into host tissue. To address functional integration, the physiological interactions between muscle cells and their microenvironment need to be further elucidated. We are using microfabricated post array detectors (mPADs) as novel functional assays to assess contractile forces transferred through focal adhesions to the extracellular environment from cardiomyocytes. mPADs utilize an array of microscale posts that deflect as cantilever springs in response to forces applied by cells cultured upon them. Contractile forces of neonatal rat cardiomyocytes (NRCs) are calculated by analyzing the deflection of the posts and multiplying by their spring constant. The measured forces are compared to vascular smooth muscle cells (VSMCs), cells with similarly disorganized contractile apparatus, and adult rat cardiomyocytes (ARCs). Using immunofluorescence of vinculin, both NRCs and VSMCs demonstrate the ability to create focal adhesions to the fibronectin coatings on the tips of the posts. Preliminary results indicate that VSMC produced a total force of 570 ± 175 nN/cell which is almost 6-fold greater than NRCs. However, NRCs produce 50% more force per unit area than VSMC. Furthermore, spontaneous beating of NRCs yielded an additional maximal contraction of 55.6 nN per post per beat above the baseline which is about two orders of magnitude lower than reported forces produced by ARCs. The use of mPADs provides a tool to further the understanding of the stress and strains created by cardiomyocytes onto their local environments through their focal adhesions. With this new technique to assess muscle adhesion and contractility, we seek to characterize the physiological interactions that implanted muscle cells must recapitulate to advance new therapies for cardiovascular regenerative medicine. HL61683 & UWRRF

2567-Pos Board B537

Integrin Response To Altered Actin-Myosin Mechanochemistry In Cardiac Myocytes

Timothy J. O'Donnell, Michael S. Carter, Mariam A. Ba, Maria L. Valencik, Josh E. Baker.
University of Nevada Reno, Reno, NV, USA.

Altered contractility in cardiac myocytes associated with motor protein mutations has been implicated in several pathologies such as familial hypertrophic (FHC) and dilated (DCM) cardiomyopathies. The mechanism(s) by which these altered contractile forces result in cell phenotype changes are poorly understood. However, they are generally believed to involve mechanotransduction of force by cell adhesion molecules such as the integrin class of proteins. Here we investigate the use of blebbistatin and other small molecule effectors of actin-myosin mechanochemistry as a chemical model for the reduced contractile force associated with the pathogenesis of DCM. In order to establish intercalated-disk cell associations in vitro that are representative of in vivo conditions, we employ micropatterned cardiac myocyte cell culture, where the extracellular matrix proteins collagen and laminin are printed onto the culture vessel surface in 10μm wide lines. Fluorescent polymer microspheres (0.25μm dia.) are embedded in the collagen layer. As the beads move from myocyte contraction, the movement amplitude is measured by nanometer-resolution position analysis, both in the presence of blebbistatin and in the rescue state where blebbistatin is removed. Relative force generation is calculated from the position analysis data and matrix modulus. Cells are then fixed and prepared with fluorescent antibodies for observation of connexin-43 and β1 integrin expression and localization by confocal microscopy. By employing micropatterned cardiac myocyte cell culture, optical contractile force measurement, and subsequent observation of the expression and localization of connexin-43 and β1 integrin, we assess the changes in integrin activity due to blebbistatin-induced reduction in contractile force.

2568-Pos Board B538

A Direct Method to Measure the Restoring Force and Slack Sarcomere Length of Intact Cardiomyocytes

Nicholas M.P. King[1], Michiel Helmes[2], Henk Granzier[1].
[1]University of Arizona, Tucson, AZ, USA, [2]University of Oxford, Oxford, United Kingdom.

