase knock-in bacterial artificial chromosome (BAC) integrated into the genome of mouse P19Cl6 pluripotent stem cells and transgenic mice. The screen yielded a promising collection of structurally diverse and chemically interesting small-molecule activators of early cardiac lineage genes, like Nkx2.5 and myocardin. Here, we describe small-molecules that could serve as a drug library for the identification of small-molecule activators of myocardial regeneration. An enrichment was identified in small molecules that bound to Sh2b, a Sh2 family Sh2 protein that regulates myogenic identity. In human HSCs, Shzs could increase BMP4 mRNA and protein expression in embryonic stem and adult stem/progenitor cells, including granulocyte colony stimulating factor (GCSF)-mobilized circulating human HSCs. Transplanted into experimentally cryo-injured rat myocardium, Shz-primed human HSCs restored contractile function to normal, correlating with persistence of human cardiac myocytes and proteins in cells of the chimeric myocardium, up to one month after injection. Shz small-molecules have also been successfully injected directly into rat myocardium. We conclude that small-molecules like Shzs, identified in a stem cell-based high throughput chemical screen, might be the starting points for potential new drugs to enhance the repair of damaged myocardium by pharmacologically regulating the cardiac gene and cell fate program in human HSCs.

Local Delivery of Statin with Biodegradable Polymeric Nanoparticle Improves Therapeutic Efficacy of Ischemic Neovascularization
Mitsuaki Kubo, Kiyushu Univ, Fukuoka, Japan; Kensuke Egashira, Shin-ichiro Oda, Kiyushu Univ, Fukuoka, Japan; Yoshiaki Kawashima, Aichi Gakui Univ, Aichi, Japan; Hiyori Kyoko; Tsumayama, Kaoi Hara; Hokkaido Powder Technology Rach Institute, Osaka, Japan; Kenji Sunagawa; Kiyushu Univ, Fukuoka, Japan

Background: We have previously shown that human bone marrow-derived mesenchymal precursor cell (hMPC) therapy following myocardial infarction (MI) improves LV dysfunction and morphometric analyses showed that single injection of statin-loaded NPs, but not FITC-loaded NPs or pitavastatin, significantly enhanced recovery of blood perfusion to the ischemic limb and increased capillary and arteriolar density. Neither group of mice showed the signs of rhabdomyolysis, such as an elevation of myoglobin.

Introduction: Mesenchymal stem cells (MSC) represent an attractive cell population for cell transplantation and tissue engineering purpose. Bone marrow-derived MSC (BMC) have special differentiation and immunomodulatory properties and might be available for allogeneic cell therapy. Aim of the present study was to search for mesenchymal stem cells in the neonatal thymus and further characterize the differentiation and immunomodulatory properties of thymic MSC. Methods: From day 2 to 12 neonatal hearts, the thymus gland and the 2 neonates undergoing cardiac surgery was removed. Tissue was minced, digested and cultured. After in vitro isolation and expansion, cells were characterized by FACS. Differentiation cultures for osteogenic, chondrogenic, and adipogen lineage were performed. Additionally, cardiomyocyte-differentiation was examined after 5-azacytidin enrichment. For immunologic investigations, the influence of nTMC on the proliferative behavior of peripheral blood mononuclear cells (PBMC) was studied as a measure of the immune response (mixed lymphocyte culture). In parallel, the expression of immunologically relevant markers (e.g. MHC-I, MHC-II, CD40, CD40L) was measured and correlations between the different sets of results were sought. Results: While the majority of isolated cells were lymphocytes or epithelial cells, we identified adherent stem cells with excellent proliferation potential. Full mesenchymal differentiation potential is maintained during this proliferation phase as confirmed by differentiation cultures. Morphologic characteristics (cytoplastic vacuoles, tropomyosin (+), α-actinin (+), etc) were achieved by differentiation culture. As MSC from other tissues, nTMC lack the expression of MHC-II. With regard to the MSC related surface antigens, the pattern of antigen expression of nTMC is the same as compared to BMC. In contrast to other MSC, some nTMC even lack the expression of MHC-I, in co-culture with allogeneic PBMC, the nTMC do not lead to stimulation. On activated PBMC, the nTMC suppress proliferation. Conclusion: Our results confirm that the neonatal thymus contains mesenchymal stem cells (nTMC) with great differentiation potential and immune modulatory properties.
Critical Role of Muscle Regeneration in Therapeutic Neovascularization
Kazuo Tateno, Junji Moriya, Tohru Minamino, Issei Komuro; Chiba Univ, Chiba, Japan

Therapeutic neovascularization by implantation of mononuclear cells (MNC) is now prevalent as a method to treat patients with critical limb ischemia. It has been believed that MNC contain vascular progenitors and also secrete angiogenic factors, and that the cells thereby induce neovascularization of the recipient ischemic tissue. More recent studies demonstrated the priority of angiogenic growth factors, rather than the processes of post natal vasculogenesis, in the mechanism of the cell therapy. Lately, we reported that angiogenic growth factors secreted from ischemic skeletal muscle cells, but not from the implanted MNC themselves, plays the major role in the therapeutic neovascularization. However, mechanisms by which implanted MNC induce muscle cells to secrete angiogenic factors remained unknown. Here we show the precise mechanism how MNC transplantation induces production of growth factors by skeletal muscle. First, we noticed that myocytes which produced growth factors were regenerating myoblasts, indicating a close relationship between MNC transplantation and skeletal muscle regeneration. To test this in vitro, we co-cultured myoblast cell line C2C12 with MNC and noticed that this strongly induced myoblasts to proliferate, and to produce various kinds of growth factors and cytokines. In vivo, MNC enhanced activation and proliferation of myoblasts, which were accompanied by significant enhancement in the production of angiogenic growth factors. These processes were observed within 24 hours of MNC transplantation, which was prior to the growth of collateral arteries into the ischemic muscle. After 9 days, animals treated with MNC showed increased number of regenerating myoblasts, enhanced production of growth factors, and marked development of collateral arteries that supplied blood flow into ischemic muscle. These effects by MNC were abolished in the aged mice, muscle dystrophy mice, in which muscle regeneration was greatly compromised. These results indicate that MNC transplantation directly enhances muscle regeneration by inducing myoblast activation and proliferation, and that this process is inevitable for the mechanism of therapeutic neovascularization.

Transendocardial Catheter Delivery of Biopolymer Matrices for Enhanced Cell Retention in Cardiovascular Cell-Based Therapy
Didier B Rouy, Aaron Miller, Daniel Rosenman, Beto Peliks, Olin Palmer, Federico Gutierrez, Peter A Altman; Biocarida inc, South San Francisco, CA

Background Cardiac regeneration strategies using stem cells have repeatedly had inefficient cell retention in the target zone. Biopolymers can enhance local cell retention by two theoretical means: First, the entrapment of cells within an in-situ cross linked or gelled biopolymer matrix providing a matrix to support tissue integration. Secondly, by acting as a sealant to the lymphatic and venous drainage routes by which cells can be flushed away from the targeted delivery site. However, life threatening embolic events can be caused by release of cell aggregates, cells suspended in viscous biopolymer gel, or controlled release biotherapeutic formulations within the left ventricular chamber during transendocardial delivery. This safety issue has not been described or addressed previously. Methods and results A new tri-lumen bipolar helical needle transendocardial catheter system has been developed which enables the delivery of advanced biotherapeutic strategies for myocardial regeneration and repair, and presents a number of design features to address embolic safety concerns. This catheter has been built on the footprint of a helical needle catheter currently in use in clinical trials. Commercially available two part biopolymers have been delivered through the catheter at various flow rates and volumes in vitro, and into swine myocardium ex-vivo. Transendo- cardiac injection was shown to be feasible, with acceptable generated pressures. The critical issue of potential leakage into the ventricular chamber was assessed with no back leak observed. This delivery system provides a means to confirm engagement to the tissue before delivery, assesses myocardial venous and lymphatic drainage at the selected delivery site prior to delivery, and inhibit back-leak of delivered agents. Theoretical considerations suggest that optimal mixing of two part biopolymers in tissue can be achieved with viscosity matching. Conclusions Catheter based tissue matrix delivery approaches have significant potential as safe and effective tools providing broad utility for cardiovascular tissue engineering strategies.
(cTNT) and GATA4 positive) distributed across the scar in both CT and TG mice. In addition, we identified small cTNT negative/ GATA4 positive cells in the innermost layer of the scar and throughout left ventricle, interpreted as cardiac resident progenitor cells. Interestingly, the beta-cad delta ex3-6/ROSA26 mice showed galactosidase-positive cells (indicating that beta-catenin is regulated), which were also GATA4 positive/cTNT negative at similar locations of the heart (as mentioned above). Quantification of GATA4 positive/cTNT negative cells in the scar (17.17 ± 1.98 vs CT 13.30 ± 1.5% of DAPI cells) showed no significant differences. We speculate that cardiac-resident progenitor cell growth account for the functional improvement. We suggest that the downregulation of beta-catenin improves LV-remodeling after cardiac ischemia, possibly by modifying cardiac beta-catenin signaling. Since the inhibition of Wnt/beta-catenin pathway is essential for cardiogenesis, we suggest that a re-activation of this pathway could play beneficial role in the healing process after MI.

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STAT3-Dependent Cross-Talk Signaling Mechanism of LIF and BMP-2 in Mouse Embryonic Stem Cell Differentiation into Cardiomyocytes and Its Therapeutic Efficacy in Mouse Model of Myocardial Infarction

Johnson Rajasiging, Hiromichi Hamada, Gangjian Qin, Douglas W Losordo, Raj Kishore; Northwestern Univ, Chicago, IL

Background: Pluripotent stem cell therapy may be an attractive source for post-infarction myocardial repair and regeneration. However, the specific stimuli and signal pathways that may control embryonic stem (ES) cell-mediated cardiomyogenesis remains to be completely defined. The aim of the present study was to investigate (a) the effect and underlying signal transduction pathways of leukemia inhibitory factor (LIF) and bone-morphogenic protein-2 (BMP-2)-induced mouse ES cell (mES-D3 line) differentiation into cardiomyocytes (CMC) and (b) the efficacy of mES cells, pre-treated with LIF + BMP2, for functional and anatomical cardiac repair in surgically induced mouse acute myocardial infarction (AMI) model. Methods and Results: Various doses of LIF and BMP-2 and their inhibitors or blocking antibodies were tested for mES differentiation to CMC, in vitro. CMC differentiation was assessed by mRNA and protein expression of CMC-specific markers, Connexin-43, CT, CTT, Tnnt2, Nkx2.5, GATA-4 and eMHC. LIF and BMP-2 synergistically induced the expression of CMC markers as early as 4 days in culture. Signaling studies identified STAT3 and MAP kinase (ERK1/2) as specific signaling components of LIF + BMP2 mediated CMC differentiation. Inhibition of either STAT3 or MAPK by specific inhibitors dramatically suppressed LIF + BMP2 mediated CMC differentiation. Moreover, in mouse AMI model, transplantation of lentivirus-GFP-transduced, LIF + BMP2 pre-treated mES cells, improved post-MI left ventricular functions and enhanced capillary density. Transplanted cells engrafted in the injured myocardium and differentiated into CMC and endothelial cells. Conclusion: Our data suggest that LIF and BMP-2 may synergistically enhance CMC differentiation of transplanted stem cells. Thus the LIF/BMP2 cocktail or the augmentation of their downstream signaling components may facilitate the effects of stem cell based therapy for post-MI myocardial repair and regeneration.

