Myofibrillar Architecture in Engineered Cardiac Myocytes

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Morphogenesis is often considered a function of transcriptional synchrony and the spatial limits of diffusing mitogens; however, physical constraint 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 myocytes 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 constraint of muscle cells by extracellular matrix may be an important regulator of myofibrillar organization.

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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 μm² (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). 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 2, 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. 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).

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 3 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 actin stress fibers terminated, exerting traction forces on the substrate.

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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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. 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. 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.
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 fluorescently-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, fluoresceinated 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 epifluorescent microscopic images acquired by a CCD camera mounted on a Nikon microscope and analyzed with the analysis tools of IP Lab Spectrum (Scanalytics, Fairfax, 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 $\alpha$-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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Data Supplement


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 fluorescent micrograph; Middle) Actin fibers are aligned along the diagonals of the square. The black arrow indicates the wider spacing between stress fibers along one 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.
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 fluorescent micrograph, note the circular nuclear shape; Middle) Sarcomereogenesis 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.
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.