Molecular mechanisms underlying diastolic suction are poorly understood. Several proteins have been implicated to play a role, including extra-cellular proteins, titin, and cytoskeletal proteins. An in vitro measurement of diastolic suction at the cell level is restoring force (or stiffness), a force which is difficult to measure as it requires a cell to be passive below the slack sarcomere length (SL). Previous restoring force studies were made utilizing calcium independent shortening of cardiomyocytes to below their slack length and then upon relaxation measuring the force that developed as the cell is stretched back to its slack length. However, these studies used chemically permeablized cells and, thus, eliminated the membrane and soluble intracellular proteins as possible contributors to restoring force. In the current study we developed a novel method to determine restoring force and the slack sarcomere length of mouse cardiomyocytes that were intact. Intact cardiac myocytes that were below their slack length due to a low level of active force development were attached to flexible carbon fibers. We then added butanedione monoxime (BDM) to inhibit actomyosin interactions and abolish active tension. This led to an increase in the sarcomere length and a negative force. From this new baseline force (-1.34 ± 0.34 mN/mm^2 (mean ± SE)), we stretched the cells across the physiological range from ~1.8-2.2 μm. We determined the sarcomere length at which force is zero (slack SL) to be 1.93 ± 0.019 μm. Plotting the stress-SL relationship we then determined the restoring stiffness from the slope of this plot as 16.1 mN/mm^2/μm. Thus we successfully measured the restoring force - SL relation of intact cardiac myocytes.

2569-Pos Board B539

Titin Isoform Transitions and Passive Stiffness During Skeletal Muscle Development

Coen Ottenheijm, Anna Knottnerus, Danielle Buck, Tiffany Pecor, Xiuju Luo, Henk Granzier.
University of Arizona, Tucson, AZ, USA.

During postnatal striated muscle development, multiple changes in active and passive properties occur, reflecting an altered mechanical demand. For instance, during postnatal cardiac development titin isoform expression switches from large isoforms to small stiffer isoforms, likely affecting diastolic filling behavior. In the present study, we investigated whether titin isoform transitions also take place during *skeletal* muscle development. We used gel-electrophoresis to determine changes in titin isoform size in mice and rabbits of various ages. A titin exon microarray was used to evaluate transcript expression of all of titin's exons. To investigate the mechanical effect of titin isoform transitions, passive properties of neonatal and adult skeletal muscles were determined.

Neonatal mice were found to express large titin isoforms, which are gradually replaced by smaller isoforms during skeletal muscle development. The half transition time of the isoform for tibialis cranialis (TC), soleus, extensor digitorum longus, gastrocnemius and diaphragm were 6, 17, 17, 12, and 10 days, respectively. Essentially similar findings were obtained from NZW rabbits, with the exception that the half-life of the isoform transitions was slightly longer compared to murine skeletal muscles. Titin exon analysis in neonatal murine gastrocnemius muscle revealed increased expression of a large group of exons when compared to adult muscle transcripts, with all upregulated exons coding for exons of the elastic PEVK region of titin. In line with these observations, we found ~50% lower titin-based passive stiffness of murine neonatal soleus and TC when compared to adult muscle.These data demonstrate that during skeletal muscle development titin isoform transitions occur from large compliant isoforms to smaller and stiffer isoforms in adult muscle, likely due to changed expression of PEVK exons.

2570-Pos Board B540

Vinculin Contributes to the Passive Stiffness of Myocardium

Joyce Chuang, Robert Ross, Andrew McCulloch, Jeffrey Omens.
University of California San Diego, La Jolla, CA, USA.

In cardiomyocytes, the costamere links the Z disc to the surrounding extracellular matrix. Proteins at these junctions include integrins, talin, and vinculin (Vin). Vin is also found in the intercalated discs. Cardiac-specific Vin knockout (VinKO) mice have a sudden death phenotype early in life (<12 weeks of age) with a progressive dilated cardiomyopathy leading to 100% mortality by 32 wks. At 7 weeks of age, systolic ventricular function is normal.

We hypothesize that deletion of the costameric protein Vin leads to changes in passive stiffness prior to the onset of systolic dysfunction. Vin deletion may disrupt the normal force transmission pathways from ECM to cytoskeleton through the integrin-based costamere or cell-to-cell force transmission along the myocyte axis, which may manifest in altered passive material properties of the myocardium.

To test the mechanical properties of myocardial tissue, a system was developed in which murine right ventricular papillary muscles could be passively strained in the axial direction while simultaneously measuring force. Papillary muscles from 7 week old VinKO mice and WT controls were isolated and stretched. Stress-strain analysis was used to measure passive stiffness in the direction of myocardial fibers.

Stress-strain curves were significantly different between WT and VinKO papillary muscles ($p<0.05$). The slope of the VinKO curve was less than in the WT curve, indicating that VinKO muscles are more compliant in the fiber

direction. This data suggests that Vin contributes to the passive mechanical properties of the myocardium, and disrupting the mechanical linkage between the cytoskeleton and the cell membrane reduces the overall stiffness of the myocardium.

