Published in final edited form as:

Ann N Y Acad Sci. 2010 February ; 1188: 121–127. doi:10.1111/j.1749-6632.2009.05091.x.

Cardiac Myocyte Force Development during Differentiation and Maturation

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Abstract

The maturation of cardiac myocytes during the immediate prenatal period coincides with changes in the mechanical properties of the extracellular matrix. We investigated the effects of extracellular stiffness on cardiomyocyte maturation in neonatal rat ventricular myocytes grown on collagen-coated gels. Cells on 10 kPa substrates developed aligned sarcomeres, while cells on stiffer substrates had unaligned sarcomeres and stress fibers. Cells generated greater mechanical force on gels with stiffness similar to the native myocardium than on stiffer or softer substrates. To investigate the differentiation of myocyte progenitors, we used clonal expansion of engineered human embryonic stem cells. Puromycin-selected cardiomyocytes exhibited a gene expression profile similar to that of adult human cardiomyocytes and generated force and action potentials consistent with normal fetal cardiomyocytes. These results suggest extracellular stiffness significantly affects maturation and differentiation of immature ventricular myocytes.

Keywords

cardiac myocyte; mechanotransduction; substrate stiffness; gel; stem cell; differentiation

Introduction

As the embryonic and neonatal heart develops, the mechanical environment of the developing cells changes. For example, collagen accumulation in the myocardium begins during embryonic development and continues to accumulate until several weeks after birth¹. This in turn affects the mechanical coupling between myocytes and the external viscoelastic loading against which they contract. The mechanical load on a contracting cell can be varied by altering the elastic modulus of the cell substrate. On a softer substrate, a cardiomyocyte shortens faster and further and, owing both to the force-velocity relation and the Frank-Starling relationship between force and length, produces less force acting on its neighbors and surrounding. Substrate stiffness can be modified through changes in monomer to crosslinker ratio in crosslinked polymers, including polyacrylamide hydrogels² (see Fig 1),

polydimethylsiloxane (PDMS) gels 3, alginate gel 4, polyethyleneglycol (PEG) 5, and many other crosslinked polymers 6.

Other methods of controlling substrate stiffness include varying agarose concentrations in agarose gels ⁷ and varying the porosity of porous gels such as Poly(1,8-octanediol-co-citric acid) (POC) ⁸. In addition to just controlling the overall stiffness of a cell substrate, studies have demonstrated methods of generating patterns or gradients in substrate stiffness using a microfluidic channel device ⁹ or patterned photopolymerization under a mask or partial shield ^{10, 11}. Alterations in substrate stiffness have been shown to affect the behavior of many anchorage-dependent cell types, including neurites, fibroblasts, myocytes, endothelial cells and mesenchymal stem cells, as reviewed previously ^{12, 13}.

Effects of Substrate Stiffness on Neonatal Cardiomyocyte Maturation

Our group has examined the substrate stiffness dependence of the maturation of neonatal rat ventricular myocytes (NRVMs), as quantified by cytoskeletal organization, force generation and the development of calcium stores ¹⁴.

Using a modification of traction force microscopy for dynamically contracting myocytes adhered to a collagen-coated polyacrylamide gel (see Fig 2), we found that NRVMs formed aligned striations (Fig 3), generated the greatest peak force and had the largest calcium transient peaks when cultured for 7 days on polyacrylamide hydrogels with an elastic modulus of 10 kPa.

Cells cultured on both softer and stiffer hydrogels generated lower contractile forces with lower calcium transients (see Fig 4).

The concentration of intracellular calcium correlated with the amount calcium stored in the sarcoplasmic reticulum and expression of the sarcomeric calcium pump SERCA2a, both of which were also greatest on 10 kPa gels. As a reference, the elastic modulus of the left ventricle of healthy adult Lewis rats was estimated as 18 ± 2 kPa, and this increased to 55 ± 15 kPa in infarcted areas ¹⁵.

