Using polyacrylamide hydrogels to explore differentiation and measure contractility of cardiomyocytes generated from human pluripotent stem cells

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Abstract

Human pluripotent stem cells (hPSCs) have the capacity to infinitely self-renew or differentiate into any somatic cell type. Since heart failure is the world’s leading cause of death, a great clinical need exists to differentiate hPSCs into cardiomyocytes, enabling exploration of new drugs or cell-based therapies. Various factors in the cellular microenvironment are known to influence self-renewal or differentiation trajectories of stem cells. In this work, we focused on substrate stiffness, as stiffening of the heart is a physiological phenomenon during embryonic development and progression of heart disease.

To investigate effects of mechanics on differentiation to cardiomyocytes, we cultured hPSCs and their derivatives on polyacrylamide hydrogel substrates. In embryoid body and directed differentiation culture systems, hPSC differentiation to cardiomyocytes peaked on 49.4 kPa hydrogels. This was linked to an early peak in mesendoderm specification in hPSCs undergoing directed differentiation on hydrogels. Next we initiated differentiation on tissue culture polystyrene (TCPS) surfaces, split cells to hydrogels at the cardiac progenitor cell stage,
and observed no ultimate difference in cardiomyocyte purity with stiffness. To manipulate cellular mechanics internally, we primed hPSCs on TCPS with Y27632, an inhibitor of cytoskeletal organization, and observed significant increases in cardiomyocyte purity. This work indicates that hPSCs are sensitive to mechanical modulation at early stages of differentiation, and proper mechanics can drastically alter their propensity to become cardiomyocytes.

Although factors to obtain cardiomyocytes from hPSCs have been elucidated, the task remains to characterize the functionality of these beating cells. To this end, we developed an assay to quantify contraction stress of cardiomyocytes. We differentiated hPSCs to cardiomyocytes on TCPS and split them to polyacrylamide hydrogels embedded with fluorescent beads. Through traction force microscopy, we tracked bead displacements beneath contracting cardiomyocytes and converted them to contraction stresses. We found that, in hPSC-derived and neonatal rat cardiomyocytes, contraction stress increased with substrate stiffness. Next we demonstrated that hPSC-derived cardiomyocyte contractility responded appropriately to isoprenaline, a cardioactive drug, and remained stable in culture over a period of two months. This assay demonstrates appropriate functional responses of hPSC-derived cardiomyocytes and serves to motivate their use in drug studies and regenerative medicine.
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CHAPTER 1: INTRODUCTION

1.1 Origin of human pluripotent stem cells (hPSCs)

In recent years, human pluripotent stem cells (hPSCs) emerged and have begun to deliver on their promise to revolutionize modern medicine. In 1998, James Thomson and researchers at the University of Wisconsin-Madison derived the first human embryonic stem cell (hESC) lines from excess blastocysts generated at in vitro fertilization clinics [1]. These cells possess two key characteristics. First, they are capable of unlimited self-renewal; second, they are pluripotent, capable of differentiation into any somatic cell type. hESCs elicited excitement from researchers who recognized their great potential for developmental studies, drug and toxicity testing, and regenerative medicine. They also ignited controversy since their derivation required the destruction of human embryos.

In 2007, teams of researchers at the University of Wisconsin-Madison and Kyoto University in Japan reported the derivation of human induced pluripotent stem cell (hiPSC) lines from somatic cells [2, 3]. This was achieved by transducing human fibroblasts with a set of four genes (Oct4 and Sox2 plus Nanog and Lin28 or Klf4 and c-Myc) to reprogram these differentiated cells to a pluripotent state. The resulting hiPSCs, similar to hESCs, were capable of unlimited self-renewal or differentiation to any of the three germ layers. Their advantages over hESCs include the lack of controversy over their derivation source and potential applications in personalized medicine. Progress has since been made in converting other easily extractable somatic cell types into hiPSC lines, including peripheral blood cells and adipose
stem cells [4, 5]. While the first reports of hiPSC derivation utilized viral vectors that permanently integrate into the genome, a technique was soon developed that utilized nonintegrating episomal vectors [6]. The resulting hiPSCs did not contain vectors or transgenes, making them less prone to mutations and potentially safer for regenerative medicine applications. Researchers are currently working to achieve reprogramming through addition of small molecules, which is expected to be faster and more efficient than gene overexpression [7].