2571-Pos Board B541

Quantitative Assay of Skeletal Muscle alpha-actin Expression In Normal and Pathological Human and Mouse Hearts

O'Neal Copeland[1], Gianna Ravenscroft[2], Kristen Nowak[2], Nigel Laing[2], **Steven Marston[1]**.
[1]Imperial College London, London, United Kingdom, [2]University of Western Australia, Perth, Australia.

We have developed a polyclonal antibody specific to skeletal muscle actin (ACTA1) in the presence of cardiac actin (ACTC) and have used it to quantify the skeletal actin content of human and mouse cardiac muscle. Heart muscle myofibrils were separated by SDS-PAGE and Western blotted. The membrane was stained first with MemCode total protein stain and then probed in with the anti-skeletal muscle actin antibody, visualised by ECL. The ECL band signal was normalised to MemCode-stained actin band. Skeletal muscle myofibrils were used as a 100% skeletal actin standard. For the negative control we used myofibrils from skeletal muscle of a skeletal actin knockout mouse crossed with a transgenic mouse over-expressing cardiac actin in skeletal muscle. There was no detectable signal from the skeletal actin antibody in the pure cardiac actin control.

Human non-failing donor heart muscle contained $21 \pm 2\%$ skeletal actin (n=9). This is comparable to previous estimates using N-terminal sequencing or Mass spectroscopy. In both end-stage failing heart muscle and in myectomy samples from HCM muscle the skeletal actin content was much higher ($58 \pm 5\%$ in both cases, n=12, 11). The increase in skeletal actin content of myopathic muscle was highly significant ($p < 0.001$). Mouse heart muscle (C57BL/6 strain) contains $26 \pm 3\%$ skeletal actin (n=7). This is similar to human heart. ACTC DCM mutation E361G expressed at 50% in mouse heart has $16 \pm 3\%$ (n=8) skeletal actin but ACTC HCM mutation E99K expressed at 50% is not significantly different from NTG $24 \pm 2\%$ (n=5). We conclude that in human heart, acquired heart failure or failure secondary to HCM is associated with an increased content of skeletal muscle actin. In contrast, in mouse genetic models of HCM and DCM skeletal muscle actin content may be lower than normal.

2572-Pos Board B542

Structural and Functional Characterization of Cardiac Troponin T Mutations in the TNT1 Domain That Cause Familial Hypertrophic Cardiomyopathy

Pia J. Guinto, Edward P. Manning, Rachel K. Moore, Steven D. Schwartz, Jil C. Tardiff.
Albert Einstein College of Medicine, Bronx, NY, USA.

FHC is a primary cardiac muscle disorder that is one of the most common causes of sudden death in young people. FHC ''hotspot'' mutations at residue 92 in cardiac troponin T (cTnT) flank the proposed α-helical TNT1 tail domain whose flexibility has been suggested to be important in normal protein-protein interactions within the thin filament. Through Molecular Dynamics (MD) simulations, we showed that FHC mutations Arg92Leu, Arg92Trp, and Arg92Gln cause local α-helical structural changes and increased flexibility at a critical hinge region 18 Angstroms distant from the mutation. We have extended this MD analysis via the use of a self-defined coordinate to measure localized bends in the helix and found that forces acting on this bending coordinate are lower in mutants than wildtype. This quantitatively suggests a less restrictive bending motion in mutants explaining the increased flexibility of the hinge region. To determine how primary biophysical changes induced by these mutations cause complex cardiomyopathies we hypothesize that flexibility alterations and changes in force within compaction-expansion regions in mutational segments lead to electrostatic perturbations, possibly interfering with cTnT-TM complex formation and thin filament function. *In vitro* motility assays with wildtype cTnT and hotspot FHC-cTnT mutants are in progress to directly correlate predicted alterations in electrostatic properties with resultant functional changes. Moreover, contractile and Ca^{2+} transient measurements on isolated myocytes address downstream myocellular responses to the mutation's primary perturbation on structure and function. Data showed normal percent shortening in Arg92Leu myocytes while Arg92Trp percent shortening was significantly impaired compared to Non-Tg ($4.740 + 1.165$ vs. $6.971 + 2.098$, $p<0.001$). Completion of these studies will directly address the links between thin filament structure/function, downstream myocellular responses, and resultant distinct cardiovascular phenotypes.

