Myofibrillar Architecture in Engineered Cardiac Myocytes

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Report

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Morphogenesis is often considered a function of transcriptional synchrony and the spatial limits of diffusing mitogens; however, physical constrainment by the cell microenvironment represents an additional mechanism for regulating self-assembly of subcellular structures. We asked whether myocyte shape is a distinct signal that potentiates the organization of myofibrillar arrays in cardiac muscle myocytes. We engineered the shape of neonatal rat ventricular myocytes by culturing them on microfabricated fibronectin islands, where they spread and assumed the shape of the island. Myofibrillogenesis followed, both spatially and temporally, the assembly of unique actin networks whose architecture was predictable given the shape of the island. Subsequently, the *z* lines of the sarcomeres aligned and registered in distinct patterns in different regions of the myocytes in such a way that orthogonal axes of contraction could be distinctly engineered. These data suggest that physical constrainment of muscle cells by extracellular matrix may be an important regulator of myofibrillar organization.

Posttranscriptional processes such as translation or signaling pathways contribute to regulation of muscle growth during cardiac organo- and pathogenesis.[1,2] However, little is known about the mechanisms and signals that potentiate directional muscle growth and the self-assembly of the sarcomere and myofibril. Myofibrillogenesis during cardiac myocyte growth is marked by the serial alignment and parallel bundling of sarcomeres and is required for the temporal and spatial synchronization required for uniform contraction. Several models of myofibrillogenesis have been proposed.[3–6] These models of myofibrillogenesis focus on the temporal order of protein complex assembly at the spatial scale of the sarcomere. Thus, we asked whether increasing the length scale of interest to that of the whole muscle cell would reveal how extracellular boundary conditions can affect intracellular assembly of the contractile apparatus. Although regulation of cardiac myocyte function by excitation–contraction coupling, soluble mitogens, and mechanical stretch represents our greatest understanding of cardiac muscle development and control, recent studies have shown that cells respond to geometric cues in the extracellular matrix (ECM) with altered gene expression profiles, selective activation of intracellular signaling pathways, and microcompartmentalization of cellular processes.[7–9] These studies suggest that cell shape, controlled by ECM constrainment, can act as a distinct signal that can regulate cell growth in much the same way as the presence or absence of specific mitogens.

We set out to explore how ECM, and the alterations in cell geometry that it induces, might affect myofibrillogenesis in cardiac muscle cells. This is important because the mechanisms that regulate muscle growth and adaptation are not yet known, but changes in myocyte shape have been associated with maladaptive growth of the heart.[10,11] ECM binding via integrin proteins at the cell surface may guide both processes. We hypothesize that alterations in cytoskeletal architecture and myocyte shape during maladaptive hypertrophy are attributable, in part, to changes in the ECM, as has been demonstrated in noncardiac cells.[12–14]

Materials and Methods

Experimental methods for preparing the microfabricated substrates, myocyte harvest, fixation, staining, viewing, and statistics are discussed in detail in the expanded Materials and Methods section that is available in the online data supplement at http://circres.ahajournals.org. Briefly, freshly dissociated neonatal rat ventricular myocytes were seeded on fibronectin islands microcontact printed on glass coverslips coated with polydimethyl siloxane. After 24 to 96 hours in culture, as noted under Results, the cells were fixed and stained against proteins of interest. Subsequently, the cells and their cytoskeletons were viewed with an inverted fluorescent microscope equipped with a charge-coupled device camera.

Results and Discussion

In vivo, ventricular cardiac myocytes appear as in Figure 1A, with a high aspect ratio (myocyte length:width) and registration of sarcomeric *z* lines across their width, indicating a contractile direction along their lengthwise axis. When freshly harvested neonate cardiac myocytes are cultured on fibronectin (FN)-coated substrates (Figure 1B), they become pleomorphic, with sarcomeres located primarily in the perinuclear region, but absent in extended processes that resemble actin-based lamellipodia. We asked whether these cardiac myocytes would remodel to assume the shape of micrometer-sized islands of ECM protein and what effects the ECM geometry might have on myofibrillogenesis.