1.2 Maintenance and differentiation of human pluripotent stem cells

In order to keep hPSCs pluripotent, their culture systems have been carefully designed. Originally, hESCs were cultured on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs), which secrete unknown factors into the medium to support hESC self-renewal [1]. It was later discovered that conditioning medium over a layer of MEFs prior to culturing hESCs was sufficient to keep hESCs pluripotent and allowed for culture on Matrigel, an extracellular matrix (ECM) mixture secreted by mouse sarcoma cells [8]. In 2006 TeSR1, the first fully defined medium for hESC culture, was introduced and used to derive hESC lines free of contact with animal byproducts [9]. In 2011 E8, a simplified defined medium was introduced, containing only 8 components as opposed to 18 in TeSR1 [10]. Recent advances in substrate technology allow for replacing the undefined, animal-derived ECM Matrigel with defined protein, peptide, or polymer surfaces for maintaining pluripotency [11].

To demonstrate pluripotency in vivo, hPSCs are injected into severe combined immunodeficiency (SCID) mice and allowed to form teratomas, tumors containing cells of all
three germ layers [1]. When one desires to differentiate hPSCs in vitro, one of three methods can be employed. First, embryoid bodies (EBs) can be formed by culturing hPSC colonies in suspension in serum-containing medium [12]. Second, hPSCs can be co-cultured with a differentiation-inducing cell type, such as visceral endoderm or stromal cells [13, 14]. Third, components can be added and/or withdrawn from the culture medium. As researchers gravitate towards more defined culture systems and more efficient, reproducible methods for differentiation, the third method is highly advantageous. Its success, however, relies on knowledge of the identity, timing, and dosage of critical components to specifically induce differentiation to one of the over 200 somatic cell types. To date, protocols have been designed to obtain several clinically relevant cell types, including those of the skin, brain, blood, and heart with varying degrees of reproducibility and purity [15].

1.3 Motivation for deriving cardiomyocytes from hPSCs

Cardiomyocytes are muscle cells responsible for producing rhythmic contractions of the heart, crucial for circulation of oxygen- and nutrient-rich blood to all organs of the body. Impairment of their functionality leads to heart failure, the leading cause of death in the United States and around the world [16]. The most common cause of this is a heart attack or myocardial infarction, when a blocked artery restricts blood flow to the heart and results in death of more than 1 billion cardiomyocytes [17]. As adult cardiomyocytes have a limited proliferative capacity, the neighboring cells are unable to repopulate damaged regions of the heart [18]. Instead, tissue that underwent necrosis becomes collagenous scar tissue, changing the mechanical properties of the heart and weakening its contractile efficiency [17]. Other
causes of heart failure include congenital heart diseases such as LEOPARD syndrome and long-QT syndrome [19, 20], and in rare cases, off-target effects of pharmaceuticals. One example is the anti-inflammatory drug Vioxx, prescribed to 80 million patients before its cardiotoxic effects were discovered and prompted its withdrawal from the market in 2004 [21].

Heart transplant can be an effective treatment for patients with a failing heart; however, the number of donor hearts available is consistently insufficient to help patients in need [22]. This motivates exploring novel heart repair strategies, such as cardioactive pharmaceuticals and engineered heart tissue patches for engraftment onto damaged regions [23]. These studies rely on the ability to culture large numbers of human cardiomyocytes in vitro, an impossible feat with primary cells. Since hPSCs have unlimited expansion potential and the ability to differentiate into cardiomyocytes, they offer the promise of sufficient biological material for drug screening, toxicity testing, and regenerative medicine studies (Figure 1.1) [21].