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Structural and Functional Characterization of cTnT in Familial Hypertrophic Cardiomyopathy

Rachel K. Moore, John Wilson, Pia Guinto, Michael Riegelhaupt, Gary Gerfen, Jil Tardiff.
Albert Einstein College of Medicine, Bronx, NY, USA.

Familial Hypertrophic Cardiomyopathy (FHC) is a primary disease of the cardiac sarcomere. Many disease-causing mutations in the thin filament protein cTnT are found within the TNT1 region. Residues 160-163 represent a mutational hotspot within a highly charged region (158-RREEEENRR-166). In this region, this highly alpha helical domain may unwind to create a flexible hinge that is necessary for function, the structure and dynamics of which may be affected by FHC mutations. We are investigating the structure and function of this region using in vitro motility (IVM) assays and SDSL-EPR. The purpose of our IVM experiments is two-fold: to functionally analyze our spin labeled proteins and to gain insight into the function of TNT1 in the presence of cysteine substitutions and FHC mutations. Preliminary IVM data shows a progressive increase in the severity of the functional effects of cysteine substitution and spin labeling across the putative hinge region ($153<168<172$), suggesting that this region is dynamically important and may be making critical interactions with other components of the sarcomere. Preliminary CW-EPR spectra show an increase in isotropic rotational rate at residue 153 (upstream of the putative hinge region) between cTnT alone and in the troponin ternary complex, suggesting that there is a decrease in alpha helical character at this residue in the ternary complex. Introduction of Δ160E further increases the isotropic rotational rate, suggesting an increase in flexibility due to backbone changes induced by the mutation. To further investigate structural and functional changes caused by FHC mutations within the putative hinge region, we will continue to expand our IVM functional analyses with additional cysteine substitutions, as well as FHC mutations at residues 160 and 163. Double label SDSL-EPR are currently underway that will provide secondary and tertiary structural information.

2574-Pos Board B544

FHC-linked Mutations in the Myosin Regulatory Light Chain Interfere with RLC Phosphorylation in Transgenic Mice

Katarzyna Kazmierczak, Alexander Raytman, Michelle Jones, **Danuta Szczesna-Cordary**.
University of Miami Miller School of Medicine, Miami, FL, USA.

Previous studies have shown that MLCK-phosphorylation of the ventricular regulatory light chain (RLC) increases myofilament Ca^{2+} sensitivity, maximal level of force and rate of tension development thereby enhancing the systolic function of the heart. We have investigated the effect of FHC (familial hypertrophic cardiomyopathy) mutations in the myosin RLC on its phosphorylation measured in rapidly frozen ventricular samples from transgenic (Tg) mice. We observe that the Aspartate to Valine substitution in Tg-D166V mice and Arginine to Glutamine in Tg-R58Q mice result in a decreased phosphorylation of RLC detected in left ventricular samples from Tg-mutant compared to Tg-WT mice expressing the human ventricular RLC. The level of RLC phosphorylation was determined by Western blotting utilizing human specific phospho-RLC antibodies (gift from Dr. N. Epstein, NIH). Our data from skinned Tg-D166V and Tg-R58Q papillary muscle fibers show that both RLC mutations lead to a decreased maximal level of force and to slower kinetics of force generating myosin cross-bridges compared to WT fibers. Studies in intact papillary muscle fibers show prolonged force transients for both D166V and R58Q mutants. In addition, the hearts of aging mutant mice demonstrate histopathological changes and frequent occurrences of fibrotic lesions. Clinical studies revealed that both D166V and R58Q mutations are associated with severe FHC phenotypes with multiple cases of sudden cardiac death. Our results suggest that phosphorylation of RLC plays an important role in regulating cardiac function and its deficit may contribute to malignant FHC phenotypes. Reduced RLC phosphorylation observed in the D166V and R58Q hearts correlates with our cellular findings and could be responsible for delayed force transients, slower cross-bridge kinetics and decreased force observed in Tg-D166V and Tg-R58Q papillary muscle fibers. Supported by NIH- HL071778 (D.S-C.).

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Mouse HCM Model Expressing E99K ACTC Mutation Reproduces Phenotypes As Found In Human Patients

Weihua Song[1], Daniel J. Stuckey[2], Emma Dyer[1], Dominic Wells[3], Sian E. Harding[1], Carolyn A. Carr[2], Kieran Clarke[2], Steven B. Marston[1].