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Dedifferentiation and Epigenetic Reprogramming of Somatic Cells into Pluripotent Stem-like Cells and Its Therapeutic Efficacy in Mouse Model of Acute Myocardial Ischemia

Johnson Rajasiging, Hiromichi Hamada, Gangjian Qin, Douglas W Losordo, Raj Kishore; Northwestern Univ, Chicago, IL

Background: De-differentiation or reverse lineage-commitment of adult somatic cells might provide an attractive, oocyte-independent alternate source for therapeutic cloning to generate pluripotent, autologous stem cells for regenerative therapy. We tested the hypothesis that exposure of mES (embryonic stem cell) or mESC (mesenchymal stem cell) to mesenchymal stimuli may reprogram the somatic cells to differentiate into multiple cell types, which may potentially impact the clinical efficacy of cardiac cell-based therapy.

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In Vivo Tracking of Stem Cell Distribution Following Intracoronary Delivery in the Setting of Myocardial Infarction

Hung G Ly, Kozo Hoshino, Irina Pomerantseva, Ryuichi Yoneyama, Yoshiaki Kawase, Yoshiaki Takewa, Jacqueline L Buros, Massachusetts General Hosp, Boston, MA; Alec DeGrand, John V Frangioni, Beth Israel Deaconess Med Ctr, Boston, MA; Roger J Hajjar; Massachusetts General Hosp, Boston, MA

Background: Cell-based therapy for ischemic heart disease has emerged as a promising novel therapeutic strategy. Little is known about the fate of different populations of stem cells (SC) delivered by the intracoronary (IC) route. Methods: Using a clinically-relevant swine model (a 60-minute balloon occlusion MI model) and near-infrared fluorescence to provide high sensitivity in vivo tracking of SC after IC delivery, the intramyocardial distribution patterns of mesenchymal (MSC), bone marrow-mono-nuclear (BM-MNC) and peripheral blood-mono-nuclear (PB-MNC) SC were quantified in the presence and absence of myocardial injury. Quantification consisted of fluorescence signal intensity (SI) analysis. Results: Major findings from comparisons were (Figure): (1) within-cell groups: whereas all 3 cell fractions maintained optimal SI in non-infarcted hearts, only SI from MSC remained unaltered in infarcted hearts; (2) between-cell groups: in normal hearts, non-significant differences in terms of SI were found among all cell fractions; but in infarcted hearts MSC, SI not only remained elevated but were significantly different relative to the other 2 cell fractions; (3) differences were maintained throughout the 60-min period. Conclusion: This study provides the first in vivo evidence of different intramyocardial distribution patterns of SC delivered by IC route following an MI. These findings suggest that stem cell distribution during the first 60 min post-injection could potentially impact the clinical efficacy of cardiac cell-based therapy.
Influence of Redox Processes in the Progression of Aortic Valve Calcification

Marcel Liberman, Marina K Martiattini, Estevao Bassi, Fabio C Lario, Carlos E Rochitte, Francisco R Laurindo; Heart Inst (InCor), Univ of Sao Paulo, Sao Paulo, Brazil

Pathogenesis of degenerative aortic valve (AV) stenosis is analogous to atherosclerosis, for which oxidative stress is relevant. We hypothesize that oxidative stress contributes to progression of AV calcification/stenosis. In a rabbit AV calcification model, we investigated topography of reactive oxygen species (ROS) production and effects of antioxidants tempol and lipoic acid in calcification progression. Male rabbits were fed Vit.D3 10iU/kg/day plus tempol or lipoic acid. Heart valves were retrieved at 0.5% for 12 weeks in the absence (HCD, n=34) or presence tempol 100 μmol/kg/day (HCDT, n=15) or lipoic acid 120 μmol/kg/day (HCDL, n=11). HCD rats demonstrated macrophage infiltration and calcification. Superoxide and H2O2, detected respectively by hydroethidine or dichlorofluorescin microtopography, and 3-nitrotyrosine immunoreactivity, were increased not only in inflammatory cells but preferentially around calcifying nuclei. Tempol increased, while lipoic acid decreased ROS. Tempol abolished calcification of vascular cells in vitro. HCD rats >5 years (n=15) or lipoic acid 120 μmol/kg/day (HCDL, n=11) or tempol 100 μmol/kg/day (HCDT, n=15) were used. Increased oxidative stress was observed in aortic plaques of young individuals (n=15), elderly with valve sclerosis (n=6), and authopsied young individuals with normal valve (n=5). We found analogous increases in ROS production and protein expression around calcifying nuclei. An in vitro model of vascular smooth muscle cell calcification showed increased Ca2+ deposit with tempol but not with lipoic acid. Thus, ROS production is markedly increased around calcifying nuclei in human or experimental AV calcification, associated with at least Nox2 and Nox4 expression. However, the specific ROS involved may possibly dictate the effect in calcification progression.

The Heme Oxygenase System Protects Cardiac Tissue by Suppressing Angiotensin II

Joseph Fomusi Ndiang, Nina Lane, Ashok Jadhav; Univ of Saskatchewan College of Medicine, Saskatoon, Canada

Upregulating the heme oxygenase (HO) system with HO inducers like hemin removes prooxidant heme and thus cytoprotective. Importantly, the products from the HO pathway including carbon monoxide, bilirubin and biliverdin scavenge reactive oxygen species, inhibit lipid peroxidation and suppress tissue inflammation, while the iron formed enhances the synthesis of the antioxidant, ferritin. Here we report the enduring cardioprotection by hemin (15 mg/kg p.o. daily for 3 wks), that was however, accompanied by elevated anti-oxidant capacity (1.74 ± 0.01), as well as blood pressure (202.2 ± 1.8 vs. 136.5 ± 1.6 mmHg, n = 6, p < 0.01), in adult spontaneously hypertensive rats (SHR). Hemin HO production was accompanied by increased HO activity and cGMP levels. Since angiotensin-II stimulates phospholipase C (PLC), we assayed and detected hemin therapy also reduced cardiac PLC activity (452.7 ± 9.2 vs. 263.1 ± 3.6 mmol/min/mg, n = 6, p < 0.01), and intracellular calcium levels in SHR. Similarly, reduced mRNA expression of the inflammatory transcription factor, NF-kB, that was however, accompanied by elevated anti-oxidant capacity (1.74 ± 0.02 vs. 0.73 ± 0.01 TEAC/mg protein; n = 4 p < 0.01) was detected in hemin-treated SHR. Our findings unveil for the first time the effect of hemin on cardiac angiotensin-II production and level, as well as the cardioprotective potential of hemin (15 mg/kg p.o. daily) in SHRs.

Withdrawn

Isolation and Expansion of Cardiac Precursor Cells from the Neonatal Heart Using c-kit+EGFP Transgenic Mice

N Yvonne Tailini, Kai Su Greene, Michael Craven, James Smith, Aiyson Spearman, Ashley Woods, Andy Ven, Michael Kollkoff; Cornell Univ, Ithaca, NY

Signaling through the receptor tyrosine kinase c-kit plays critical but distinct roles in early migration, differentiation, and self-renewal of numerous cell lineages, including germ cells, hematopoietic stem cells, melanocytes, and mast cells. Recently an Nkx2.5/c-kit+ cell population was isolated from differentiated embryonic stem cells and has bipotential differentiation capacity (Wu SM, et al Cell 2006), but these cells have not been clearly identified in the developing heart. To characterize resident c-kit+ heart cells we developed transgenic mice in which enhanced green fluorescent protein (EGFP) replaces the kit coding sequence within a bacterial artificial chromosome spanning the ckit gene locus. To determine the developmental potential of resident c-kit+ cardiac cells, hearts were removed from postnatal day 0–5 pups, dissociated, and cells collected by fluorescent activated cell sorting. Approximately 85% of EGFP+ cells are nestin positive and include a variety of morphologies ranging from small spherical cells with minimum cytoplasm to large flat cells with long processes. Acutely isolated cells lacked endothelial (CD31 and Von Willebrand Factor), skeletal (myogenin), and hematopoietic (CD34, Sca1, and CD45) cell lineage markers, but expressed markers for smooth muscle (α smooth muscle actin) and cardiac myocytes (Troponin T), determined by colabeling immunohistochemistry. EGFP+ cells expanded and gradually lost EGFP expression, but a portion exhibited spontaneous, rhythmic contractions after 7–10 days in differentiating culture media. Contracting cells displayed spontaneous action potentials and cytosolic Ca2+ transients consistent with previous recordings from embryoid body cardiac cells and embryonic cardiomyocytes, with burst -type activity, a spontaneously depolarizing phase 0, and a variable or nonexistent plateau phase. Action potentials were either reduced in frequency, or abolished by ZD 7288 (100 μM), tetrodotoxin (10 μM), or nifedipine (1 μM), suggesting a role for the cardiac funny current, fast sodium current, and L-type Ca2+ current in these differentiated cells. We conclude neonatal c-kit+ cells include a population of cardiac progenitor cells with expansion capacity and cardiac potential.