1.4 Research objectives

Several barriers exist before the promise of hPSC-derived cardiomyocytes can be fully realized. First of all, we must be able to efficiently and reproducibly differentiate hPSCs to cardiomyocytes, which can be achieved by identifying crucial parameters for this transition. Second, we must fully characterize hPSC-derived cardiomyocytes to demonstrate not only phenotypical but functional similarities to native cardiomyocytes. We developed a research objective to address each of these challenges.
1.4.1 Investigate impact of substrate mechanics on differentiation of hPSCs to cardiomyocytes

Since the first report of cardiomyocyte differentiation from hESCs via formation of EBs, methods have become drastically more efficient and reproducible [23, 24]. Researchers have identified proteins and small molecules which, when applied in sequence, drive differentiation of hPSCs to cardiomyocytes [25-27]. Although these chemical factors have been identified, less is known about other culture parameters which impact the transition of hPSCs to cardiomyocytes. These include the substrate on which cells are adhered, applied mechanical or electrical forces, and the 3D architecture of the culture. For cardiogenesis, we suspect that substrate stiffness may be of particular importance, as the stiffness of heart tissue changes throughout development and progression of heart disease [28, 29]. We investigated the effect of substrate stiffness on hPSC cardiogenesis in three different culture contexts, work further described in Chapter 3. We also explored the use of an inhibitor of cytoskeletal tension to modulate cardiac differentiation. Applying the correct combination of chemical and mechanical factors during differentiation may further improve the purity or quality of resulting cardiomyocyte cultures.

1.4.2 Develop an assay to quantify the functionality of hPSC-derived cardiomyocytes

hPSC-derived cardiomyocytes are commonly characterized by expression of cardiac-specific genes and proteins, calcium handling analysis, and measures of action potential, the electrical activity of the cells [30, 31]. These cells also spontaneously beat in culture beginning approximately 8 days post-induction of differentiation. While beating provides a qualitative indicator that hPSC-derived cardiomyocytes are functional, a quantitative measure of beating
strength would be more instructive and allow for tracking changes in functionality upon changes in culture parameters. Our efforts to develop and demonstrate a quantitative assay to this end are described in Chapter 4.

1.5 Outline of chapters

**Chapter 1** introduces hPSCs and the clinical need to derived cardiomyocytes from them, and outlines the objectives of the work detailed in later chapters.

**Chapter 2** lists the components of the cellular microenvironment and methods for manipulating them to achieve desired cellular responses. It also provides a historical perspective on culture systems to report cellular-level forces.

**Chapter 3** details our investigation into how intracellular and extracellular mechanical modulation at different stages impacts hPSC differentiation to cardiomyocytes.

**Chapter 4** describes the development of an assay to quantify contractile activity of hPSC-derived cardiomyocytes and demonstrates the effectiveness of this assay through proof-of-concept examples.

**Chapter 5** summarizes the findings of this work and suggests avenues for future work.
1.6 Figures and tables

**Figure 1.1.** Applications of cardiomyocytes generated from human pluripotent stem cells. Current applications for these cells include drug screening and safety testing, while cell therapy applications remain a long-term goal. Reprinted from [21].
1.7 References


CHAPTER 2: MANIPULATING THE CELLULAR MICROENVIRONMENT

2.1 Introduction to the microenvironment

The cellular microenvironment consists of all stimuli acting on a cell at a given time in its culture. These stimuli include soluble factors, mechanical forces, surface properties, electrical stimulation, and cell-cell contacts. These signals act collectively to elicit a cell’s fate decision, which may be self-renewal, differentiation, quiescence, or apoptosis. In the culture of human pluripotent stem cells (hPSCs), the spatio-temporal presentation of these cues is often just as important as their identity (Figure 2.1).

Significant research has been devoted to understanding how specific microenvironmental cues can induce stem cells to differentiate to a cell type of interest. Section 2.2, summarized in Table 2.1, will describe efforts to engineer the various microenvironmental factors to produce hPSC-derived cardiomyocytes. Section 2.3, summarized in Table 2.2, will focus on using substrate stiffness to promote desired stem cell fates. In section 2.4, we describe the use of substrates to report forces exerted by adherent cells, presumably in response to microenvironmental variations.

