Stiffness of the substrate influences the phenotype of embryonic chicken cardiac myocytes

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Abstract: We examined the effect of substrate stiffness on the beating rate, force of contraction, and cytoskeletal structure of embryonic chicken cardiac myocytes by culturing them on laminin-coated polyacrylamide (PA) substrates. Cells cultured on PA substrates with elasticity comparable to that of the native myocardium (18 kPa) exhibited the highest beating rate during the first few days of culture. The initial beating rate of individual cells on all the substrates varied significantly but began to converge within 5 days. We also examined the focal adhesions (FAs) and cytoskeletal structure on different substrates via confocal microscopy and found a higher percentage of FAs on tissue culture (TC) plastic dishes compared with the softer PA gels. Furthermore, highly aligned sarcomeric striations were clearly visible on 18 kPa, 50 kPa, and TC dish, whereas cells on 1 kPa only showed nonaligned diffused striations. The force of contraction on these substrates was measured using a micro-electromechanical system force sensor, which showed that the force of contraction for the cells on TC dishes (\(F = 71.30 \pm 6.38\) nN) was significantly larger than those cultured on the 18-kPa PA gel (\(F = 30.16 \pm 3.83\) nN). This is most likely due to the formation of higher percentage of FAs on the TC dishes compared with fewer FAs on the softer gels. Our cumulative findings can have a significant impact on the design of 3D cardiac tissue engineered scaffolds. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 95A: 1261–1269, 2010.

Key Words: substrate stiffness, focal adhesions, beating force, embryonic chicken, cardiac myocytes

INTRODUCTION
Living cells can sense their physical microenvironment and respond to mechanical cues with changes in their morphology, migration, division, and gene expression. Previous studies have shown that mesenchymal stem cells can differentiate to specific lineages/fates based on the stiffness of the substrates.1 Neurons have been shown to display increased branched actin structures on soft brain-like substrates compared with stiff substrates, whereas glial cells (i.e., astrocytes) from the central nervous system grow better on rigid substrates than on flexible ones.2 Fibroblasts have been shown to adjust their intracellular cell stiffness by changing their cytoskeletal structure to match the stiffness of the substrate on which they are cultured.3 Moreover, when NIH/3T3 fibroblasts were cultured on a substrate with varying rigidity along the surface, they readily migrated from the soft side of the substrate to the rigid side, which is called “durotaxis.”4 Myoblasts, which are the precursors of myotubes, differentiate optimally and show actomyosin striations only on substrates with stiffness typical of a normal muscle.5 Wang et al.6 demonstrated that stiffness of the substrate can also dictate whether a cell will grow or undergo apoptosis. Nontransformed NIH/3T3 fibroblasts cultured on soft substrates showed a decrease in the rate of DNA synthesis and an increase in the rate of apoptosis compared with the same cells cultured on rigid substrates.

The stiffness of the substrate also plays an important role in the development and the normal functioning of the myocardium.7 On average, the heart beats about 72 times a minute, which roughly translates to about 2.5 billion beats over the human lifetime. During this period, the heart responds to a variety of mechanical stresses based on the physiological conditions of the individuals. These mechanical stresses can be increased as a result of heart disease. The stiffness of healthy myocardium lies between the soft brain cells and the relatively hard cartilage/bone cells.8,9 Previous studies have indicated that after myocardial infarction, scar formation begins in the area around infarction and increases the stiffness of the myocardium locally by several folds.10 As a result, the force of contraction decreases, thereby affecting the performance of the left ventricles as well.11

Additional Supporting Information may be found in the online version of this article.
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In this study, the beating rate, force of contraction, and cytoskeletal structure of embryonic chicken cardiac myocytes (ECCMs) were quantified using a micro-electromechanical system (MEMS) force sensor by plating them on laminin-coated polyacrylamide (PA) substrates with stiffnesses of 1–50 kPa and tissue culture (TC) dishes. The beating rate of the cells cultured on the 18-kPa PA gel was the highest during the first few days. However, by the fifth day, the cells showed similar beating rates on all the substrates. The cells cultured on the rigid substrates showed higher percentage of focal adhesions (FAs) compared with those cultured on the softer substrates. Highly aligned sarcomeric striations were visible on the stiff substrates, whereas cells on the soft substrate only showed nonaligned diffused striations. It was also found that cells on rigid TC dishes generated larger forces most likely by forming higher number of FAs compared with 18-kPa PA gel. Because the stiffness of the biomaterial/substrate dictates the behavior of the cells, understanding it better can result in improved therapies for many heart-related diseases and for engineered heart tissue.