Characterization of Circulating Stem/Progenitor Cells During Postnatal Human Development

Stefan Rupp, Masamichi Koyanagi, Masayoshi Iwasaki, Corinna Schütz, Philip Bushoven, Univ of Frankfurt, Frankfurt, Germany; Gonzalez Galvez, G Cossu, Stem Cell Rsch Institute, Milan, Italy; Dietmar Schranz, Pediatric Heart Cntr, Justus-Liebig Univ, Giessen, Germany; Andreas Zeher, Stefanie Dimmelmer; Univ of Frankfurt, Frankfurt, Germany

Endothelial progenitor cells (EPC) - characterised by the co-expression of haematopoietic stem cell markers and endothelial markers - contribute to neovascularization and have been successfully used for cell therapy of ischemia. Recent experimental findings suggest that ischemic stimuli additionally mobilize cells independent from bone marrow. The origin and gene expression of these circulating non-bone marrow-derived cells so far are unclear. One may speculate that tissue resident stem cells and/or vessel-associated cells, such as mesangioblasts may be mobilized after injury. In order to assess phenotype and functional properties of circulating progenitor cells during postnatal cardiac development, we isolated circulating progenitor cells from peripheral blood of children aged between 8 days and 2.5 years (n=8). Isolated mononuclear cells were plated on fibronectin-coated dishes in EBM medium plus supplement. At day 14, isolated cells showed mesenchymal markers CD13 and CD73, endothelial markers CD10, KDR and VE-cadherin, but were negative for haematopoietic marker as shown by FACS analysis and RT-PCR. In contrast, EPC from adults express haematopoietic and endothelial markers but lack the expression of mesenchymal markers. The morphological presentation resembles the profile of vessel associated resident mesangioblasts (CD44+ - CD145- CD34+ - CD29+). Investigating progenitor cells from children shows prominent proliferation (27.6 +/- 1.1 passages, 64.8 +/- 3.6 population doublings) before entering a senescent state. Consistently, these cells display a high telomerase activity (0.58 +/- 0.03). Children derived circulating progenitor cells express several transcription factors such as Isl1 and Nkx2.5, which were recently described as important factors for mesodermal cell determination. These cells differentiate into cardiomyocytes after co-culture with cardiomyocytes. Furthermore, after appropriate stimulation an adipocyte, smooth muscle cell or osteoblast phenotype can be acquired. These data indicate that children derived circulating progenitor cells resemble previously described mesangioblasts, have a high proliferation capacity and are multipotential. These cells might be suitable for cell therapeutic strategies.

The Contribution of Second Heart Field Cells to the Jaw Muscle Reveals the Multipotential Nature of the Cardio-Craniofacial Mesoderm

Eldad Tzahor; Weizmann Institute of Science, Rehovot, Israel

Recent studies demonstrated that common progenitor populations of mesodermal cells in the head contribute to both cardiac and skeletal muscle lineages, suggesting that developmental multipotency is more pervasive than previously expected. In this study, we utilized several fate-mapping techniques, along with gene expression analyses in chick embryos, to systematically track both cardiac and skeletal muscle precursors. We demonstrate that the cardiac progenitor population, known as the second heart field (SHF), lies between the cranial paraxial mesoderm (CPM) and the lateral splanchnic mesoderm (first heart field or FH) at cardiac crescent stages. During gastrulation, these cells are segregated with the lateral mesoderm, and marked by the expression of Isll, Nkx2.5, Tbx20 and Fgfl0. We further provide cellular and molecular insights into the regionalization of the pharyngeal mesoderm within the branchial arches by both CPM and SHF cells; CPM cells contribute to the proximal region of the mesoderm, whereas SHF cells populate the distal portion of this core. Moreover, our findings reveal distinct developmental programs for CPM and SHF–derived branchiogenic muscles. While both muscle types express Myf5 and MyoD, CPM–derived muscle anlagen (e.g., the Mandibular Adductor complex) expresses Pax7 and SHF–derived muscles (e.g., the Intermandibularis) express Isl1. Furthermore, the late differentiation marker MHC, was significantly delayed in the SHF-derived Isl1+ myoblasts. In addition, ectopic activation of the Wnt5a/catenin pathway resulted in a cardiac looping phenotype, along with inhibition of Nkx2.5 and Isl1 expression in the SHF, suggesting a role for this signaling pathway in the regulation of the second heart field. These experiments provide the first insights into the processes underlying the development of the cardio-craniofacial mesoderm by embryogenesis.
High-Mobility Box 1 Protein Induces Cardiac Stem Cell Activation in a Paracrine Manner

Alessandra Rosso, Cinr Cardiologico Monzino, Milan, Italy; Antonella Zacheo, Istituto Dermopatico dell’Immacolata, Rome, Italy; David Mocini, Ospedale San Filippo Neri, Rome, Italy; Pierangela Totta, Antonio Facchiano, Istituto Dermopatico dell’Immacolata, Rome, Italy; Raffaella Castoldi, Nerviano Med Science, Milan, Italy; Paolo Sordini, Ospedale San Filippo Neri, Rome, Italy; Giulio Pompilio, Cinr Cardiologico Monzino, Milan, Italy; Maurizio Capogrossi, Istituto Dermopatico dell’Immacolata, Rome, Italy; Antonia Germani; Cinr Cardiologico Monzino, Milan, Italy

Introduction. High Mobility Box 1 Protein (HMGB1) is a non-histone chromatin protein which modulates gene transcription. HMGB1 is released into the extracellular space by necrotic cells and activated macrophages in response to injury; under these conditions HMGB1 acts as a cytokine. We recently demonstrated that HMGB-1 administration into the mouse heart during acute myocardial infarction resulted in cardiac tissue regeneration by stimulating proliferation and differentiation of resident cardiac stem cells and significantly improved ventricular function. In the present study we analyzed the mechanism involved in HMGB1-mediated effect on CSCs. Specifically, it was examined the hypothesis that human cardiac fibroblasts (cFbs) exposed to HMGB1 may exert a paracrine effect on CSCs.

Methods and Results. Human cFbs were exposed to HMGB1 (10 ng/ml); the conditioned medium (CM) was collected after 72 hr and analyzed for the presence of growth factors, cytokines and chemokines using a multiplex-27 bead assay and Luminex technology. It was found that HMGB1 enhanced cFbs secretion of VEGF, Mip-1α, IFN-γ, GM-CSF, IL-10, IL-8, IL-6, IL-1ra, IL-9 and TNF-α. CMs obtained from untreated- and HMGB1-treated cFbs were then used to evaluate their effects on CSC function. CSCs were isolated from the non-myocyte population of the mouse and human hearts by magnetic selection for the antigen c-kit. CM from HMGB1-treated cFbs enhanced CSC migration 2 fold (p<0.05) and induced CSC differentiation towards the endothelial lineage as detected by CD31/CD34 immunostaining (23.7%±2.5% vs 14.5%±1.8%, n=6; p<0.01). Finally CM from HMGB1 treated cFbs stimulated CSC proliferation, evaluated by BrdU incorporation assay (6.9%±1.5% vs 2.1%±0.2%; n=3; P<0.05). Importantly, HMGB1 administration directly to CSCs did not affect their proliferation and differentiation into endothelial cells. Conclusions. HMGB1 stimulates growth factor cytokine and chemokine release by cFbs which, in turn, enhance CSC proliferation, migration and differentiation. This paracrine effect could account, at least in part, for the ability of HMGB1 to induce regeneration after myocardial infarction.

Serum Levels of Interleukin-18 After Percutaneous Coronary Intervention Are a Strong Predictor of Adverse Cardiac Events in Patients with Unstable Angina Pectoris

Parangkita Vavoulis, Konstantina Bouki, Dinossios Chagouris, Dimitris Theodoridis, Michael Katsikis, Anna Loukaipouli, Evaggelia Kapsali, Thomas Apostolou, General Hosp of Nikaia, Piraeus, Greece

BACKGROUND - Interleukin-18 (IL-18) is a strong predictor of cardiovascular death in patients (pts) with coronary artery disease (CAD) and is positively correlated with the severity of aging trends included an increase in GAG sulfation (especially 4-sulfation) and DS relative to our previous works demonstrating HSC contribution to other cell populations with fibroblastic properties (kidney mesangial and brain microglial cells), we evaluated fibroblastic phenotype in our work, we find that IL-4 and IL-13 enhanced cPLA2 activity and PGI2 synthesis, which leads to the arginase induction through cAMP and PKA in bCAEC.

Serum levels of IL-18 were then measured in pts undergoing percutaneous coronary intervention (PCI). METHODS: In a prospective study of 158 patients (pts), age 63±12, male 116) with unstable angina pectoris treated by PCI, serum levels of IL-18 and other markers of inflammation [hs-CRP, ESR, fibrinogen (FIB), C-reactive protein (CRP), IL-6, IL-18] were measured. The study was divided into two groups, those that developed MACE during the follow-up period and those that did not. During the follow-up period, 28 pts (20.3%) developed MACE (cardiac death, myocardial infarction or repeat revascularization). The value of IL-18 in predicting MACE was assessed using receiver operating characteristic (ROC) curves. RESULTS: The area under the ROC curve for IL-18 was 0.70 (95% confidence interval 0.58-0.82). The optimal cut-off value was 97 pg/ml (p<0.002), with sensitivity of 2.5% vs 14.5% and a specificity of 97.5% vs 85.5% (p<0.05). Importantly, HMGB1 administration directly to CSCs did not affect their proliferation and differentiation into endothelial cells. Conclusions. HMGB1 stimulates growth factor cytokine and chemokine release by cFbs which, in turn, enhance CSC proliferation, migration and differentiation. This paracrine effect could account, at least in part, for the ability of HMGB1 to induce regeneration after myocardial infarction.