Our initial studies indicated that single cardiac myocytes isolated on an island of larger than 900 μm^2 would not spontaneously remodel and spread to assume the shape of the island, as previously observed with nonmuscle cells. By comparison, myocytes on islands of 900 μm^2 would spread enough to fill and assume the shape of the island. To facilitate

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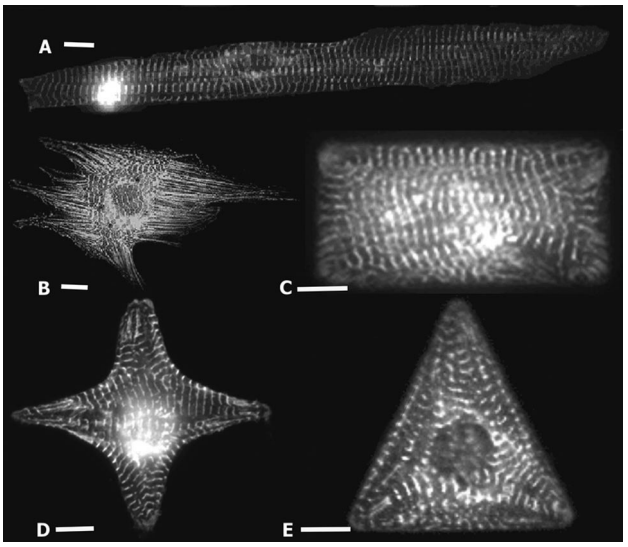

Figure 1. Neonatal cardiac myocytes immunostained against sarcomeric α-actinin. A, In vivo morphology. B through E, Pleomorphic on an unpatterned, FN-coated surface (B) and on a micropatterned FN rectangle (C), star (D), and triangle (E). All images are single, mononucleated ventricular myocytes. Patterned myocytes were cultured for 72 hours. Scale bar=10 μm.

muscle growth and to study the effects that the ECM might have on myofibrillogenesis, we coaxed the muscle cells to grow by stimulating beating with epinephrine (see the expanded Material and Methods section in the online data supplement for details), which resulted in autonomous contractile activity and the growth of the myocytes such that they often times occupied islands of 2500 μm^2 (Figure 1C and 1D). Beating myocytes cultured on micropatterned FN island displayed myofibrillogenesis throughout their volume. When myocytes cultured on unpatterned FN-coated substrates were quantitatively compared with myocytes cultured on square micropatterned FN islands, the 2D myofibrillar area of shape-controlled myocytes was significantly higher than that of the pleomorphic myocytes of the same area (Figure I in the online data supplement). Myocytes cultured on islands whose geometry included corners produced repeatable patterns of myofibrillogenesis that appeared similar to diffraction patterns emanating from the corners (Figure 1C, 1D, and 1E).

Myocytes whose growth in culture was limited to 2 days showed α-actinin fibers collocated with the actin fibers that were oriented toward the corners with sarcomeres arrayed in the perinuclear area (Figure 2 and supplemental Figure II). Thus, the *z*-line patterning that appears to emanate from the corners actually does the opposite: it converges on the corners. The alignment of actin toward the corners suggests that cardiac myocytes recognize angular cues as reference points for actin network assembly. This network then serves as a scaffold for myofibrillogenesis. This result is further illustrated by reorganization of the underlying matrix by the myocyte, where striations in the FN at the corners are observed where first actin fibers and, later, myofibrils terminate (supplemental Figure III). Previously, similar striations were reported in shape-controlled cells and demonstrated to be associated with vinculin-based adhesion plaques, where

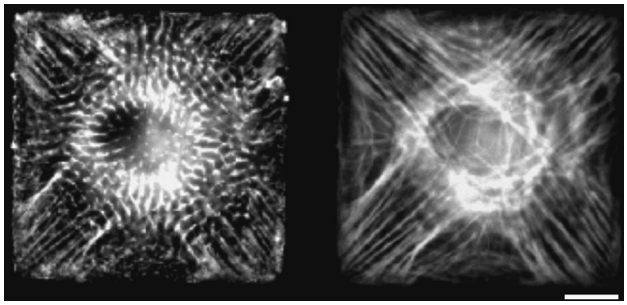

Figure 2. Neonatal cardiac myocyte cultured on a micropatterned FN island for 48 hours. Left, Fluorescent micrograph of immunostained sarcomeric α-actinin. Right, Fluorescent micrograph of actin stained with fluorescein isothiocyanate–conjugated phalloidin. Scale bar=10 μm.

actin stress fibers terminated, exerting traction forces on the substrate.[9]