2.2 Strategies to generate cardiomyocytes from hPSCs

2.2.1 Embryoid body/aggregate size control

The earliest reports of hESC and hiPSC differentiation to cardiomyocytes employed the use of embryoid bodies (EBs), which are spherical aggregates of cells cultured in suspension for
several days in serum-containing medium [2, 3]. EBs are then plated and exhibit spontaneously contracting regions as cardiomyocytes are generated. The name for these 3D bodies reflects that they mimic the microenvironment of the developing embryo; however, like an embryo, EBs produce a heterogeneous mixture of cell types with low yield of cardiomyocytes. Efforts have been made to engineer embryoid bodies to increase the percentage of cardiomyocytes they can generate.

Pluripotent stem cells are typically grown in colonies of various sizes, and this size heterogeneity is propagated when colonies are made into embryoid bodies. Two strategies have commonly been employed to control the size of hPSC colonies and thus, to control embryoid body or aggregate size: microwells and micropatterning. Our lab used a cuboidal polyurethane microwell system with lateral dimensions ranging from 100-500 µm to demonstrate that all sizes of 3D microwell culture enhanced cardiogenesis relative to 2D tissue culture polystyrene (TCPS) culture [4]. A starting colony size of 300 µm was optimal for cardiogenesis in hESCs, although cardiogenic 100 µm aggregates contained more cardiomyocytes per EB. This effect was linked to upregulation of Wnt/β-catenin signaling in EB derivatives of hESCs cultured in microwells [5]. The Zandstra lab used micropatterned features of 200-800 µm in diameter to control hESC colony size prior to making EBs and demonstrated that cardiogenesis depended both on EB size and the ratio of endoderm-biased (high Gata6/Pax6 ratio) to neural-biased (low Gata6/Pax6 ratio) cells in the EB [6]. Large endoderm-biased EBs and small neural-biased EBs exhibited high levels of cardiogenesis. When differing numbers of hESCs were seeded into 3D microwells, intermediate-sized aggregates of 1000 cells
were found to have the highest cardiogenesis, and this effect was linked to signals from neighboring extraembryonic endoderm cells [7]. A concave PDMS microwell system has been used to demonstrate that, when comparing 200-1000 µm diameter aggregate sizes, larger aggregates of mESCs exhibited higher α-actinin expression and beating frequency [8]. However, when mESCs were seeded and differentiated on micropatterned islands of 100-400 µm in diameter, 200 µm aggregates exhibited the highest cardiogenesis, based on flow cytometry data from an α-MHC EGFP reporter line [9]. These examples, with their seemingly conflicting findings, illustrate the importance of context when interpreting the effects of a single cue on lineage commitment.

2.2.2. Co-culture with non-cardiomyocytes

Another technique used to induce cardiomyocyte differentiation is co-culture with non-cardiomyocytes. One early example involved co-culture of hESCs with mouse visceral endoderm-like cells to induce hESC differentiation to functional cardiomyocytes [10]. Another study demonstrated the role of non-cardiomyocytes in facilitating functional maturation of hESC-derived cardiomyocytes [11]. When hESC-derived cardiomyocytes were cultured in isolation from non-myocytes, they did not mature, but maturity could be rescued by adding non-myocytes back into culture. Although co-culture can produce a desired outcome, its mechanism of action is unclear and could be attributed to direct cell-cell contacts or secreted factors between heterogeneous cell types.
2.2.3. Addition of soluble factors