We further observed that the percentage of beating cardiomyocytes increased with decreasing elastic modulus ¹⁴. This result agrees with another study of cardiomyocytes on much softer materials that used PEGylated fibrinogen gels with varying concentrations of reactants and a diacrylate crosslinker in order to create varying elastic modulus with a shear modulus range from 8 to 340 Pa (tensile modulus around 20-1,000 Pa, depending on the material Poisson ratio). After four weeks of culture on those gels, neonatal rat ventricular myocytes on the softest moduli had the highest percentage of beating cells and the highest correlation of beating times and frequencies across the constructs ¹⁶. One subsequent study has confirmed this result using cardiomyocytes from quail chick embryos ¹⁷. This study found that the spontaneous beating frequency, as well as the percentage of beating cells, increased as the stiffness of the underlying substrate decreased.

Effects of Substrate Stiffness on Mesenchymal Stem Cell Differentiation

No study has linked substrate stiffness directly to cardiomyocyte differentiation from stem cells or other precursors. However, research has shown that substrate stiffness alone can affect the differentiation of mesenchymal stem cells into myogenic cells, as shown through cell morphology, presence of striations and the expression of several myogenic markers including Myogenesis Differentiation Protein 1 (MyoD1), which has a peak in expression in cells on gels with an elastic modulus of 10 kPa and is nearly undetectable in cell on gels with elastic moduli above 20 kPa or below 2 kPa ¹⁸. Additionally, another group reported that striations in C2C12 myotubes form only when cells are plated in a very small elastic

modulus range on polyacrylamide gels, centered at 12 kPa 19, while later research confirmed that C2C12 cells on alginate gels with an elastic modulus below 10 kPa do not differentiate and form myotubes, but found no reduction in myotube formation or activity of the myogenic marker muscle creatine kinase (MCK) in cells grown on stiffer substrates, up to 50 kPa 20.

Differentiation of Embryonic Stem Cells into Cardiac Myocytes: Effects of Stretch

Embryonic stem cells can spontaneously differentiate into cardiomyocytes in serum-containing media and can be driven toward differentiation into the major components of heart muscle tissue or the conduction system. In general, cardiogenesis in embryonic stem cell cultures is indicated by spontaneous beating, the shape of action potentials and calcium transients, the presence of specific ion currents, and by the expression of specific cardiac cell markers. The differentiation into cardiac tissue is denoted by the termination of certain pluripotency markers (such as Oct-3/4, fibroblast growth factor-5 (FGF-5) and Nodal), the expression of early cardiac markers (such as the transcription factors Nkx2.5 and GATA-4, and sarcoplasmic/endoplasmic reticular calcium ATPase 2a (SERCA2a)) and the expression of some late-stage cardiac markers (such as α - and β -MHC, the ryanodine receptor, cardiac troponin-T, and calsequestrin). An overview of differentiation times and markers has been previously reviewed for mouse embryonic stem cells ²¹.

One recent study showed that mouse embryonic stem cell embryoid bodies increased the percentage of beating cells and the percentage of cells expressing sarcomeric α -actinin when statically stretched for 2 hours and that this effect was graded over 5%, 10%, 15% and 20% radial strain. These cells also increased expression of cardiac markers MEF2c and GATA-4 when stretched by 10%. The demonstrated cardiogenesis was inhibited with free radical scavengers vitamin E and N-(2-mercapto-propionyl)-glycine, though these treatments further enhanced the upregulation of GATA-4. Interestingly, angiogenesis, indicated by the formation of capillary-like structures and the expression of PECAM-1, increased with increasing strain up to 10%, then decreased with further strain back to basal levels at 20% strain ²².

Mechanical stretch has been shown to inhibit differentiation as well. At low frequencies of stretch (10 cycles/min), 10% stretch tended to decrease the differentiation of human embryonic stem cells and keep them in a pluripotent state ²³. Furthermore, the application of shear stress has been shown to induce the early cardiac and smooth muscle cell markers vascular endothelial growth factor receptor 2 (VEGFR-2), smooth muscle actin, smooth muscle protein 22- α , MEF2c, α -sarcomeric actin, and PECAM, all downstream of a remodeling of chromatin structure ²⁴.