MATERIALS AND METHODS

PA gel preparation and calibration

PA gels were prepared following the protocols described by Wang and Pelham. In brief, PA gels of increasing stiffness were obtained by changing the concentration of acrylamide solution (BioRad Laboratories, Hercules, CA) from 5 to 8% and the concentration of bis-acrylamide solution (BioRad Laboratories) from 0.04 to 1.2% in 10 mM HEPES (Sigma, St. Louis, MO). These gels were then activated with 1 mM Sulfo-SANPAH (Thermo Scientific, Rockford, IL) and conjugated with 5 μg/mL of laminin (Sigma). To test the attachment of the protein as a function of substrate stiffness, fluorescently tagged bovine serum albumin (Invitrogen, Carlsbad, CA) was coated on the gel, and it was found that the protein concentration was independent of the stiffness (see Supporting Information Fig. S1). The coating concentration has also been shown to be independent of the stiffness of the gel with other proteins such as collagen I, collagen IV, laminin, and fibronectin.

To mimic a wide range of mechanical microenvironments, gels with physiologically relevant stiffnesses were produced. Soft PA gels with stiffness 1.05 ± 0.17 kPa were used to mimic mammary gland, brain, and breast tissue, which have physiological stiffness ranging from 0.1 to 2 kPa. Intermediate hard gels with stiffness 18.31 ± 0.19 kPa were used to mimic chicken embryonic myocardium, which have physiological stiffness ranging from 9 to 20 kPa. Hard gels with stiffness 50.68 ± 0.92 kPa were used to mimic cartilage and bone. TC dishes served as the control for the study (stiffness of TC ~ 3 GPa).

An Asylum atomic force microscope with precalibrated silicon nitride tip was used to characterize the stiffness of PA gels in phosphate-buffered saline (PBS, pH 7.4; Invitrogen) solution.

Cardiac cell isolation and culture

Cardiac cells were obtained from chicken embryos during the 32–35 Hamburger Hamilton stage, which is approximately 8 days after conception. After sterilizing the eggshell with 70% ethanol, the embryo was removed, placed in a petridish containing Puck’s saline, and quickly decapitated. The heart was removed, rinsed twice in PBS solution to remove the blood, and incubated in a solution containing 0.05% trypsin/PBS (Invitrogen) for ~ 15 minutes at 37°C. Following enzymatic digestion, the solution containing the heart was pipetted gently to dissociate the tissue. About 100,000 cells/cm² were seeded on the laminin-coated substrates and cultured in minimum essential medium with alpha modification (Sigma), supplemented with 10% fetal bovine serum (Sigma) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Invitrogen). During data acquisition, the gels and the culture dishes were kept on a heated microscope stage to maintain a temperature of 37°C, and a tube releasing 5% CO₂ was kept over the dishes to maintain a physiologically relevant pH.

Immunofluorescence and imaging

Cells cultured on the PA gels and TC dishes were immunolabeled on the second and the fifth day in the study. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 min and then permeabilized with 0.2% Triton X-100 (Sigma) for 15 min. Cells were then blocked with Image-IT FX (Invitrogen) for 30 min. They were incubated in monoclonal mouse anti-vinculin (Sigma) at a 1:200 dilution in PBS at 4°C overnight. These cells were permeabilized again in 0.2% Triton X-100 for 15 min and incubated in fluorescein isothiocyanate-conjugated goat anti-mouse (BioRad Laboratories) at a 1:200 dilution in PBS at 37°C for 2 h. Cells were then incubated for 2 h in 6.6 μM rhodamine-phalloidin (Invitrogen) and finally in 500 nM of DAPI (Invitrogen) for 5 min at 37°C. The immunolabeled cells were mounted in ProLong gold (Invitrogen) antifade reagent to prevent photobleaching. The cell preparations were imaged by a Zeiss LSM 710 confocal scanning laser microscope at 40×, NA 1.4 water immersion objective.