Phase-contrast microscopy indicated wrinkles of the myocyte lipid membrane in the corners of myocytes cultured on islands with internal angles of 90° or less. This is illustrated in Figure 3, where wrinkles in the membrane are observed along the diagonals of larger square myocytes, indicating lines of mechanical stress, as we observed previously in fibroblast cells with atomic force microscopy and predicted by theoretical models of nonbiological membranes with mechanical stress applied at the corners.[15] In these myocytes, the myofibrillar patterning is striking in that it is repeatable and throughout the cell volume, as indicated by the multiple planes of myofibrils evident in the center of the myocyte, under the nucleus (Figure 2, right). Sarcomeric *z* lines register along the internal angle of corners until the nucleus is reached in the vicinity of the island and myocyte center. This data are interesting because where the healthy in vivo cardiac myocyte has all sarcomeres aligned for a preferential axis of contraction, those myocytes cultured in vitro on micropatterned islands could be engineered such that several contractile orientations, spatially distinct and ordered, could be spontaneously formed, suggesting that the corner geometry of the myocytes potentiated a distinct microcompartment whose contractile apparatus was assembled with respect to the local cue, rather than the global condition of the myocyte itself. This is particularly evident when examining star-shaped cardiac myocytes (Figure 1D).

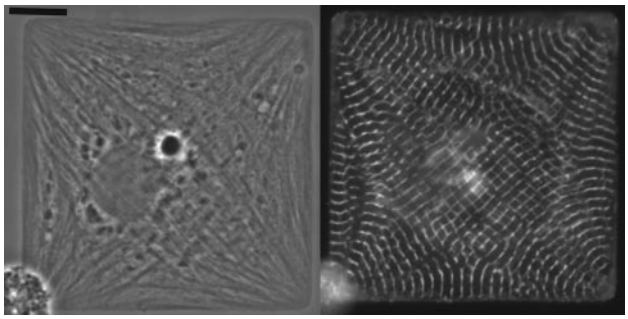

Figure 3. Cardiac myocyte cultured on a 50-μm square FN island for 72 hours. Left, Phase image, with membrane wrinkles apparent in the corners. Right, Fluorescent image revealing stained sarcomeric α-actinin at the *z* lines. Scale bar=10 μm.

We tested this hypothesis by culturing myocytes on circular FN islands. These myocytes lacked regular myofibril patterning, with *z* lines that appeared in a variety of patterns, such as a meshwork, or registered as secants within the myocyte or as spokes on a wagon wheel. The inability of circular cardiac myocytes to reproduce unique cytoskeletal architectures and myofibrillar patterning in response to their confinement, as illustrated in supplemental Figure IV by 2 immunostained myocytes cultured on adjacent FN islands, is reminiscent of previous results with capillary endothelial cells on circular islands that extended lamellipodia randomly from points around their perimeter and were also unable to assemble unique actin cytoskeletal networks.[9] These results suggest that an external cue is required to polarize the contractile cytoskeleton of cardiac myocytes.

Conclusion

Our data suggest that sarcomeres assemble sequentially along an actin fiber and that the actin network is a template whose topology is determined by the myocyte boundary conditions. Although sarcomeregenesis proceeds from the perinuclear region, geometric cues on the myocyte periphery dictate the alignment of the forming sarcomeres, suggesting that the cue, like the internal angle of a corner, encodes information and communicates it from the myocyte boundary to deep within the cell volume. From previous studies, we know the conduit for this information is the cytoskeleton and the signal is encoded as mechanical stress.[9] This pathway is suggested in the present data by the reorganization of the ECM in these internal angles and the eccentricity of myocyte nucleus. These results may offer insight to muscle tissue morphogenesis and the pathological consequences of myocyte shape change, specifically those observed in the cardiomyopathies characterized by contractile dysfunction.

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Disclosures

None.

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KEY WORDS: cardiac myocyte ■ myofibril ■ sarcomere ■ cytoskeleton

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Materials and Methods

All methods for handling laboratory animals were approved by Animal Use and Care Committee at The Johns Hopkins University School of Medicine.

Cell Culture

Ventricular myocytes were harvested from the hearts of two day old neonatal Sprague-Dawley rats as previously described and cultured in M199 culture medium supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 3.5 g/L glucose, 2mM L-glutamine, 2 mg/L vitamin B-12, and 50 U/ml penicillin.[1] Dissociated ventricular myocytes were cultured on micropatterned coverslips for two to seven days. The dissociated ventricular myocytes did not generally beat autonomously and were stimulated with a single dose of epinephrine (0.2 μM final concentration, Sigma) was administered after the myocytes had been seeded (100,000 cells/well in six well dishes) for 24 hours.