Several studies also found effects of mechanical activation on the maturation of cardiomyocyte-like cells that already differentiated from embryonic stem cells. One research group used mouse embryonic stem cells that were hand-selected for beating colonies, which were then verified for expression of cardiac α -MHC, cardiac α -actin, GATA-4 and Nkx2.5 mRNA. These cells were then seeded onto poly(lactide-co-caprolactone) (PLCL) elastic scaffolds. Cells on scaffolds that had been cyclically stretched for 2 weeks at 10% strain and 1.0 Hz had increased expression of cardiac α -MHC, cardiac α -actin, GATA-4 and Nkx2.5 mRNA compared to control unstretched cultures. These stretched cultures also integrated electrically into the myocardium of infarcted rat hearts, beating in synchrony with the heart, while unstretched cultures did not have synchronous beating ²⁵. Another report found that contractile markers in murine embryonic stem cell-derived cardiomyocytes, selected by transfection of an α -myosin heavy chain (MHC)-promoter-driven gene conferring resistance to Genetecin (G418) and embedded in a collagen-fibronectin scaffold are highly sensitive to the frequency of 10% mechanical stretch. While the expression of α -cardiac actin increased

with frequency of stretch of 1, 2, or 3 Hz, the expression of α -skeletal actin, α -MHC, and β -MHC decreased after 3 days of 1 Hz stretch, but increased after 3 days of 3 Hz stretch. The transcription factor GATA-4 decreased with 1 Hz stretch, but was not significantly different after higher stretch frequencies 26. One study used stretch in order to both condition and align stem cell-derived cardiomyocytes, though these were not compared to unstretched samples so the added benefit of stretch is difficult to determine 27.

Studies of myocytes cultured from embryos have shown that stretch can both aid in proliferation of cells and maturation of functional properties of these myocytes. Embryonic (day 7) white Leghorn chicken cardiomyocytes attached to collagen-coated rubber and radially stretched by 20% at 2 Hz doubled their proliferation, measured by cell number and BrdU uptake 28. Embryonic (day 7) or fetal (day 14) White Leghorn chicken ventricular cells embedded in Type I collagen gel and uniaxially stretched at 0.5 Hz by 8% (embryonic) or 4% (fetal) had increased active stress compared to unstretched cells. The constructs also had decreased cross-sectional areas and increased passive stress in fetal constructs and proliferation in embryonic constructs. Stretch did not increase the calcium sensitivity, response or isoproterenol or upregulation of the cardiac markers α -actinin or β -actin in these cells 29.

Effects of Substrate Stiffness on Embryonic Stem Cell-Derived Cardiomyocyte Progenitors: Preliminary Investigation

Recently, Kita-Matsuo *et al* 30 described new methods to isolate and visualize large numbers of fluorescently labeled, functional cardiomyocytes obtained by clonal expansion of engineered human embryonic stem cells expressing Puromycin resistance protein under the control of the cardiomyocyte-specific α -myosin heavy chain (α MHC) promoter. Drug selection yielded beating embryoid bodies (“cardiospheres”) 96% pure in cardiomyocytes that could be cultured for over four months. To characterize the contractile function of these embryonic stem-cell derived cardiac myocytes, cardiospheres were isolated at day 12-13.5 and cultured until day 48 when they were dispersed and deposited onto gelatin-functionalized surfaces of polyacrylamide cast with fluorescent beads and analyzed for force generation at day 50 using traction force microscopy (Fig 5)30. We found that human embryonic stem cell-derived cardiomyocytes generated axial and total traction forces on 4 kPa gels comparable in magnitude to those generated on similar soft gels by neonatal rat ventricular myocytes, but significantly lower than the maximal forces seen in neonatal myocytes cultured on stiffer gels. Corresponding average contractile peak stresses were 220 ± 70 Pa.