Image processing and image analysis

Images were processed using Imagej version 1.42q. Area of the cell was found by first creating a maximum z-projection from the confocal images and then thresholding it so that the entire cell area was covered. Any object smaller than 3 μm in radius was eliminated using the software to get rid of any cellular debris and then the percentage of the area covered by the cells was computed by the software. This percentage was converted to an area in square microns. To find the percentage of FAs, one confocal section per image was used, which is approximately 0.50 μm from the interface between the cell membrane and the coverslip. The images were thresholded, and a Watershed segmentation algorithm was applied to the images to see only the FAs. Imagej was also used to create fluorescence intensity line profiles over FAs of actin and vinculin images around
different regions of the cell. The cells were found independently for computing the FA percentage.

Statistical analysis
Statistical analysis was performed using one-way ANOVA in OriginPro 8.1. Unless otherwise mentioned, the values reported are mean ± standard error of the mean (SEM), and the results were considered statistically significant when the p value was less than 0.05 (p < 0.05).

RESULTS
Beating rate of cardiac cells versus substrate rigidity
ECCMs were seeded on laminin-coated PA gel substrates of varying stiffnesses, and the beating rate of the cells was monitored as a function of time over 5 days. Cells were independently chosen on all the substrates, and a 30-s video of the beating cells was taken every day for 5 days in culture. Figure 1(A) shows the variation in the average beating rate of the cardiac cells over this period. Note that the initial average beating rate varies significantly ranging from <20 beats per minute on the rigid TC dishes to 100 beats per minute on the 18-kPa gel. However, the average beating rate converges to a more narrow range around 60–80 beats per minute by the fifth day. Figure 1(B) shows the beating rate of the cardiac myocytes as a function of substrate stiffness after 1 and 5 days post-seeding. The 18-kPa substrate, which mimics the stiffness of the myocardium, shows the highest beating rate on the first day of culture. However, by the fifth day, the beating rate of the cells converge to a narrow range of 60–80 beats per minute and no longer follows a bell curve.

FA formation and cytoskeletal organization of cells on the different substrates
Formation of FAs and the cytoskeletal organization of the cell were examined via confocal microscopy on the various substrates on the second and fifth day. FAs are specialized sites of adhesion developed by many cells in culture. They serve as the pivots to assist the intracellular pre-stress build-up and are the sites at which cell traction is transmitted to the substrate. They link the extracellular matrix components to intracellular cytoskeleton (actin filaments) via integrin receptors. FAs can be thought of as a sensory organ capable of responding to a variety of diverse external features such as ligand density, topography, and rigidity. FAs are composed of many different types of proteins, such as paxillin, vinculin, and talin. However, it has been proposed that vinculin depletion leads to drastic changes in the motility of cells and FA sizes. Furthermore, vinculin is the most abundant FA protein. Hence, the cells on these substrates were labeled with anti-vinculin antibody.