Preparation of Micropatterned Substrates

Micropatterned substrates were created with a previously published method.[2] Briefly, coverslips were cleaned in a UVO cleaner, oxidizing the PDMS surface and making it more hydrophobic and thus more receptive to stamped protein and subsequent treatment with F127 Pluronics to block protein adsorption on nonstamped regions. The elastomeric stamps are cleaned with 70% EtOH and blown dry under a nitrogen stream for 10s, and then coated with fibronectin (50 micrograms/ml) in PBS at room temperature for 1 hr. The stamps are then rinsed in sterile water and blown dry under a nitrogen stream for 10s, and placed gently on the oxidized PDMS face of the coverslip for 5-10 s. The stamp is then removed from the substrate and the coverslip is immediately immersed in a solution of F127 Pluronics (1% W/V) in sterile water for not less than 5 minutes. The coverslips are then rinsed in PBS and are ready for use.

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Immunoflouresence Microscopy and Image Analysis

Light and fluorescence microscopy was used to examine cell morphology and cytoskeletal architecture. Cytoskeletal proteins and fibronectin are immunostained as follows: Samples are fixed and permeabilized in 3.7% paraformaldehyde with 0.5% TritonX100 for 15 min and washed with PBS. To prevent nonspecific binding of secondary antibodies, a blocking procedure includes incubation for 15 min in 5% serum from the species source of the secondary antibody, 1%BSA in PBS. The samples are then incubated with primary antibody to the desired target in PBS for 1 hr, washed, incubated in flourescently-labeled secondary antibody in PBS for 1 hr, and washed. Phase and fluorescent microscopy were used to assess cell type, geometry, and intercellular architecture, as well as intercellular connections between myocytes and alterations to the micropatterned fibronectin island upon which the myocytes where cultured. Fibronectin, actin, sarcomeric α-actinin, and nuclear DNA were visualized using rabbit anti-fibronectin antibody, flouresceinated phalloidin, mouse anti-α-actinin antibody, and DAPI staining (all from Sigma, with secondary antibodies from Molecular Probes), as previously described.[3]

Quantitative morphometric analysis of myocyte shape and architecture was accomplished with phase contrast and epiflourescent microscopic images acquired by a CCD camera mounted on a Nikon microscope and analyzed with the analysis tools of IP Lab Spectrum (Scanalytics, Farifax, VA). The total projected area of the myocyte containing sarcomeres will be quantitated in a manner described previously.[9] Using computerized image acquisition and analysis tools of IP Lab Spectrum and RatioPlus software (Scanalytics, Fairfax, VA), images of cardiac myocytes cultured on micropatterned islands and immunostained against sarcomeric α-actinin were registered with phase and images of fluorescent actin. Mononucleated myocytes that covered the entire surface of each island were included in the analysis of patterned myocytes and compared

with unpatterned myocytes that were not in physical contact with adjacent cells. Any region of the myocyte with a sarcomere, defined as two parallel lines of sarcomeric α-actinin, was included in the area calculation. The pixel occupancy at each position relative to the cell shape was determined using IP Lab Spectrum software.

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3 Parker KK, Brock AL, Brangwynne C, Mannix RJ, Wang N, Ostuni E, Geisse NA, Adams JC, Whitesides GM, Ingber DE. Directional control of lamellipodia extension by constraining cell shape and orienting cell tractional forces. *FASEB J* 2002; 16: 1195-2004.