A more defined strategy to engineer cardiac differentiation involves addition of growth factors or cytokines to medium to mimic the signaling environment of cardiac specification. Activin/Nodal and BMP signaling are important in cardiomyocyte differentiation. In one report, BMP-4 was added to the medium of EBs in suspension and resulted in up to 97.7% of contracting EBs, 4-fold higher than control samples without BMP-4 [12]. In a monolayer system developed in the Murry lab, sequential addition of Activin A and BMP-4 was sufficient to drive cardiogenesis in H7 hESCs [13]. The Keller lab has developed protocols utilizing BMP-4, bFGF, Activin A, vascular endothelial growth factor (VEGF) and dickkopf-1 (DKK-1) for efficient cardiogenesis in mouse and human PSC aggregates, but these protocols require optimization for each cell line of interest, which could be problematic in the advent of patient-specific hiPSC lines [14, 15]. A universal protocol was recently arrived upon by optimizing 4 different stages of differentiation: hPSC growth, EB formation/mesoderm induction, cardiac specification, and cardiomyocyte development [16]. This approach highlights the dynamic nature of the niche, and effective protocols must offer temporal changes that mimic different developmental stages.

Researchers at the University of Wisconsin-Madison have made major advances in monolayer-based directed differentiation in recent years. Building on the Murry lab’s Activin A/BMP-4 protocol, the Kamp lab developed a Matrigel “matrix sandwich” method, which induced hPSCs to undergo an epithelial-to-mesenchymal transition and efficiently differentiate to cardiomyocytes [17]. Our lab modified the Activin A/BMP-4 protocol to include a pre-
treatment step with the Wnt activator 6-bromoindirubin-3’-oxime (BIO), resulting in > 90% purity of cardiomyocytes (GiAB protocol) [18, 19]. We next developed a fully defined, growth-factor free protocol for cardiomyocyte generation from hPSCs [19]. This protocol relies on temporal modulation of Wnt signaling through sequential application of CHIR99021 and IWP4 small molecules (GiWi protocol).

2.2.4. Fluid flow and bioreactor culture

*In vivo*, forces generated by proximal blood flow play a role in organogenesis. Culture methods which incorporate a dynamic flow element have been shown to improve cardiogenesis when compared to traditional static culture methods. For example, mESCs exposed to laminar shear stress showed several histone modifications, which paralleled upregulation of early cardiac markers [20]. Bioreactors offer a 3D, dynamic fluid environment ideal for embryoid body culture. Bioreactor culture was shown to increase beating percentage in size-controlled hESC-derived EBs, with additional benefits shown in hypoxic conditions representative of the embryonic environment [21]. Rotary suspension culture has been demonstrated to increase beating percentage as well as cardiac gene and protein expression in mESC-derived EBs [22]. A bioreactor study in the Tzanakakis lab demonstrated increased cardiac gene expression in mESCs and hESCs encapsulated in liquid core poly-L-lysine-coated alginate capsules cultured in spinner flasks [23]. In addition to benefitting cardiogenesis, bioreactors provide a scalable system that may prove useful for producing large numbers of cardiomyocytes for therapeutic applications.
2.2.5. Electrical stimulation

Electrical fields in the developing embryo also play a role in cardiogenesis, and incorporating electrical stimulation into culture systems can increase yield of cardiomyocytes. When electrical fields were applied to mESC-derived EBs, increasing electrical field strength was found to increase both the number and size of beating regions [24]. Addition of 1 nM H$_2$O$_2$ had a similar positive effect, implying that electrical fields were acting through the generation of reactive oxygen species (ROS). hESCs have also been shown to generate ROS upon electrical stimulation; however, ROS were not shown to improve cardiac differentiation in this study [25]. Another study utilized point-source electrical stimulation with a controlled current, which better mimics the electrical microenvironment of the heart [26]. After investigating various mESC-derived EB differentiation stages and stimulation amplitudes, stimulating day 7 EBs for 4 days at 30 µA was found most beneficial for cardiogenesis, evidenced by increased cardiac gene expression.