The formation of FAs is a highly orchestrated event starting from: (1) Rho-stimulated contractility, (2) the generation of isometric tension in adherent cells, (3) bundling of actin filaments, (4) aggregation of integrins, and (5) activation of the focal adhesion kinase. To produce a more visual illustration of the relative intensity of actin and vinculin, fluorescence intensities in 1D line profiles drawn over FAs was compared and is shown in Figure 2. It can be seen from the line profile that the intensity of actin and vinculin present on the soft (1 kPa) gel is small compared with the other substrates. The cells are not able to form many FAs because there is not enough actomyosin-based contractions on the soft substrate. Because one of the first events in the formation of FAs is reduced on the soft gel, other downstream events are also hindered and hence few FAs form on this substrate. This is consistent with previously published results that a smaller force is required to peel off cells from soft gels compared with the rigid glass. On the other hand, the pixel intensity values for actin and vinculin is high on the stiff (50 kPa) gel and the rigid (TC dish) substrate. Furthermore, there is...
colocalization of actin and vinculin, which leads to aggregation of integrins at the FA sites.\textsuperscript{30} On these stiff surfaces, FA development is possible by enhanced tyrosine phosphorylation on multiple FA proteins.\textsuperscript{33} In addition, a higher degree of organized cytoskeleton emerges with the increase in stiffness as shown in Figure 2. On the soft 1-kPa substrates, formation of tissue-like aggregates can be seen and only small nascent FAs are visible, whereas on the other substrates, cells acquire a more spread morphology and show well-defined prominent FAs of larger size.

Small nascent FAs, also referred to as focal complexes (FXs), are formed on soft 1-kPa substrates.\textsuperscript{34} These small FAs cannot provide the necessary adhesion to propel the cell forward. As a result, these FAs quickly disassemble, and the cells on the soft 1-kPa gel acquire a tissue-like morphology, where a lot of cells are present in the aggregate. However, on the comparatively stiffer substrates, small FAs quickly mature and form larger FAs, providing the necessary traction forces for the cells to acquire a more spread morphology.\textsuperscript{33,35,36} There is not a statistical difference in the percentage of FAs present on fibroblasts ($p = 0.49$) and cardiac cells ($p = 0.95$) for the two different days, suggesting that after the first 2 days, properties of FAs do not change much. This also suggests that with time, FAs change their main function from the transmission of strong propulsive forces to a more passive function of providing adhesion sites for...
the maintenance of a well spread cell morphology. Fibroblasts were identified from cardiac cells based on the sarcomeric striation patterns seen on the cardiac cells. Figure 3(C) shows that the number of FAs per cell ($p < 0.05$) and the FA area per cell ($p < 0.05$) are much higher on the rigid (TC) substrate compared with the soft (1 kPa) gel substrate. The means of all these parameters are statistically different for the rigid and soft gels at $p < 0.05$.

Figure 4(A) shows the actin cytoskeleton on the soft 1-kPa PA gel. It can be seen that even though there are striations visible on the cells on this soft gel, which is a hallmark of any striated muscle cell, these striations are not aligned as seen in the sarcomere in vivo. On the other hand, highly aligned sarcomeric striations are seen in Figure 4(B,C,D) on the 18-kPa, 50-kPa PA gel and the rigid TC substrate.

**Measurement of the contraction force for cardiac cells**

A novel MEMS force sensor was used to perform a force spectroscopy on cells that were cultured on 18-kPa PA gels and TC dishes for the second and fifth day. The fabrication process and other applications of this MEMS force sensor have been described previously. The resolution of the
MEMS sensor was \(~0.25\) nN. The MEMS force sensor has two beams, a fixed (reference) beam and a free (measurement) beam, which touches the cell cluster as seen in Figure 5(A). The MEMS sensor was kept in a solution of 70% ethanol for 2 h under UV light to sterilize the sensor before starting the measurements. The MEMS sensor was cleaned by sterilized DI water multiple times before experiments to remove any ethanol residue or micro-physisorptions on device. To eliminate the external mechanical indentation, the forward movement of the beam was stopped right after contacting the cell body (see Fig. S2). The cyclic contraction force, \(F\), was quantitatively obtained from, \(F = k \Delta x\), where, \(k = 48.96\) nN/\(\mu m\), the spring constant of the force sensor, and \(\Delta x\) is the deformation of the sensor spring. Figure 5(B) shows the phase-contrast image of the MEMS probe in contact with the 18-kPa gel, with the sensor probe contacting the cell body along the direction of contraction.