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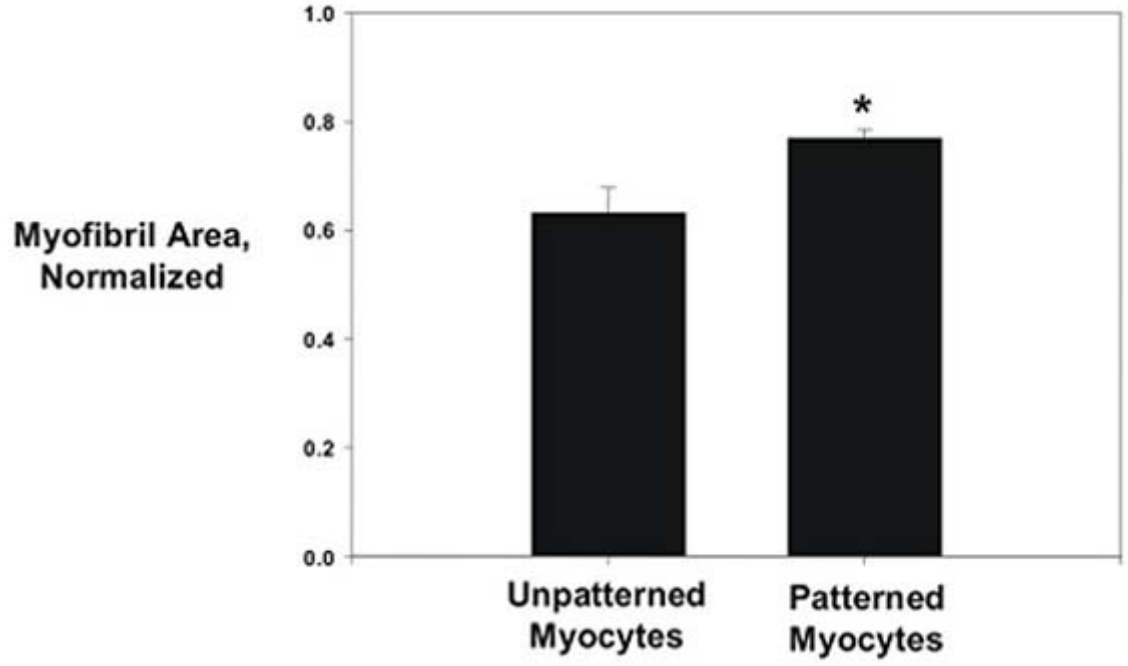

Fig. S1. Comparison of the myofibrillar content of patterned (n=31) versus unpatterned (n=18) mononucleated cardiac myocytes after 2 days in culture. * indicates $p<0.05$ with student T-test for unpaired samples.

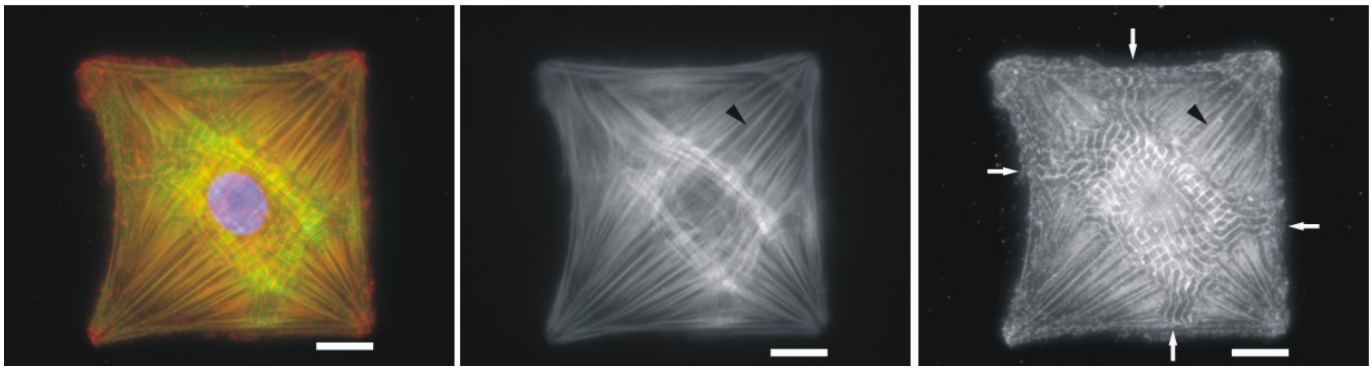

Fig. S2. Neonatal cardiac myocyte cultured on a micropatterned FN island for 48 hrs and stained against nuclear DNA (blue, DAPI), actin (green, FITC-phalloidin), and sarcomeric α-actinin (red, anti-sarcomeric α-actinin). Left) Merged flourescent micrograph; Middle) Actin fibers are aligned along the diagonals of the square. The black arrow indicates the wider spacing between stress fibers along on of the diagonals. Right) Sarcomeric α-actinin shows sarcomeres in the perinuclear region and fibers collocated with actin fibers on the diagonals. White arrows indicate that myofibrillogenesis has proceeded from the perinuclear region and reached the periphery, saddling the stress fibers that were more tightly bunched along the opposite diagonal. Note the eccentricity and orientation of the nucleus, along the diagonal with the greatest number of sarcomeres. Scale bar is 10 microns.

