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Temporal impact of substrate mechanics on differentiation of human embryonic stem cells to cardiomyocytes

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ABSTRACT

A significant clinical need exists to differentiate human pluripotent stem cells (hPSCs) into cardiomyocytes, enabling tissue modeling for in vitro discovery of new drugs or cell-based therapies for heart repair in vivo. Chemical and mechanical microenvironmental factors are known to impact the efficiency of stem cell differentiation, but cardiac differentiation protocols in hPSCs are typically performed on rigid tissue culture polystyrene (TCPS) surfaces, which do not present a physiological mechanical setting. To investigate the temporal effects of mechanics on cardiac differentiation, we cultured human embryonic stem cells (hESCs) and their derivatives on polyacrylamide hydrogel substrates with a physiologically relevant range of stiffnesses. In directed differentiation and embryoid body culture systems, differentiation of hESCs to cardiac troponin T-expressing (cTnT+) cardiomyocytes peaked on hydrogels of intermediate stiffness. Brachyury expression also peaked on intermediate stiffness hydrogels at day 1 of directed differentiation, suggesting that stiffness impacted the initial differentiation trajectory of hESCs to mesendoderm. To investigate the impact of substrate mechanics during cardiac specification of mesodermal progenitors, we initiated directed cardiomyocyte differentiation on TCPS and transferred cells to hydrogels at the Nkx2.5/Isl1+ cardiac progenitor cell stage. No differences in cardiomyocyte purity with stiffness were observed on day 15. These experiments indicate that differentiation of hESCs is sensitive to substrate mechanics at early stages of mesodermal induction, and proper application of substrate mechanics can increase the propensity of hESCs to differentiate to cardiomyocytes.

1. Introduction

Cues from the mechanical microenvironment play a critical role in many cell fate decisions. Adherent cells interact with their substrate through integrin adhesion receptors, and they are capable of sensing the stiffness of their substrate and responding through alterations in contractility, spreading and migration [1,2]. In recent years, several studies have demonstrated the influence of physiologically relevant (1–100 kPa) substrate stiffness on differentiation of mesenchymal stem cells and neural stem cells [3,4] and maintenance of mouse and human pluripotent stem cells [5–8]. Less is known about the effects of stiffness on the differentiation of pluripotent stem cells to specific lineages.

As heart failure is the leading cause of death in the USA, there is great interest in deriving cardiomyocytes from human pluripotent stem cells (hPSCs) to explore novel strategies for heart repair [9,10]. Early methods for the differentiation of human embryonic stem cells (hESCs) [11] and human induced pluripotent stem cells [12,13] to cardiomyocytes employed embryoid bodies (EBs), spherical aggregates formed in suspension culture with serum [14,15]. Recent defined methods involve the application of small molecules or proteins to monolayers of hPSCs to efficiently direct differentiation to cardiomyocytes [16–19].

EB and monolayer-based differentiation to cardiomyocytes typically occurs on rigid tissue culture polystyrene (TCPS) surfaces, which are not representative of physiological tissue mechanics in the developing or adult heart. Culture systems which incorporate elements representing the mechanical microenvironment of the heart may benefit cardiogenesis. For example, applying mechanical strain to mimic cyclic contractions increased expression of early cardiac markers in mouse ESCs [20]. A hydrogel designed to stiffen ninefold, representing the transition from mesoderm tissue to myocardium, enhanced maturation of chicken pre-cardiac cells [21]. In contrast, the absence of biophysical signals is detrimental for cardiogenesis; blocking blood flow and its resultant shear stresses led to phenotypic defects in zebrafish hearts [22]. These examples illustrate that developing cardiomyocytes can sense mechanical cues, and their temporal presentation is significant.

In this study, we used polyacrylamide hydrogels to present physiologically relevant (4–80 kPa) substrate stiffnesses to hESCs subjected to directed differentiation, EBs and cardiac progenitor

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cells (CPCs), and we quantified effects of stiffness on cardiomyocyte purity in each culture context. Taken together, our results demonstrate that hESC differentiation to mesendoderm progenitors is sensitive to mechanical modulation, but cardiac progenitor differentiation to cardiomyocytes is not affected by matrix mechanics during multiple distinct cardiac differentiation protocols.

2. Experimental

2.1. hESC maintenance

For embryoid body experiments, tissue culture polystyrene (TCPS) 6-well plates (Corning) were coated with 0.1% gelatin (Sigma) and irradiated mouse embryonic fibroblasts (MEFs) were seeded at a density of 19,500 cells cm^{-2} in MEF medium. MEF medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% minimal essential medium (MEM) non-essential amino acid solution (all components from Life Technologies). hESCs (H9 or H9-hTnnTZ-pGZ-D2 cTnT reporter) were passaged onto the feeder layers every 5 days by exposure to 1 mg m^{-1} collagenase type IV (Life Technologies) in DMEM/F12 (Life Technologies) for 3 min at 37 °C, followed by mechanical dissociation and centrifugation. hESCs were maintained in UM/F+, which consisted of DMEM/F12 culture medium supplemented with 20% KnockOut serum replacer (Life Technologies), 1% MEM non-essential amino acid solution, 1 mM L-glutamine (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma) and 4 ng ml^{-1} human recombinant bFGF (Waisman Biomanufacturing).