Compositional Changes in Glycosaminoglycan Content with Age in the Heart Valve

Elizabeth H Stephens, Kai Chu, K. Jane Grand-Allen, Rice Univ, Houston, TX

While the need for a tissue-engineered heart valve for treatment of pediatric valve disease is well established, the creation of an age-specific valve has been precluded by limited characterization of pediatric valve composition. As such, the use of young adult heart valves in the treatment of young patients with congenital heart disease and the glycosaminoglycans (GAGs) that are critical to tissue differentiation, structural assembly, and mechanical properties. To this end, we used fluorophore assisted carbohydrate electrophoresis to analyze the concentration and sulfation of the GAGs within various porcine mitral (MV) and aortic valves (AV). The mitral valve anulus was divided into the central clear zone (MCZ) and free edge (MVF). Valves were also Results were compared to findings in immunohistochemically (IHC) stained for chondroitin-4-sulfate (CS), chondroitin-6-sulfate (CS6), hyaluronic acid (HA), the proteoglycans (PGs) versican (VC), biglycan (BGN), and decorin (DC). The MCZ contained higher concentrations of vimentin, contained the highest density of glycosaminoglycans, and was particularly rich in the young valvular fibroblast population. In the mitral valve, the compressive-bending MVF contained more GAGs, particularly the fraction of HA, which increased with age (p<0.01). The AV contained high proportions of CS, particularly COS and HA (p<0.01). Furthermore, the AV contained significantly more GAG content compared to MVAC and MFV, confirming an increase in PS seen by IHC (p<0.006). With age, the AAs showed reduced proportions of COS, CS6, total CS, and HA, but a greater proportion of DC (p<0.005). The increase in DAS suggests an increase in DC and BGN, as found in IHC. General aging trends included an increase in GAG sulfation (especially 4-sulfation) and DS relative to CSCs (CD34+, bone marrow HSC into lethally irradiated congenic mice), we analyzed the contribution of HSCs to the cardiac fibroblast lineage. Methods: To investigate the contribution of circulating cells of bone marrow origin to cardiac fibroblasts, we transplanted mice with a single EGP2+ lineage labeled with a single EGP2+ cell and CD34- bone marrow cells into lethally irradiated congenic mice. This strategy allowed us to differentiate the contribution of HSCs to the cardiac fibroblast population. Methods: To investigate the contribution of circulating cells of bone marrow hematopoietic stem cell (HSC) origin to the adult cardiac fibroblast population, we transplanted cloned animals derived from a single EGP2+ lineage labeled with a single EGP2+ cell and CD34- bone marrow cells into lethally irradiated congenic mice. This strategy allowed us to differentiate the contribution of HSCs to the cardiac fibroblast population. Results: Analysis of cardiac tissues from mice with high levels of multilineage hematopoietic reconstitution revealed numerous EGP2+ cells within the cardiac interstitium and the valve leaflets. Further, in response to myocardial infarction, HSC-derived cells populated the infarct zone at high density compared to the viable myocardium. Based on our analysis of the efficacy of mouse MSCs to work as a source of HSCs to cardiac fibroblasts, we conclude that the potential for use of MSCs in the treatment of cardiac diseases is limited by the proliferation of differentiation of MSCs into fibroblasts. Our results suggest that the potential for use of MSCs in the treatment of cardiac diseases is limited by the proliferation of differentiation of MSCs into fibroblasts.
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**Interruption of Transforming Growth Factor-β Signaling Attenuates Cardiac Remodeling and Fibrosis**

Bryan J Wells, Jason Lucas, Erum Hassan, Rosa Serra, Suzanne Oparil, Yu-Fai Chen, UAB, Birmingham, AL

**Introduction:** Transforming growth factor-β (TGF-β) is a profibrogenic/proliferative factor that is overexpressed in the heart under stressful conditions. Transgenic mice overexpressing TGF-β have cardiac enlargement accompanied by interstitial fibrosis. Pressure overload stress induces myocardial interstitial and/or endocardial fibrosis (TAC) associated with enhanced cardiac expression of TGF-β. **Objective:** To test the hypothesis that interruption of TGF-β signaling attenuates cardiac remodeling and fibrosis in the pressure overload stressed heart. **Methods:** Our experimental model was a novel transgenic mouse that expresses an inducible negative point mutation of the TGF-β type II (Smad2) receptor gene and thus lacks functional TGF-β signaling. Eight to 10 wk old male Smad2−/− and wild type (WT) control mice were given 25 mg ZnSO4 to drink 1 wk prior to TAC to induce the expression of Smad2−/− mice. One wk after TAC, hearts were excised, weighed and processed for histological and immunohistochemical analysis. Quantitative assessment of cell proliferation and/or apoptosis and interstitial collagen content was performed in WT and TAC and immunohistochemical analysis. Using the immunohistochemical analysis, we performed quantitative assessment for cell proliferation and/or apoptosis and interstitial collagen content was performed in WT and TAC groups. **Results:** TAC significantly increased intracellular collagen content (%) in WT mice only (7.4±2.3 vs. 5.1±1.4 for WT and TAC, respectively, p<0.05). TAC increased LV weight equally in all 4. experimental groups. **Conclusion:** TGF-β signaling may result in fibrosis proliferation of non-myocyte cells and increases interstitial collagen content in the mouse heart in response to pressure overload stress. These pro-fibrotic effects of TGF-β were not reflected in LV weight, which likely was dependent on cardiomyocyte hypertrophy.

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**Molecular Tracking of Bone Marrow Progenitor Differentiation in Vitro**

Jeffrey Schmecpecker, Noel Caplice, Univ College Cork, Cork, Ireland, Yasuhiro Ikeda; Mayo Clinic, Rochester, MN

**Background:** While indirect immunohistochemical evidence suggests bone marrow cells home to sites of vascular injury, incorporate to vessel walls and differentiate, this methodology is limited by antibody specificity, signal loss and reactive tissue effects. Lentivectors encoding for identifiable marker gene controlled by lineage specific promoters can be used to track differentiation bone marrow progenitors into endothelial and/or smooth muscle like cells. Methods: Minimal human vascular Cdn2 progenitor and human SmoothMus-B promoter was cloned into a self-inactivating SIN second generation lentiviral vector (VECA3-eGFP and SMTHB-eGFP) and used to drive expression of enhanced green fluorescent protein (eGFP) specifically in endothelial cells and smooth muscle cells respectively in vitro. To demonstrate activation of these promoters in cells differentiating down either an endothelial or smooth muscle lineage, a specific bone marrow progenitor cell (BMPC) subtype was used. BMPCs were treated with the angiogenic growth factors VEGF (25 mg/mL), bFGF (25 mg/mL) or Angiopoetin (200 ng/mL) and evaluated for eGFP expression. When transduced with the VECad-eGFP lentiviral vector, the promoter was initially silent but became activated by angiogenic factor treatment. Treatment with PDGF-BB significantly increased expression of SmoothMus promoter. **Conclusion:** These experiments demonstrate our ability to selectively target cell populations and track their ability to activate marker genes. This may allow for specific expression of eGFP. **Conclusion:** These experiments demonstrate our ability to selectively target cell populations and track their ability to activate marker genes. This may allow for specific expression of eGFP. **Conclusion:** These experiments demonstrate our ability to selectively target cell populations and track their ability to activate marker genes. This may allow for specific expression of eGFP.

**P94**

**Effect of Chronic Insulin Therapy on Mitochondrial Function in an Ex Vivo Animal Model of Diabetes and Hypercaloric Diet Submitted to Global Myocardial Ischemia-Reperfusion**

Pedro Monteiro, M Paiva, R Carreira, A Duarte, L Providencia, Coimbra Univ Hosp, Coimbra, Portugal

**Introduction:** Obesity diabetes have more and worse ischemic heart disease; this may be improved by insulin (INS) therapy. Mitochondria play an important role in cardiac ischemia. If these subjects are treated with INS do they have better mitochondrial function? Aim: To evaluate, in a model of diabetes + hypercaloric diet + ischemia-reperfusion (IR), if chronic therapy with human recombinant insulin improves cardiac mitochondrial function. Material and methods: Ob/Ob (KO) diabetic rats (fed with an hypercaloric diet between 2 and 6 months) were divided in 4 groups (n=15/group): A-DHDK control (no medication) or IND-B-INS control (insulin bid -as needed- between 5 and 6 months) or C-DHDK insulin (as DHDK control and then INS) or D-B-INS (as INS control and then IR). At 6 months, hearts were removed and submitted to 15 min perfusion (control) or 10 min perfusion + 35 min ischemia + 120 min reperfusion (IR). Mitochondrial parameters assessed were: oxidative stress (colorimetric thiobarbituric acid colorimetry test - TBARS), mitochondrial swelling and calcium uptake (fluorimetry). Results: INS-treated rats had significantly lower oxidative stress levels, both in control (0.72±0.01 vs. 0.81±0.04 nmol TBARS/mg protein;p<0.05) and in IR (0.93±0.02 vs. 1.05±0.02 nmol TBARS/mg protein;p<0.05). Figure 1. INS-treated animals also showed a significant decrease in mitochondrial swelling, both in IR (2.1±2.6 vs. 88.1±1.8 arbitrary units; p<0.05) and in control (0.1±0.2 vs. 650±44 arbitrary units; p<0.05). INS therapy induce a significant improvement in calcium uptake in IR (63.0±2.8 vs. 53.9±8.0 nmol/mg protein;p<0.05). Conclusion: In our model of diabetes and IR, INS improves cardiac mitochondrial function, due to less oxidative stress and better ischemia tolerance (higher calcium uptake and lower mitochondrial swelling).

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**Proteomic Analysis of Mitochondrial Proteins in Preconditioned Mouse Myocardium: Effects of Endosomal Trafficking**

Renee P Wong, Elizabeth Murphy; NHBLI, NIH, Bethesda, MD

**The mechanisms by which ischemic preconditioning (PC) decreases tissue injury during a subsequent sustained ischemic insult are yet to be understood. It has been proposed that PC initiates signaling cascades that converge on mitochondrial end-effectors and result in cardioprotection. We have previously observed that inhibition of endosomal recycling by addition of bafilomycin blocks the protection afforded by PC. We propose that endosomal signaling may result in the translocation of signaling proteins to the mitochondria. To test this hypothesis, we utilized two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) to detect differences in protein expression levels and post-translational modifications of mitochondrial fractions from Langendorf mouse hearts preconditioned (+/-/bafilomycin) for 4 cycles of 5 min ischemia and 5 min reflow and time matched control perfused hearts. Using comparative gel analysis, we focused on proteins that were altered (+/-1.5-fold difference in visible abundance) in PC hearts but not in control or PC + bafilomycin treated hearts. Spots corresponding to two known PC-induced mitochondrial proteins (Tfam and PtdIns5P3) were detected in 9 PC hearts but not in 6 control hearts. When compared to control and PC + bafilomycin, expression level of beta-tocopherolase was decreased in PC compared to control and PC + bafilomycin. These differences appear to be due to post-translational modifications as evident by protein isoelectric point shift.**

**P96**

**Screening Nuclear Factor of Activated T-Cells Gene in Lebanese Patients with Valvular Congenital Heart Diseases**

Amin Yehya, Georges Nemover, Fadi Bitar; American Univ of Beirut-Med Cntr, Beirut, Lebanon

**Most forms of congenital heart diseases (CHD) result from aberration in cardiac morphogenesis including errors in septation, valve formation, and proper patterning of the great vessels. Transcription factors are key proteins that dictate mRNA synthesis rate and subsequently protein production. Nuclear factor of activated T-cells (NFATc) belongs to the Rel family of transcription factors. In mice, it is expressed in the embryonic heart and is restricted to the endocardium where it plays a major role in valve formation. In order to establish role of NFATc1 in patients with CHD, we started screening for possible mutations in patients and their families (parents and siblings) registered at the Children’s Cardiac Registry Center (COCR) at the American University of Beirut Medical Center (AUB-MC). DNA was extracted from 38 patients with pulmonary stenosis, tricuspid atresia and ventricular septal defects. PCR amplification and DNA sequencing were done on the patients and their parents/sibling. Our findings revealed different single nucleotide polymorphisms (SNPs) that were not previously reported. Moreover, in exon 2 we have found a novel missense mutation in a patient with tricuspid atresia, leading to a change in amino acid position 66 from proline to leucine. None of the 80 control individuals included in the study did show this mutation suggesting it might be disease-causing. In addition, we have found a duplicated segment in exon 7 in 57% of the patients enrolled in our study. This duplication may play a role in RNA splicing and could be a predisposing factor for a sub category of congenital heart diseases. All together our results do point to a role for NFATc1 in heart malformations in humans. 