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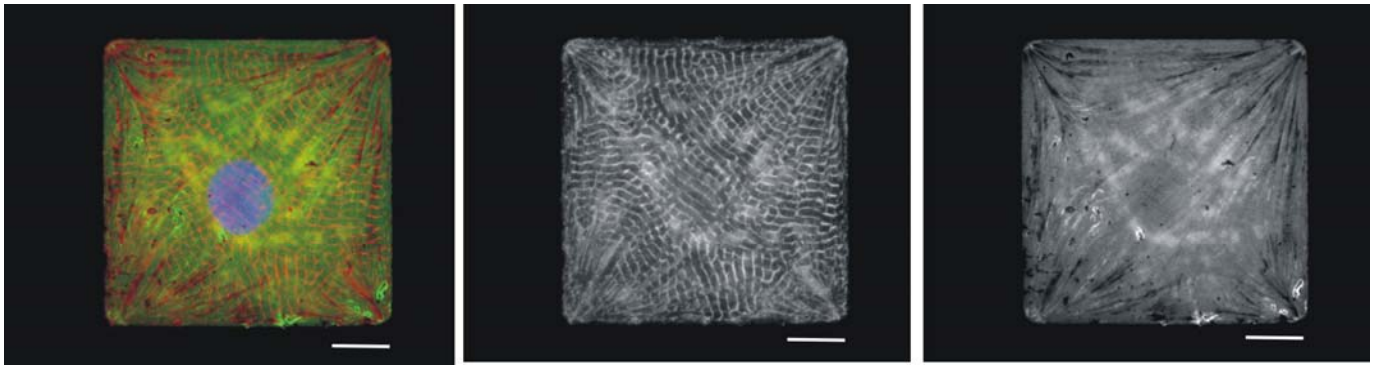

Fig. S3. Neonatal cardiac myocyte cultured on a micropatterned FN island for 48 hrs and stained against nuclear DNA (blue, DAPI), sarcomeric α-actinin (red, anti-sarcomeric α-actinin), and fibronectin (green, rat anti-human fibronectin). Left) Merged flourescent micrograph, note the circular nuclear shape; Middle) Sarcomeregenesis is complete throughout the myocyte. Right) Underlying fibronectin island with striations extending from the corners and mimicking the orientation of the myofibrils. Scale bar is 10 microns.

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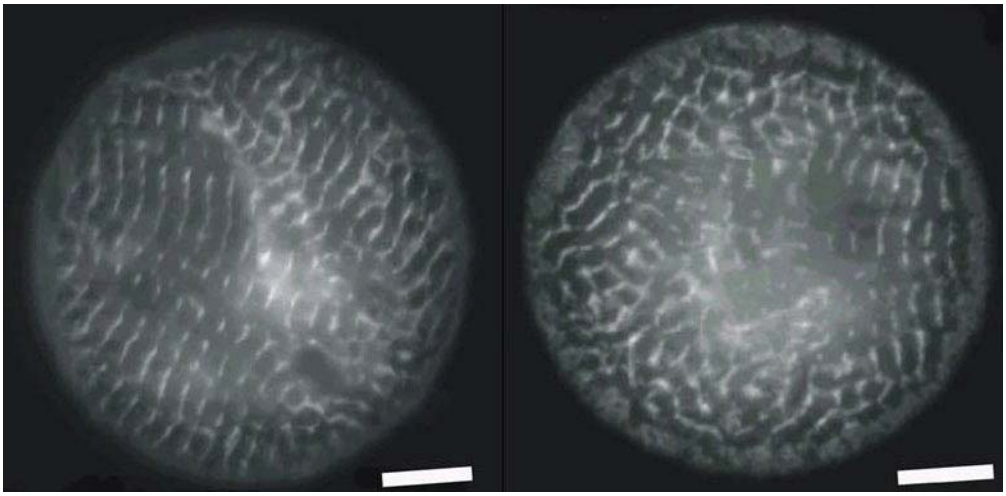

Fig. S4. Cardiac myocytes cultured for 72 hrs on 50 μm diameter FN islands and stained for sarcomeric α-actinin at the Z-lines. Scale bar is 10 microns.