For directed differentiation experiments, TCPS 6-well plates were coated with 8.3 μg cm^{-2} growth factor reduced Matrigel (BD Biosciences) by resuspending 0.5 mg of Matrigel in 6 ml cold DMEM/F12, adding 1 ml to each well of a 6-well plate and incubating overnight at 37 °C. hESCs (H9) were passaged every 4 days by exposure to Versene (Life Technologies) for 3 min at 37 °C, followed by mechanical dissociation. hESCs were maintained in mTeSR1 medium (STEMCELL Technologies).

2.2. Polyacrylamide hydrogel substrate fabrication and characterization

Polyacrylamide substrates were fabricated as previously described, with minor modifications [17]. Stock solutions of 10% acrylamide (Acros Organics) and 0.03–0.5% bis-acrylamide (Fisher) in deionized water were generated and stored at 4 °C in amber glass vials. Prior to polymerization, aliquots of each stock solution were brought to room temperature and degassed under vacuum. Polymerization was initiated by 1:100 addition of 5% (w/v) ammonium persulfate (APS, Fisher) in deionized water and 5% (v/v) N,N,N′,N′,-tetramethyletylenediamine (TEMED, Sigma) in deionized water. 2500 μl of pre-polymer was pipetted onto the inverted lid of a glass Petri dish (Pyrex) and covered with its base. Both faces of the glass Petri dish which contacted the pre-polymer were coated with Rain-X (ITW Global Brands), and 1 mm PDMS (Dow Corning) spacers were employed to control gel thickness. After 75 min, polymerization was halted by flooding each dish with 50 mM HEPES (Sigma) buffer, pH 8.5. Gels were allowed to swell in HEPES buffer for 1–3 days before continuing.

Circular gels with 1.59 cm diameters were generated from the polymer slabs using a punch cutter (McMaster-Carr). To functionalize the gels for protein adhesion, 60 μl of 1 mM N-sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] (Sulfo-SANPAH; Pierce) in HEPES buffer was dried onto all gel surfaces in a 60 °C oven for 1.5 h. Gels were exposed to UV light (OmniCure) at 365 nm, 90 mW cm^{-2} for 2 min. The Sulfo-SANPAH addition, drying and UV exposure steps were repeated once. Gels were transferred to individual wells of 12-well plates, hydrated in phosphate-buffered saline (PBS) and exposed to germicidal UV light for 20 min to sterilize. Gels were coated with 0.6 μg cm^{-2} fibronectin (Life Technologies) or 8.3 μg cm^{-2} growth factor reduced Matrigel at 37 °C overnight. If not used the next day, gels were transferred to 4 °C. Methods for characterizing the elastic moduli of these hydrogels were previously reported [17] and are duplicated here.

A pre-polymer of each bis-acrylamide concentration was prepared as described above, and 400 μl of pre-polymer was pipetted to fill a dogbone-shaped Teflon mold. Glass beads with diameters of 30–50 μm (Polysciences, Inc.) were sprinkled over the pre-polymer to allow for optical strain measurement during the test, and the pre-polymer was covered with a polyethylene terephthalate transparency film. After 75 min of polymerization, the mold was disassembled and samples were stored in HEPES buffer for 1–3 days before mechanical testing to allow the gels to reach hydrostatic equilibrium.

Prior to testing, additional glass beads with diameters of 30–50 μm were adhered to the surface of the samples. The samples were tested in an Instron 5548 MicroTester mechanical testing machine. The samples were secured using self-aligning grips with an abrasive surface at either end to prevent slipping of the sample during testing. The displacement rate of the test was 1 mm min^{-1}, which correlated to a strain rate of ~0.0025 s^{-1}. This rate was fast enough that evaporation of the surrounding PBS was negligible, but slow enough to reduce inertial and viscous effects. A 10 N load cell was used to measure load data at a rate of 1 Hz. The entire system was placed on a pneumatic air table to eliminate noise caused by environmental vibrations.

A temperature-controlled environmental chamber was used during the tests to match in vivo conditions as closely as possible. The temperature during the tests was maintained at a constant 37 °C via a water jacket surrounding the chamber. The samples were also fully hydrated prior to testing and submerged in PBS during the test to simulate the salinity that the gels would typically experience in vivo. Corrections were made to account for the buoyancy of the submerged portions of the testing apparatus. A "buoyancy test" was conducted after each real test to measure the amount of buoyant force that the Instron experienced. This was done by simply removing the sample and running an identical test without any tension between the grips.

Due to the compliant nature of the samples, an optical strain measurement technique was chosen in which the relative displacements of small glass beads embedded within the material or on its surface were measured and used to calculate the strain experienced by the samples. Previous studies have shown that embedding beads within the samples has no effect on the measured modulus of the samples, provided that the beads are small enough, comprise less than 1% of the volume and are evenly distributed within the material [23]. Time-lapse microscopy was used to observe the locations of the beads at designated increments during the tensile test. By measuring the vertical (axial) distance between pairs of beads during these increments, the strains at these times were calculated with Matlab using particle-tracking software developed by Prof. John C. Crocker of the University of Pennsylvania.

The rectangular cross-sections of the samples were measured before and after testing. Before the test, they were measured in multiple places using calipers. After the test, cross-sections were cut from the neck region of the samples and measured optically using a 1.25× microscope objective. Both tests resulted in nearly identical cross-sectional areas, which were then used to calculate stress data. The elastic modulus for each sample is the slope of its stress vs. strain curve.