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**A Novel Cardiac Apoptotic Pathway: The Dephosphorylation of Apoptosis Repressor with Caspase Recruitment Domain by Calcineurin**

Pelfeng Li, Univ of Illinois at Chicago, Chicago, IL; Wei-qi Tan; Chinese Academy of Sciences, Beijing, China

**Apoptosis repressor with caspase recruitment domain (ARC) is cardiac abundant, constitutively activated, and can block apoptosis through multiple manners. Nevertheless, cardiomycocytes still undergo apoptosis that is related to cardiac diseases such as myocardial infarction, cardiomyopathy and heart failure. The occurrence of apoptosis indicates that the interplay between pro- and anti-apoptotic factors is imbalanced. It remains enigmatic as to whether this imbalance can be contributed by ARC when it loses its anti-apoptotic function under pathological conditions. Both isoproterenol (I)os and aldosterone (Aldo) promoted cardiomycocyte apoptosis with a decrease in the phosphorylation levels of ARC. Direct irradiation or co-stimulation of ARC with calcium resulted in the reduction of ARC phosphorylation levels. Inhibition of calcineurin could attenuate the decrease in ARC phosphorylation levels. These data indicate that the decrease in ARC phosphorylation levels induced by Ios and aldol was due to...**
its dephosphorylation by calcineurin. Further study revealed that ARC could prevent iso- and Aldo-induced apoptosis. However, such an effect depended on its phosphorylation status. While ARC was dephosphorylated and lost its anti-apoptotic function, the phosphorylation levels of FOXO3a, a transcriptional factor, were reduced upon stimulation with iso and Aldo. Concomi-
tantly, Fox ligand was upregulated, and the death-inducing signaling complex (DISC) was formed. Inhibition of FOXO3a by its RNAi could attenuate both Fox ligand upregulation and cardiomyocyte apoptosis. ARC could block DISC formation depending on its phosphorylation. Our study identifies a novel cardiac apoptotic pathway in which ARC is dephosphorylated by calcineurin. This pathway may constitute a component in the cardiac apoptotic cascades.

Diminished GATA4 Protein Levels Contribute to Hyperglycemia-Induced Cardiomyocyte Injury
Sau-Ton Kobayashi, Sanford Rusvd, Sioux Falls, SD; Hangiao Zhang, Xuejun Wang, Univ of South Dakota, Vermillion, SD; Sam Patterson, Univ of North Carolina Sch of Medicine, Chapel Hill, NC; Timothy D’O Connell, Qiangrong Liang; Sanford Rusvd, Sioux Falls, SD

Hyperglycemia is an independent risk factor for diabetic cardiomyopathy and heart failure. However, the mechanisms that mediate hyperglycemia cardiomyopathy cardiac damage remain poorly understood. The transcription factor GATA4 is essential for cardiac homeostasis, and its protein levels are dramatically reduced in the heart in response to diverse pathologic stresses. In this study, we investigated if hyperglycemia affects GATA4 expression in cardiomyocytes and if enhancing GATA4 signaling could attenuate hyperglycemia-induced cardiomyocyte injury. In cultured rat cardiomyocytes, high glucose (HG; 25 or 40 mM) markedly reduced GATA4 protein levels as compared to normal glucose (NG; 5.5 mM). However, the GATA4 mRNA content, either steady-state or polysome associated, remained unchanged. Increased culture medium osmolarity did not explain the reduction in GATA4, because normalizing osmolarity with mannitol (Man) did not alter GATA4 protein levels (NG 100 ± 12%, Man 97 ± 8% vs HG 43 ± 16%; p < 0.05). HG-induced GATA4 reduction was reversed by MG262, a specific proteasome inhibitor, suggesting that the ubiquitin proteasome system (UPS) is likely responsible for HG-induced GATA4 degradation. Nevertheless, HG did not activate the UPS in cardiomyocytes as indicated by a GFP UPS reporter, nor did it increase the peptidase activities or protein expression of the proteasomal subunits. However, the mRNA levels of E3 ubiquitin ligase CHIP (Carboxyl terminus of Hsp70-interacting protein) were markedly increased in HG-treated cardiomyocytes. Adenovirus-mediated gene transfer of CHIP promoted GATA4 protein degradation, while small interfering RNA (siRNA)-mediated CHIP knockdown prevented HG-induced GATA4 depletion. Moreover, overexpression of GATA4 blocked HG-induced cardiomyocyte injuries. Importantly, GATA4 protein levels were diminished in the hearts of streptozotocin-induced type 1 diabetic mice and db/db type 2 diabetic mice (44 ± 7% and 67 ± 13% of control, p < 0.05), which correlated with increased CHIP mRNA abundance. In summary, our findings suggest that enhanced GATA4 protein degradation may be an important step in the pathogenesis of diabetic cardiomyopathy and heart failure.

Cardiac Protection of Heat Shock Protein 27 (Hsp27) Has Been Observed in Myocardial Infarction
Hany Li, Xiaoyan Min, Xiang Zhang, First Affiliated Hosp with Nanjing Med Univ, Nanjing, China; Chuanfu Li, East Tennessee State Univ, Johnson City, TN; Bo Qian, Sisi Peng, First Affiliated Hosp with Nanjing Med Univ, Nanjing, China; Xiang Gao, Model Animal Resc Cntr, Nanjing Univ, Nanjing, China; Yinfeng Cheng; First Affiliated Hosp with Nanjing Med Univ, Nanjing, China

Cardiac protection of heat shock protein 27 (Hsp27) has been observed in myocardial ischemia/reperfusion injury and doxorubicin-induced heart failure. However, the role of Hsp27 in endotoxin-induced cardiac dysfunction, a lethal complication during sepsis/septic shock, has not been investigated. We hypothesized that overexpression of Hsp27 will attenuate endotoxin-induced cardiac dysfunction. To evaluate this hypothesis, transgenic mice overexpressing Hsp27 solely in heart (TG) and wild type littermates (WT) were challenged with LPS.
Activated Transient Receptor Potential Vanilloid Receptor Type 1 Improves Vasodilation by Inhibition of RhoA/Rho Kinase Pathway

D Y Liu, L D Ma, Z D Luo, T B Cao, L J Wang, Z M Zhu; Cntr for Hypertension and Metabolic Disease, Daping Hosp, Third Military Med Univ, Chongqing Institute of Hypertension, Chongqing, China.

The RhoA/Rho kinase (ROCK) pathway plays an important role in the pathophysiology of many diseases, including hypertension, myocardiopathy and heart failure. Activation of the RhoA/ROCK pathway has been demonstrated in vascular tissue from type 2 diabetic db/db mice. Transient receptor potential (TRP) channels were found to be involved in the regulation of vascular function. It is unclear whether activation of vanilloid receptor type 1 (TRPV1) can influence the vascular function. Objective: aim of this study was to determine whether TRPV1 agonist, capsaicin, has a beneficial effect on vascular function in mice on high fat diet/HD.

Methods: Male C57BL/6J mice were fed with normal diet (ND) or high fat diet for 12 weeks. Isometric force of agonist-induced relaxations was measured in aortic rings from mice. At the presence or absence of capsaicin. TRPV1 and RhoA/ROCK proteins expression were identified using western blot assay in aorta from mice. Isometric force of agonist-induced contraction and relaxation of mice aortic rings were measured using a force transducer connected to a polygraph. Results: Mice on HD had a markedly increased in blood pressure compared with mice on ND (135 ± 5 vs 126 ± 5 mmHg, p < 0.01). Nitroglycerin-induced relaxation of mice aortic rings was significantly decreased in mice on HD compared with mice on ND (32 ± 8 vs 169 ± 12, p < 0.01). Similarly, acetylcholine-induced relaxation of mice aortic rings was also decreased in mice on HD compared with mice on ND (26 ± 12 vs 108 ± 11, p < 0.001). However, administration of capsaicin in mice on HD for 4 months, both of impaired endothelium dependent and independent relaxation in mice on HD were improved compared with mice on ND (acetylcholine: 69 ± 15, Nitroglycerin: 78 ± 6, p < 0.05). Next, we determined whether capsaicin could effect on TRPV1 and RhoA/ROCK expression of aorta in mice on ND or HD. We found that capsaicin significantly enhanced TRPV1 and inhibited Rho / ROCK expressions of aorta from HD mice and ND plus capsaicin compared to mice on ND without capsaicin (< 0.05). Conclusions: It concluded that activation of TRPV1 by capsaicin improving the vasodilation in HD mouse may be associated with the inhibition of RhoA/ROCK signaling pathway supported by 793 program NO:2006CE503804).

Knockdown of Cyclin-Dependent Kinase Inhibitors by RNAi Induces Cardiomyocyte Mitosis

Valeria Di Stefano, Policlinico San Donato-IRCCS, San Donato Milanese, Milano, Italy; Marco Crescenzi, IBS-IRCCS, Rome, Italy; Maurizio C Capogrossi, Fabio Martelli; IDI-IRCCS, Rome, Italy.