Figure 5(C) shows the force profile of the cardiac cell on both the 18-kPa gel and the TC substrate as a function of stiffness on both the second and fifth days. The force exerted on the MEMS sensor by the cells cultured on TC dish (\(F_\text{5th day} = 71.30 \pm 6.38\) nN) was statistically higher than that by the cells cultured on the soft 18-kPa gel (\(F_\text{5th day} = 30.16 \pm 3.83\) nN) at \(p < 0.05\), and the same trend was seen for FAs as well. The isometric contractile stress that cardiac myocytes applied on their fibroblast surrounding can be estimated by considering that the beating force measured by the force sensor is only sampled from a portion of the cell membrane (contact area of \(~25 \mu m^2\)). The value of the stress on the TC dish was calculated to be \(3.2 \) nN/\(\mu m^2\), whereas that on the 18-kPa gel was \(2.7 \) nN/\(\mu m^2\) on the second day. These values are close to the contractile stress of neonatal rat cardiac myocytes (5.5 \(\pm 2\) nN/\(\mu m^2\)), which has been reported elsewhere.40

**DISCUSSION**

ECCMs were seeded on laminin-coated PA gels with a range of stiffness from 1 to 50 kPa and their beating rate was monitored as a function of time over 5 days. It was found that the cells beat with the highest frequency on the substrate, which mimics the stiffness of the myocardium (\(~18\) kPa) as previously shown.41 However, in our study, the cells had the highest beating rate on the 18-kPa PA gel only during the first few days. After that, the beating rate for the cells on all the substrates converged to a narrow band between 60 and 80 beats per minute. A plausible mechanism for this is that during the initial days, when the cells are seeded on the substrate, no cell–cell interactions are possible because a confluent cell sheet is not formed. However, by the second day, cells started forming cell–cell junctions, and a cell network was seen forming on the stiffer substrates (18 kPa, 50 kPa, and TC). Fibroblasts started proliferating between neighboring cardiac cells, which linked them together. It has been shown that the beating rates of cardiac cells within 300 \(\mu m\) can be synchronized through the fibroblast connection,42 and a similar phenomenon was
observed during our experiment when two cardiac cells were connected via a fibroblast (see Supporting Information Movie S1). It is known that fibroblasts do not extend their processes on soft substrates (1 kPa) and remain rounded.36 So how do the cells on the soft 1-kPa gel beat around the same frequency as well? One of the reasons could be that the beating frequency of the cells reverts to a natural beating frequency that is inherent to the cell as seen in previous studies.43 Another possible reason could be that when the cells are close to each other, the substrate itself may serve as a mechanical link between the substrates. Contraction of a cell during beating results in stretching of the nearby substrate, which in turn stretches the neighboring cells’ membrane and a cascade of signaling mechanisms start in this neighboring cell (see Supporting Information Movie S2). It would be interesting to see if this convergence phenomenon is still seen on the stiffer substrates when an enrichment of the cell mixture is done to limit the number of fibroblasts by pre-plating this cell mixture before seeding them on the different substrates.

Previous reports have shown that optimum actomyosin striations were seen on substrates that mimic the stiffness of the myocardium.5,44,45 However, even on the rigid TC substrate, we were able to see highly aligned sarcomeric patterns. This could be because cell–cell contacts might have a similar effect as cell-on-gel effect when a cellular network is formed. This was also seen in another study when endothelial cells had indistinguishable morphology on the stiff and the soft substrates when they reached confluence.36 Furthermore, fibroblasts, which generally cannot extend their processes and remain rounded on the soft gels, are seen to have a well spread morphology when cell–cell contacts are established.36 Myofibrillogenesis involves the precise stacking of multiple linear array of units into a structured sarcomere.46 It starts with the formation of pre-myofibrils at the periphery of spreading cardiomyocytes and get bundled into mature myofibrils over time.37 However, because the soft 1-kPa substrate cannot provide enough actomyosin-based contractions, formation of aligned sarcomeric structure is not seen on this substrate. On the other three substrates, enough tension is developed from the mature FAs that the entire process of myofibrillogenesis is possible.