We also used this system to study the effects of substrate stiffness on differentiation of human embryonic stem cell-derived cardiomyocytes purified at day 12 with puromycin. α MHC positive cells were plated on polyacrylamide substrates at day 16 post-differentiation and fixed on day 23. With increasing gel stiffness between 1 and 50 kPa, the cells become more spread and exhibit signs of stress fiber formation similar to observations in neonatal ventricular myocytes (Fig 6). We are continuing to investigate the role of substrate stiffness in cardiomyogenesis.

Acknowledgments

This study was supported in part by grants from the National Institutes of Health (5P01HL46345) and the National Science Foundation (BES-0506252) to ADM and a University of California Biotechnology, Research and Education Program (UCBREP) GREAT award (2006-016) to ADM and KW. Parts of this work were supported by grant support from the California Institute for Regenerative Medicine (CIRM) (RC1-00132-1) and NIH (R37HL059502, R33HL088266) to MM.

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Figure 1.

The elastic moduli of polyacrylamide gels can be controlled by varying the concentration of monomer (%PAAm) up to 7% with a 1:20 monomer:crosslinker ratio. Shear moduli shown here in the range 1 to 16 kPa correspond to Young's moduli for tensile stiffness of 2 to 50 kPa for this gel material. These values span the range of developing and adult resting myocardium at low strain.

Figure 2.

Dynamic traction force microscopy of isolated neonatal rat ventricular myocytes grown on polyacrylamide gels coated with type I collagen. Phase contrast images define the cell outline. Fluorescence microscopy reveals microbeads embedded in the gel just below the surface. Automated image processing with an optical flow algorithm using the cross-correlation between a reference image and subsequent frames during stimulated cell contractions was used to compute displacement vector fields. The solution of a Boussinesq problem for deformation of a linearly elastic half-space was used to compute corresponding traction vector fields that are integrated over the cell area to give resultant axial and transverse cell forces during each cell twitch. See Jacot *et al*¹⁴ for detailed methods.

Figure 3. NRVMs on polyacrylamide gels and labeled for α -actin have poorly defined striations on soft 1 kPa substrates, well defined and aligned striations on 10 kPa substrates and unaligned striations with long, large stress fibers on stiff 50 kPa gels. Modified from Jacot *et al*¹⁴ with permission.

Figure 4.

Calcium transients (insert) were measured as peak fluorescence divided by baseline fluorescence, in Fura-2 or Fluo-4 labeled NRVMs. The magnitude of calcium transients (bar graph) on 10 kPa gels was significantly greater than transients on 1 kPa and 50 kPa gels ($P < 0.05$). See Jacot *et al*¹⁴ for details.

Figure 5.

Cardiospheres were isolated at day 12-13.5 and cultured until day 48 when they were dispersed and deposited onto gelatin-functionalized surfaces of polyacrylamide cast with fluorescent beads and analyzed for force generation at day 50. Human embryonic stem cell-derived cardiomyocytes generated axial and total traction forces on 4 kPa gels are comparable in magnitude to those generated on similar soft gels by neonatal rat ventricular myocytes, but significantly lower than the maximal forces seen in neonatal myocytes cultured on stiffer gels. Corresponding average contractile peak stresses were 220 ± 70 Pa. Modified with permission from Kita-Matsuo *et al.*³⁰

Figure 6.

Human embryonic stem cell-derived cardiomyocytes purified at day 12 with puromycin for α MHC positive cells were plated on polyacrylamide substrates at day 16 post-differentiation and fixed on day 23. Immunostaining was performed for cardiac-specific α -actinin (Green) and nuclear DAPI (Blue) to image cardiomyocytes (40 $\times$) and compare cellular morphology as a function of substrate stiffness: (A) 1 kPa gel, (B) 10 kPa gel, (C) 50 kPa gel, and (D) glass. With increasing stiffness, the cells become more spread and exhibit signs of stress fiber formation.