Background. Mammalian cardiomyocytes (CM) have limited proliferation potential, and acutely injured mammalian hearts do not regenerate adequately. Indeed, mouse CM stop proliferating rapidly after birth and become terminally differentiated, at least in part, by the up-regulation of the Cyclin-Dependent Kinases Inhibitors (CKI) p21Waf1/Cip1/Sdi1 (p21) and p27Kip1 (p27). In this report, we investigated whether simultaneous p21 and p27 knock-down by RNAi induced CM proliferation. Methods and results. Newborn mice CM were transfected with small interfering RNAs (siRNA) targeting both p21 and p27, yielding a >70% decrease in protein expression compared to scrambled-siRNA. After 2 days, CKI knock-down CM identified by alpha-sarcomeric actin staining, were almost twice the control (1.8 ± 0.1; p < 0.001). To corroborate these data indicating CM proliferation, we measured whether markers of different cell cycle phases increased accordingly. It was found that: 1) CKI knock-down increased the rate of cells undergoing DNA synthesis after transfection (Brdu cells: control – 8.2% ± 1.5; CKI siRNA – 45.0% ± 4.2; p < 0.0002); interestingly, Brdu + CM were both mono-and bi-nucleated; 2) mitotic figures were detectable only in CKI-siRNA transfected cells; 3) increased the rate of cells undergoing DNA synthesis after transfection (BrdU). Our findings suggest that Hep27 plays an important role in attenuation of endothelin-induced cardiac dysfunction and the mechanism involves down-regulation of p53-induced activation of TLR4/NF-kappaB pathway.

MicroRNA Role in Endothelial Cell Response to Hypoxia

Pasquale Fasano, IDI-IRCCS, Rome, Italy; Yuri D’Alessandra, Cntr Cardiologico Monzino-IRCCS, Milan, Italy; Valeria Di Stefano, Policlinico San Donato-IRCCS, San Donato Milanese, Milano, Italy; Roberta Melchionna, Sveva Romani, IDI-IRCCS, Rome, Italy; Giulio Pomplio, Cntr Cardiologico Monzino-IRCCS, Milan, Italy; Maurizio C Capogrossi, Fabio Martelli; IDI-IRCCS, Rome, Italy.

Background. MicroRNAs (miRNAs) are small non-protein-coding RNAs that act as negative gene regulators by inhibiting mRNA translation in mammalian cells. In this study, we investigated miRNAs regulation and functional role in endothelial cell response to hypoxia. Methods and results. Human Umbilical Vein Endothelial Cells (HUECs) were cultivated in normoxic conditions or in the presence of 1% oxygen for 4–8 hours. Then, a selection of 157 miRNAs was measured using a real-time PCR assay designed to quantitively only mature miRNAs. The expression of miR-210 and miR-150 progressively increased upon exposure to hypoxia. Interestingly, neither acidification nor oxidative stress, two components of the cell response to hypoxia, were sufficient to modulate miR-210. miR-210 expression in normoxic cells stimulated with formamidines, like structures and Cu/Fe-dependent miR-210 blockade, inhibited the formation of capillary-like structures stimulated by hypoxia, decreased cell migration in response to VEGF and induced apoptosis. We found that one relevant target of miR-210 in hypoxia was Ephrin-A3, since miR-210 was necessary and sufficient to down-modulate the Ephrin-A3 activity. To test whether Ephrin-A3 was a direct target of miR-210. Conclusions. miR-210 is a pivotal element of endothelial cell response to hypoxia.

A Novel Nuclear Function for GRK5 and Its Potential Role in the Hypertrophic Response

Jeffrey Martin, Philip Rakke, Leif Vinge, Brent DeGeorge, Kurt Chuppin, David Harris, Andrea Eckhart, Thomas Jefferson Univ, Philadelphia, PA; Julie Pitcher, Univ College London, London, United Kingdom; Walter Koch; Thomas Jefferson Univ, Philadelphia, PA.

G-protein coupled receptor kinases (GRKs) are critical regulators of adrenergic signaling in the heart. During heart failure (HF) GRK2 and GRK5 protein are elevated, leading to a diminished cardiac function. Mice with cardiac-specific overexpression of GRK5 have a unique phenotype in response to overload hypertrophy. GRK5 mice, but not GRK2 overexpressors or non-transgenic control (NLO) mice, rapidly decompensate within 4 weeks after banding (TAC) with signs of HF. GRK5, unlike GRK2 can reside in the nucleus and contains a nuclear localization (NLS) and export sequence (NES). Our hypothesis is that cardiac decapsulation after TAC in GRK5 mice is due to its unique activity in the nucleus. Histone deacetylases (HDAC) reside in the nucleus and acetylate histones. Inhibition of nuclear HDACs reduces cardiac hypertrophy at the level of ME20. Phenotype deacetylation is triggered by Gq activation and we found that expression a constitutively active mutant Gq7 in myocytes leads to significant GRK5 nuclear translocation and Gq7 and GRK5 overexpression enhances ME2 in myocytes. Finally, significant HDAC activity in vivo can be found after immunoprecipitation of GRK5 from mouse hearts and this is increased after TAC. We conclude that GRK5 possesses nuclear HDAC kinase activity and this novel non-G-protein coupled receptor activity of this GRK may play a key role in cardiac hypertrophy and HF.
anti-hypertrophic effect of Rassf1A. These results suggest that Rassf1A acts as a negative regulator of cardiac hypertrophy. Furthermore, the anti-hypertrophic effect of Rassf1A is mediated through direct interaction with Mst1.

Oxidative Genotoxicity Reverts the Antipapoptotic Function of Laminar Flow: Opposite Roles for p21Waf1 and p53

Stefania Mattussi, Silvia Trutta, Maurizio C Capogrossi, Carlo Gaetano; Laboratorio di Patologia Vascolare, Roma, Italy

Abstract: Laminar shear stress (SS) provides powerful antipapoptotic signals protecting endothelial cells from a large variety of damaging conditions/agents including oxygen peroxide, oxidized lipoproteins and serum starvation. The present work provides evidences that under specific circumstances SS signaling, surprisingly, activates an intense pro-apoptotic program in human umbilical vein endothelial cells (HUVEC) exposed to the DNA damaging agent Bleomycin, Adriamycin or to a cycle of hypoxia/re-oxygenation. In brief, cells were treated with a laminar SS of 12 dyn/cm² for 4 to 16 hours in presence of absence of Bleomycin (10 μg/ml), Adriamycin (10 ng/ml) or after 3 hours of hypoxia (1% O2) followed by 1 hour of reoxygenation. In all these conditions SS elicited a strong induction of reactive oxygen species (ROS) and cells underwent apoptosis. This process was characterized by an increased production of the inducible nitric oxide synthase (iNOS). HUVEC treated with the INOS inhibitor GW274150 reduced their intracellular levels of NO and were protected from apoptosis in the presence of SS Bleomycin, Adriamycin or hypoxia/re-oxygenation. Biochemical analyses revealed that in presence of Bleomycin p53 was phosphorylated on serine-15 and acetylated on lysine-382 reflecting the transactivation of this molecule along a pro-apoptotic pathway. Experiments with the antioxidant N-acety-cysteine (NAC) or with the small hairpin RNA interference of p53 completely abolished apoptosis in the presence of SS indicating that both ROS and p53 were important in this process. In addition, in an in vitro experimental setting, SS failed to stimulate p21Waf1 expression. However, in spite of the increased intracellular level of oxidative stress the adenosine-activated p21Waf1 delivery restored the SS antipapoptotic effect. Taken altogether these results provide the evidence that oxidative stress may alter the function of laminar flow and suggest, under specific circumstances, a possible negative role of SS in the pathogenesis of cardiovascular diseases.

Fo xo Transcription Factors Activate Akt and Attenuate Insulin Signaling Through Inhibition of Protein Phosphatases

Yan G Ni, Merck & Co Inc, Rahway, NJ; Na Wang, Nita Sachan, David J Morris, Robert D Gerard, Makoto Kuro-o, Beverly A Rothermel, Joseph A Hill; UT Southwestern Med Ctr, Dallas, TX

Insulin resistance and metabolic syndrome are rapidly expanding public health problems. Acting through the PISK/Akt pathway, insulin and insulin-like growth factor-1 (IGF-1) inactivate Foxo transcription factors, a class of highly conserved proteins important in numerous physiological functions. However, whether Foxo is a downstream target of insulin, Foxo factors also control upstream signaling elements governing insulin sensitivity and glucose metabolism. Here, we report that sustained activation of either Foxo1 or Foxo3 in cardiac myocytes increases basal levels of Akt phosphorylation and kinase activity. Foxo-activated Akt directly interacts with and phosphorylates Foxo, providing feedback inhibition. We recently reported that Foxo factors attenuate cardiomyocyte calcineurin (PP2B) activity. We now show that calcineurin forms a complex with Akt, and inhibition of calcineurin enhances Akt phosphorylation. In addition, Foxo activity suppresses protein phosphatase 2A (PP2A) and disrupts Akt-PP2A and Akt-calcinuerin interactions. The Foxo-mediated repression of Akt-PP2A interactions and phosphatase activities contributes, at least in part, to increased Akt phosphorylation and kinase activity. Importantly, Foxo-dependent increases in Akt activity diminish insulin signaling, as manifested by reduced Akt phosphorylation, reduced membrane translocation of Glut4, and decreased glucose uptake in response to insulin. Conversely, genetic inactivation of Foxo3 enhances insulin-dependent Akt phosphorylation. Finally, reverterant, an activator of Sirt1, increases nuclear localization of Foxo1 in cardiac myocytes, and thus increases its transcriptional activity. Taken together, this study demonstrates that changing Foxo activity have a dose-responsive inhibitory effect on insulin signaling in cardiomyocytes through altered basal Akt phosphorylation levels, culminating in reduced insulin sensitivity and impaired glucose metabolism.