2.2.6. Engineering expansion and sorting to increase yield and purity

One approach to improve the yield of cardiomyocytes from hPSCs is to enhance cell survival and expansion throughout the differentiation process. Incorporation of pro-survival factors can be beneficial to this end. For example, the anesthetic isoflurane has been shown to improve survival of Nkx2.5+ cardiac progenitor cells derived from hESCs by offering protection from oxidative stress [27]. Ghrelin, a peptide with cardioprotective activity, has recently been demonstrated to increase cardiogenesis in hESC-derived EBs through a yet undefined mechanism [28]. We can also attempt to mimic the niche of cardiac progenitor cells (CPCs)
which are intermediates in the journey from hPSCs to cardiomyocytes. One elegant study examined the *in vivo* CPC niche and sought to reconstruct it *in vitro* [29]. A 3D gelatin/polycaprolactone electrospun scaffold, collagen IV coating, and IQ1, a small molecule to specifically inhibit p300-dependant β-catenin signaling, increased CPC proliferation.

In order to improve cardiomyocyte purity it is possible to genetically engineer hPSC lines to facilitate sorting and selection. This has been demonstrated with an hESC-derived α-MHC GFP reporter line, where cardiomyocytes were isolated at early stages of differentiation [30]. Cardiomyocytes may also be selected for using antibiotics, such as an example utilizing hESCs with α-MHC-driven puromycin resistance [11]. The exciting recent identification of a cardiomyocyte surface marker, SIRPA, facilitated their sorting without the need for genetic modification [31]. Ultimately cardiomyocytes may need to be mixed back with other cell types present in the heart, such as endothelial cells and smooth muscle cells, but sorting enables mixing at consistent ratios.

### 2.3 Modulating substrate stiffness to control cell fate

Traditionally, adherent cell culture takes place on rigid tissue culture polystyrene (TCPS) surfaces. In recent years, researchers have experimented with soft surfaces which more closely mimic the stiffness of tissues in the body. One material frequently used for these studies is polyacrylamide, which gained popularity as a cell culture substrate after a 1997 study of fibroblast locomotion by Pelham and Wang [32]. By changing the concentration of acrylamide monomers and/or bis-acrylamide crosslinker in the pre-polymer solution, the stiffness of polyacrylamide can be controlled to fall within the range of native tissues, approximately 1 kPa.
for brain tissue to $15 \times 10^6$ kPa for cortical bone [33]. These biologically inert gels require surface modification to allow for protein coupling and cell attachment, achieved through the UV-activated crosslinker Sulfo-SANPAH or other chemistries [34]. This approach works with other synthetic biomaterials including polydimethylsiloxane (PDMS) and polyethylene glycol (PEG), as well as natural materials such as chitosan, collagen, and hyaluronic acid (HA) [35, 36]. This section will outline efforts to control stem cell differentiation and pluripotency by modulating the stiffness of various biomaterial substrates.

2.3.1 Effects of substrate stiffness on multipotent stem cell differentiation

The premiere study in this area was published by the Discher lab in 2006 [37]. In this study, human mesenchymal stem cells (hMSCs) were seeded onto polyacrylamide hydrogels with varying stiffness in the presence of serum to induce differentiation. Soft (0.1-1 kPa) substrates had a neurogenic effect on cells; intermediate (8-17 kPa) substrates were myogenic, while stiff (25-40 kPa) substrates were osteogenic. This was the first demonstration that mechanical cues could be sufficient to instruct cells to differentiate to a lineage of choice and ignited great interest in field of stem cell biomechanics. A later study using hMSCs derived from umbilical cords supported these findings by demonstrating that osteogenic differentiation was enhanced on stiff (> 26 kPa) polyacrylamide substrates [38].

The effects of stiffness on neural stem cell (NSC) differentiation have also been examined. When rat NSCs were cultured on interpenetrating networks of polyacrylamide and PEG with variable moduli, soft (100-500 Pa) gels promoted neural differentiation while stiff (1-10 kPa) gels promoted glial differentiation [39]. A follow-up study demonstrated that
mechanosensing of NSCs acts through RhoA and Cdc42, which are activated on stiff substrates and suppress neurogenesis [40]. These findings were verified in a later study, where rat neural stem/progenitor cells (NSPCs) were cultured on methacrylamide chitosan gels [33]. In this study, soft (< 1 kPa) gels encouraged differentiation to neurons while stiff (> 7 kPa) gels encouraged oligodendrocyte differentiation. The authors noted, however, that oligodendrocyte maturation was better on soft gels, likely a paracrine effect of the neurons in this environment.