It has been well known that most normal cells in cell culture are anchorage dependent.31 FAs are the sites of attachment from the intracellular cytoskeleton via integrin receptors to the extracellular matrix. Formation and development

FIGURE 5. MEMS force measurement on the substrates (A). The principle of operation (B). Phase-contrast image showing a cardiac cell aggregate with MEMS force sensor attaching from right side to measure the beating force during contraction–relaxation cycle. Cell is on 18-kPa gel on the fifth day of culture. (C) The force profile of the cardiac cell cluster sensed by the MEMS force sensor as a function of time on the 18-kPa PA gel and the TC dish. (D) Bar graphs showing the average peak force exerted by the cardiac cell cluster on the 18-kPa PA gel and the TC dish on the second and fifth day. Asterisks indicate statistical significance between the average peak force on day 5 between 18 kPa PA gel and TC dish (3E6 kPa). At \( p < 0.05 \), the means are statistically different. Data shown are the mean ± standard deviation (SD; \( n = 3 \)). Scale bar: 50 \( \mu \)m.
of FAs require generation of isometric tension by movement of myosin II motors on the actin filaments. Because cells on the soft substrate (1-kPa PA gel) cannot develop this isometric tension by the actomyosin contractions, few FAs are formed on this substrate. Furthermore, the FAs that are formed on this substrate are mostly immature because aggregation of integrins is not possible on the soft substrate because of the reduced actomyosin contractions. As a result, cells on this substrate resemble tissue-like morphology where a lot of cells are present in the aggregate. On the other hand, cells on stiffer substrates are able to generate isometric tension by actomyosin contractions leading to their maturation. It should be noted that the morphology of cells on the 50-kPa PA gel is similar to that on the rigid TC dish. Furthermore, as seen in Figure 3(B), there is a small increase in the percentage of FAs from the 50-kPa substrate to the TC substrate, and the graph seems to reach a steady value. This means that after a certain threshold, the stiffness of the substrate is no longer the dominant factor in the formation of FAs. Any further increase in the stiffness of the substrate after the threshold stiffness will only lead to small changes in the overall morphology of the cells and growth of FAs. However, for the cells to perform their functions optimally, a stiffness matching between the cell and the substrate is desired.41

A novel MEMS-based force sensor was used to measure the force of contraction of the cardiac cell cluster on the 18-kPa PA gel substrates and the TC dish. It was found that the force of contraction as seen by the MEMS sensor was larger on the TC dish than on the 18-kPa gel. This higher force on the rigid TC dish could be because of the formation of higher percentage of FA on this substrate compared with the 18-kPa gel as shown in Figure 3(B). It should be noted that the force exerted on the MEMS sensor might not be the total beating force of the cardiac cell cluster. Part of the force is lost in the displacement of the PA gels when the cell is contracting. In addition, the size of the cluster chosen might have an important effect on the force seen by the MEMS sensor. The force seen by the MEMS probe on the rigid TC dish on days 2 and 5 was not different as shown in Figure 5(D). Also, the percentage of FAs remained similar during this period on the TC dish as shown in Figure 3(B). The sensor is based on the principle of Hookean mechanics, $F = k\Delta x$; therefore, if the placement of the sensor is not optimum for sensing the maximum contraction, a big difference in the force is possible. A shortcoming for the sensor is that it is dependent on the size of the cluster and the location of the sensor with respect to the cluster. If both these parameters are not optimized, an error in the force measurement is possible. Although these are some of the uncertainties associated with the MEMS sensor, it can be used effectively to give a firsthand approximation for the force of a beating cardiac cell/cluster.

CONCLUSIONS

We have shown that ECCMs beat with the highest beating rate on the substrates with tissue-like stiffness only during the first few days after plating, and later the beating rate becomes independent of the stiffness. However, the cells form bigger and higher number of FAs with an increase in the stiffness of the substrate resulting in higher forces on the stiffer substrates. Furthermore, highly aligned sarcomeric striations are only seen on the stiffer substrates, whereas the soft substrate shows nonaligned striations. Given the profound effect of stiffness of the substrate on the behavior of cardiac cells, future scientists and engineers must take into account the physical parameters of the materials, such as rigidity, in designing the next generation of biomaterials.

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