Protein Expression of Stem Cell Markers Is Increased in Left Ventricular Myocardium of Patients and Dogs with Heart Failure

Sharad Rastogi, Victor G Sharov, Hani N Sabbah; Henry Ford Health System, Detroit, MI

Background: Sca1 and c-Kit are key stem cell markers that, when observed in tissue, provide evidence for the presence of bone marrow stem cells (BMSC). We previously showed that ongoing cardiomyocyte injury, degeneration and loss occur in the failing left ventricle (LV). This ongoing myocardial injury creates an ideal substrate for colonization of the myocardium by circulating BMSC; the latter are decreased in dogs with chronic heart failure. In this study, we tested the hypothesis that protein expression for Sca1 and c-kit may be increased in the failing LV myocardium in response to ongoing injury, degeneration and loss of cardiomyocytes. Methods: Protein expression for Sca1 and c-kit was measured in LV myocardium of explanted failed human hearts due to ischemic cardiomyopathy (ICM, n=6), idiopathic dilated cardiomyopathy (IDC, n=6), normal donor human hearts which were deemed not suitable for transplantation (NL-Human, n=6), dogs with intracoronary microembolization-induced heart failure (HF-Dogs, n=6) and in LV myocardium of normal dogs (NL-Dogs, n=6). Tissue homogenates was prepared from all samples. Protein expression of Sca1 and c-kit was measured using Western blots and bands quantified in densitometric units (du). Results: Data are shown in the table. Protein expression for Sca1 and c-kit was significantly increased in LV myocardium of patients with ICM and IDC compared to LV myocardium from NL-Human donor hearts as well as in LV myocardium of HF-dogs compared to NL-Dogs. Conclusions: The results of this study indicate that in LV myocardium of explanted failed human hearts, regardless of etiology, as well in LV myocardium of dogs with experimentally-induced HF, protein expression for Sca1 and c-kit is increased. This upregulation of key stem cell markers in HF support the possibility that colonization of the myocardium by circulating BMSC occurs in HF and may also explain the decrease in circulating BMSC observed in dogs with HF.

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<th>Protein Expression of Sca1 and c-Kit</th>
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<td>Sca1 (du)</td>
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*p<0.05 vs NL-Human; †p<0.05 vs NL-Dog

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Large Tumor Suppressors 2 (Lats2), a Downstream Effector of Mammalian Sterile 20-Like Kinase 1 (Mst1), is a Critical Negative Regulator of Biventricular Mass

Noritugu Nakano, Yutaka Matsui, Junichi Sadoshima; Univ of Medicine and Dentistry of New Jersey, Newark, NJ

Mst1 is an evolutionarily conserved serine threonine kinase, which is a critical regulator of growth and death of cardiac myocytes. Mst1 forms a complex with hWW45 and Lats2, thereby antagonizing both basal and BMP4-induced expression of SM-specific genes including a-smooth muscle actin, SM22, and calponin. Overexpression of Mst1 in failed human hearts due to ischemic cardiomyopathy (ICM, n=6), idiopathic dilated cardiomyopathy (IDC, n=6), normal donor human hearts which were deemed not suitable for transplantation (NL-Human, n=6), dogs with intracoronary microembolization-induced heart failure (HF-Dogs, n=6) and in LV myocardium of normal dogs (NL-Dogs, n=6). Tissue homogenates was prepared from all samples. Protein expression of Sca1 and c-kit was measured using Western blots and bands quantified in densitometric units (du). Results: Data are shown in the table. Protein expression for Sca1 and c-kit was significantly increased in LV myocardium of patients with ICM and IDC compared to LV myocardium from NL-Human donor hearts as well as in LV myocardium of HF-dogs compared to NL-Dogs. Conclusions: The results of this study indicate that in LV myocardium of explanted failed human hearts, regardless of etiology, as well in LV myocardium of dogs with experimentally-induced HF, protein expression for Sca1 and c-kit is increased. This upregulation of key stem cell markers in HF support the possibility that colonization of the myocardium by circulating BMSC occurs in HF and may also explain the decrease in circulating BMSC observed in dogs with HF.

Four and a Half LIM Domains 2 (FHL2) is a Negative Regulator of the Contractile Phenotype of Vascular Smooth Muscle Cells

Nicole A Newman, Tufts Univ, Boston, MA; Giorgia Lagna, Tufts New England Med Cntr, Boston, MA; Akiko Hata, Tufts Univ, Boston, MA

Vascular smooth muscle cells (VSMCs) retain phenotypic plasticity, which allows them to switch from a fully differentiated and quiescent “contractile” phenotype to a dedifferentiated and proliferative “synthetic” phenotype. This phenotypic change plays a role during vascular remodeling and development of vascular disorders, such as atherosclerosis and diabetic pulmonary arterial hypertension. The molecular mechanism of regulating this switch, however, is not fully understood. We investigated the role of FHL2 in the regulation of SM phenotype in VSMCs. It has been reported that overexpression of FHLL2 suppresses Rhoa-stimulated induction of SM-specific gene promoters using reporter assays. We show that FHL2 antagonizes both basal and BMP4-induced expression of SM-specific genes including a-smooth muscle actin, SM22, and calponin. Overexpression of FHL2 in pulmonary artery smooth muscle cells (PASMCs) by recombinant adenovirus reduced expression of SM genes and promoted a morphological change that mimics the “synthetic” phenotype. Consistently, downregulation of endogenous FHL2 by shRNA in PASMCs increased the basal expression of SM genes. We also found that FHL2 is, at least in part, required for suppression of SM genes by P09F-BB treatment. These results suggest that FHL2 may play a critical role in dedifferentiation of SMs.
PKCe Mediates Nonischemic Preconditioning-Induced Bradykinin-2 Receptor Signaling

Xiaoping Ren, Siyun Liao, Jo El J Schultz, Maria Brown, Walter K Jones; Univ of Cincinnati, Cincinnati, OH

Introduction: We previously demonstrated a cardioprotective effect of non-ischemic surgical trauma (abdominal incision) against I/R injury that has both a late and early phase, similar to ischemic preconditioning (IPC). We further have shown that this novel phenomenon, remote preconditioning of trauma (RPCT), requires bradykinin signaling. Hypothesis: The late phase of RPCT elicits a cardioprotective response by activating a signaling pathway that includes BK2R, PKCe and NF-κB. Methods: RPCT was initiated in vivo in mice, 24 h (late RPCT) before 45 min I/R and infarct size determined. Blockade of NF-κB (ibodan transgenic mice) and iNOS gene ablation were employed to determine the effect upon MI after RPCT. RPCT was performed with or without pretreatment with chelerythrine (1 μM), a non-selective PKC inhibitor. Translocation of PKC-isoforms from cytosol to the particulate fraction were measured by quantitative immunoblotting in wild type and bradykinin 2 receptor knockout (BK2R KO) mice 24 h after RPCT. Results: Late RPCT significantly reduced infarct size (55.3 ± 3.4 vs. 53.2 ± 6.3%, P<0.05). NF-κB blockade prevented development of late RPCT (40.4 ± 1.8% vs. 38.6 ± 2.2%, P<0.05). iNOS is not required for the protective effect of late RPCT (39.2 ± 4.1% in sham vs. 15.4 ± 2.7% in iNOS knockout mice, P<0.05). The cytosolic to particulate ratios were shifted for PKCe and PKCd 24 h after late RPCT (PKCe, 2.03 ± 0.27 to 2.81 ± 0.15, P<0.05; for PKCd, 2.3 ± 0.5 vs. 0.9 ± 0.17, P<0.05). Moreover, the effects of RPCT upon PKK isoform localization were abrogated in BK2R KO mice. PKCa activation was not affected by RPCT. Conclusions: The protective effect of RPCT against MI involves bradykinin signaling upstream of PKC modulation.

Glu1-Mediated Cardiac Electrophysiological Remodeling in Hypertrophic Cardiomyopathy

Hongmei Ruan, Scherrie Mitchell, Joshua I Golhaber, Yibin Wang; David Geffen Sch of Medicine, Univ of California, Los Angeles, CA

Cardiac hypertrophy is a major risk factor for arrhythmias and sudden cardiac death. However, the underlying signaling mechanisms involved in the induction of arrhythmia and electrophysiological remodeling in cardiac hypertrophy are unclear. Using an inducible gene-switch approach, we achieved tissue-specific and temporally regulated induction of a well-established hypertrophic pathway, the Ras-Raf-MAPKs pathway, in adult mouse heart. Upon Ras activation, the transgenic animal developed ventricular hypertrophy and arrhythmias. The development of ventricular arrhythmias was temporally correlated with electrophysiological remodeling in isolated ventricular myocytes, including action potential prolongation, increased inward sodium calcium exchange activity, reduced outward potassium currents, SR Ca2+ deficits and loss of PKA dependent phospholamban phosphorylation. From genome-wide expression profiling, we discovered a selective induction of Glu1 subunit 1 expression in the Ras transgenic heart. Treating transgenic animals with the Glu1 inhibitor pertussis toxin normalized the phospholamban phosphorylation by PKA, reversed the action potential prolongation and significantly reduced the frequency of cardiac arrhythmias in Ras transgenic animals. These data suggest that selective induction of Glu1 expression and activity is a novel downstream event in hypertrophic signaling that may be a critical factor leading to cellular electrophysiological remodeling and cardiac arrhythmias in hypertrophic cardiomyopathy.

Regulation of Nitric Oxide Production by L-Ariginase Induction in the Heart Following Myocardial Infarction

Mark M Stayton, Jacquie A Keeler, Mark H Harpster, Somnath Bandypadhyay, Richard J McCormick, D P Thomas; Univ of Wyoming, Laramie, WY

Nitric oxide synthesis (NOS) catalyzes the synthesis of nitric oxide (NO) from L-arginine. NOS regulates many aspects of cardiovascular function. NOS consists with L-arginine (L-Arg) for a common substrate. ARG1 catalyzes the production of L-ornithine from L-Arg and is the single most highly induced transcript in the mouse left ventricle after acute myocardial infarction (AMI). With ischemic/infarcted tissue (IF), the ARG1 transcript is induced 121-fold over 24 h post-AMI. In addition to induction of the ARG1 transcript, arginase specific activity is elevated as early as 15 min after AMI and at 48 h by 10-fold by 48 h post-AMI. This induction is also induced along with transcripts encoding enzymes of polyamine biosynthesis and a protein inhibitor of NOS activity. Thus, NO production may be regulated, in part, by inhibition of NOS and depletion of the NOS substrate, L-arginine, by L-arginase. In addition, L-arginine may undergo conversion to polyamines as part of the stress response and/or to promote cytokine (collagen biosynthesis). To test these hypotheses, we produced antibodies against ARG1, ornithine decarboxylase, antizyme inhibitor and protein-inhibitor of NOS. As judged by western blot, ARG1 and the antizyme inhibitor polypeptides show induction in the IF. We have further evaluated the regional and cell-type specificity of their induction by injection of 15 min, 60 min, and 15 minutes, and the effects of NF-kB blockade prevented development of late RPCT (40.4 ± 1.8% vs. 38.6 ± 2.2%, P<0.05). iNOS is not required for the protective effect of late RPCT (39.2 ± 4.1% in sham vs. 15.4 ± 2.7% in iNOS knockout mice, P<0.05). The cytosolic to particulate ratios were shifted for PKCe and PKCd 24 h after late RPCT (PKCe, 2.03 ± 0.27 to 2.81 ± 0.15, P<0.05; for PKCd, 2.3 ± 0.5 vs. 0.9 ± 0.17, P<0.05). Moreover, the effects of RPCT upon PKK isoform localization were abrogated in BK2R KO mice. PKCa activation was not affected by RPCT. Conclusions: The protective effect of RPCT against MI involves bradykinin signaling upstream of PKC modulation.

β2-Adrenergic Receptors Localize to the Nucleus but Still Impact ERK Signaling at the Membrane in Adult Cardiomyocytes

Casey D Wright, Yuan Huang, Guan Hai Chen, Nichole L Baye, Chastity L Merkwan, Timothy D O’Connell; Sanford Rsrch–USD, Sioux Falls, SD

We previously demonstrated that an α1-adrenergic receptor (α1A-AR)-ERK signaling pathway mediates survival signaling in adult cardiac myocytes. Here, we investigated the localization of the two α1-AR subtypes expressed in cardiac myocytes (α1A and α1B) and how their localization impacts α1A-AR signaling. To examine localization of endogenous α1A-ARs in cardiomyocytes, we used a fluorescent analog of the non-specific α1-AR antagonist prazosin, BODIPY-prazosin, which fluoresces subsequent to receptor binding. Using BODIPY-prazosin, we found that endogenous α1A-ARs localize to the nucleus in cultured adult mouse cardiac myocytes and nuclei isolated from myocytes. To clarify α1A-subtype specific localization, we reconstituted α1A-signaling in cultured α1BKO (α1A- and α1B-AR double knockout) cardiac myocytes, which lack endogenous α1-ARs, using α1A- and α1B-FP fusion proteins. Similar to WT α1A-ARs, the α1A- and α1B-FP co-localized with the nuclear membrane protein LAP2 by confocal microscopy and biochemical fractionation of cardiac myocytes. The α1-A signaling partners Gq and PLCβ1 also localized to the nuclear membrane (and plasma membrane) suggesting that α1-A signaling might be initiated in the nucleus. In α1BKO myocytes, caveolin-3 localized to the plasma membrane and t-tubules, but did not overlap with the nuclear α1-GFPs, demonstrating that α1-A-ARs are most likely not on the plasma membrane or in caveolae as previously suggested. To understand how nuclear localization affected α1-A signaling, we examined α1A-AR activation of ERK. In α1BKO myocytes expressing the α1A-FPs, phenylephrine increased phosphorylated-ERK levels at the plasma membrane within 15 minutes, yet the α1A- and α1B-FPs co-localized with the nuclear α1-GFPs. Previous studies reported that α1-A and ERK locate to caveolae and that caveolin-3 regulates the α1-ERK signaling pathway in myocytes. However, our results indicate that the α1-ERK signaling pathway initiates at the nucleus and ends at the membrane (without receptor translocation), where caveolin-3 could mediate ERK activation. In summary, our results suggest a reconsideration of classical α1-AR signaling pathways based on our novel finding that α1-A-ARs localize to the nucleus in cardiac myocytes.

Effects of Oxidative Stress in PKCζ Modulation of Cardiac Contractile Protein Phosphorylation

Steven C Wu, R John Solaro; Univ of Illinois at Chicago, Chicago, IL

We have previously identified protein kinase C ζ (PKζ) as a novel modulator of cardiac contractile protein phosphorylation. We hypothesize that oxidative stress alters the PKζ pathway for contractile protein phosphorylation and function. In this study, we examined changes induced by hydrogen peroxide (H2O2) on PKζ and protein kinase C ζ activity in cardiac myocytes. We have previously shown that wild-type PKζ is tyrosine (Tyr)-phosphorylated and it is increased by H2O2. In addition, H2O2 induced phosphorylation of wild-type PKζ. However, Tyr phosphorylation and proteolysis was markedly reduced by constitutively activating PKζ. We conclude that activation of PKζ specifically led to threonine (Thr) dephosphorylation of cardiac troponin I (cTnI) and troponin T (cTnT) through protein phosphatase 2A (PP2A). We examined cTnI and cTnT phosphorylation by PKζ under oxidative stress. H2O2 induced PKζ-induced Thr phosphorylation of cTnI and cTnT by Tyr phosphorylation of PP2A, which is known to inhibit its phosphatase activity. In addition, we observed an H2O2-induced increase in serine (Ser) phosphorylation of cTnI and cTnT by Tyr phosphorylation and proteolysis of Thr-phosphorylated cTnT. Constitutive activation of PKζ did reduce proteolysis
activation of mitochondrial matrix targeted type 2C Ser/Thr protein phosphatase (PP2Cm) that regulates mitochondrial permeability transition pore (MPTP) and is essential for cardiac development and function. PP2Cm expression is highly enriched in mouse heart and significantly downregulated upon mechanical stresses via trans-aortic occlusion. RNAi mediated PP2Cm inactivation in isolated rat neonatal ventricular cardiomyocyte induced stress-activated protein kinase signaling and cell death associated with loss of mitochondrial membrane potential. In vivo, PP2Cm inactivation in mouse liver led to increased amount of apoptotic cell death as well as elevated sensitivity to calcium induced MPT. Moreover, PP2Cm knockdown in developing zebrafish embryos caused abnormal cardiac and neural development as well as heart failure associated with induced apoptosis. Finally, we observed that the level of reactive oxygen species was drastically increased in PP2Cm−/− mouse embryonic fibroblast compared to wild-type controls. Our data suggest PP2Cm is a novel mitochondrial protein phosphatase that has a critical function in cell death and survival, and may play a role in regulating the MPT opening by modulating the production of mitochondrial specific reactive oxygen species.

Transcriptional Regulatory Sequences Within the Intron of the Phospholamban Gene Are Regulated by MEF2 and FAK

Carla J Maria, Ana C Deckman, Alisson C Cardoso, Carolina F Clemente, Kleber G Franchini; State Univ of Campinas, Campinas, Brazil

FAK has been shown to play a prime role in the transduction of mechanical stress into biochemical signal in cardiac myocytes. Data exist indicating that FAK re-locate to the nuclei upon mechanical stresses via trans-aortic occlusion. RNAi mediated PP2Cm inactivation in isolated rat neonatal ventricular cardiomyocyte induced stress-activated protein kinase signaling and cell death associated with loss of mitochondrial membrane potential. In vivo, PP2Cm inactivation in mouse liver led to increased amount of apoptotic cell death as well as elevated sensitivity to calcium induced MPT. Moreover, PP2Cm knockdown in developing zebrafish embryos caused abnormal cardiac and neural development as well as heart failure associated with induced apoptosis. Finally, we observed that the level of reactive oxygen species was drastically increased in PP2Cm−/− mouse embryonic fibroblast compared to wild-type controls. Our data suggest PP2Cm is a novel mitochondrial protein phosphatase that has a critical function in cell death and survival, and may play a role in regulating the MPT opening by modulating the production of mitochondrial specific reactive oxygen species.
or empty plasmid. The transient transfection of C2C12 cells with pln indicated an increased activity (by 2.3 fold) of the reporter gene implying a regulatory role for the pln sequence. In conclusion, our data indicate that FAK activation regulates phospholamban gene expression possibly by an interaction with MEF2 and that the intronic of pln sequence plays a role in the expression of PLN in response to mechanical stress in cardiac myocytes.

A Novel Role for Mitochondrial-Localized Stat3 in Cardiac Mitochondria

Joanna Wegrzyn, Ramesh Potla, Young-Joon Chwae, Qifang Zhang, Magdalena Szelag, Lerner Res Institute, Cleveland Clinic Foundation, Cleveland, OH; Gun Chen, Case Western Res Louis Stokes, Cleveland, OH; Jozef Dulak, Jagiellonian Univ, Krakow, Poland; Xin-Yuan Fu, Indiana Univ Sch of Medicine, Indianapolis, IN; Edward J Lesnefsky, Case Western Res Louis Stokes, Cleveland, OH; Andrew C Larner; Lerner Res Institute, Cleveland Clinic Foundation, Cleveland, OH

Regulation of immune responses and cell growth by pro and anti-inflammatory cytokines is mediated through activation of the Stat3 transcription factor. The current model dictates that binding of cytokines including IL-6 and IL-10 to cell surface receptors induces tyrosine and serine phosphorylation of Stat3 resulting in nuclear translocation, binding to a GAS enhancer in Stat3-responsive genes, and downstream transcriptional responses. We provide the first evidence for the presence of Stat3 in mitochondria (MITO) of cultured cells and primary tissues including liver and heart. In Stat3/-/- primary pro B cells the activities of complexes I and II of the electron transport chain (ETC) are decreased 50--90%. MITO-targeted Stat3 expressed in Stat3/-/- cells restores the function of complexes I and II. Floxed Stat3 mice were crossed with transgenic mice that express Cre recombinase under the control of the α-myosin heavy chain promoter whose expression is restricted to cardiomyocytes. MITO from the hearts of 8 week old Stat3 flox/flox mice (Stat3/-/-) and Stat3 fabeled mice (WT) were isolated. Hearts are normal as assessed by histology and physiological parameters. Stat3 content in heart tissue and MITO from Stat3+/flox mice varied between 5 and 20% of Stat3 fabeled mice. Stat3/-/- mice exhibit decreased rates of oxidative phosphorylation with complex I (72% decrease, n=5 Stat3 +/- vs. n=5 WT, p<0.01) and complex II (64% decrease, n=7 Stat 3 +/- vs. n=6 WT, p<0.01) substrates. These defects are due to decreased complex I and complex II enzyme activities. Thus, MITO-localized Stat3 contributes novel functions which may orchestrate responses to cellular stress and innate immunity through modulating the activity of the ETC. Since partial inhibition of the ETC during ischemia or early reperfusion can attenuate cardiomyocyte injury, inactivation of stat 3 during these periods opens a new approach to address mitochondrial-driven cardiomyocyte injury during ischemia and early reperfusion.