A few studies have investigated the effects of substrate mechanics on precursor cells of the cardiovascular system. In a report from the Engler lab, mesoderm cells from chicken embryonic hearts were digested and seeded onto hydrogels composed of an HA-PEG mixture [41]. This material was tuned to continue crosslinking over a 2-week period, mimicking the stiffening of tissue that occurs during heart development. Cells cultured on the stiffening substrates exhibited a 3-fold increase in cardiac markers relative to cells cultured on gels with a constant stiffness. In another study, rat cardiosphere-derived cells (CDCs) were cultured on polyacrylamide gels with a stiffness gradient of 8-21 kPa [42]. Endothelial differentiation was enhanced on the intermediate stiffness range, 12-16 kPa. Taken together, these studies demonstrate that mechanical cues can be introduced to lineage-committed cells to bias their terminal differentiation.

2.3.2 Effects of substrate stiffness on pluripotency

Attention has also been devoted to studying the effects of stiffness on mouse and human embryonic stem cell pluripotency. In one study, mouse embryonic stem cells (mESCs)
were cultured on soft (0.6 kPa) polyacrylamide gels or TCPS [43]. Soft gels were more effective for maintaining pluripotency in the presence and absence of leukemia inhibitory factor (LIF), a molecule generally crucial for mESC pluripotency. The authors suggested this is because 0.6 kPa gels match the intrinsic stiffness of mESCs. In another study, mESCs were seeded onto polyacrylamide gels of 2, 7.5, or 67 kPa in the absence of LIF [44]. Relative to the soft and stiff gels, the intermediate 7.5 kPa gel induced downregulation of pluripotency genes and upregulation of mesendoderm markers.

While mESCs prefer soft (<10 kPa) substrates for pluripotency, studies with hESCs have demonstrated a preference for stiff substrates. When hESCs were cultured on PDMS micropost arrays, in which post height determines effective modulus, stiff substrates (1218.4 kPa) yielded more Oct4+ cells than softer substrates (1.92 and 14.22 kPa), a hallmark of pluripotency [45]. In another study, hESCs were cultured on polyacrylamide hydrogel substrates conjugated with peptides designed to engage glycosaminoglycans [46]. Stiff, 10 kPa substrates maintained hESC pluripotency more effectively than softer substrates of 0.7 and 3 kPa.

Less is known about the effects of stiffness on differentiation propensity of pluripotent stem cells. One study from 2009 examined gene expression and osteogenic potential of mESCs cultivated on PDMS substrates [47]. Over the 41-2700 kPa range, expression of mesendoderm genes increased with substrate stiffness in the absence of LIF. When cells underwent osteogenic differentiation on these substrates, osteogenic gene expression and mineralization again increased with stiffness. This study suggests that substrate stiffness may have roles in both priming cells to differentiate to a specific lineage and encouraging terminal differentiation.
2.4 Culture systems to measure cellular traction forces

While the previous section described the design of substrates to apply forces to adherent cells, this section will focus on the converse: designing substrates to report the forces exerted by adherent cells, termed traction forces [48]. These forces originate in the actomyosin cytoskeleton, which links to focal adhesion complexes. Integrins are transmembrane adhesion molecules which act as a bridge between focal adhesion complexes and the extracellular matrix, thus they serve as force transmitters between cells and their substrate. Traction force analysis has been used to probe cellular responses to microenvironmental factors such as ECM patterning and substrate stiffness [43, 49].

Several methods have been employed to quantify traction forces, leading to discoveries about cell migration and force transmission to neighboring cells with implications in tissue morphogenesis (reviewed in [50]). Here we will briefly describe the most commonly used methods in order to provide a historical perspective for the work described in Chapter 4.

2.4.1 Discrete methods for traction force analysis

Discrete methods obtain force information from distinct and isolated points beneath a cell. The first system of this type used for traction force calculation was a micro-electrical-mechanical system (MEMS) device reported by Galbraith and Sheetz in 1997 [51]. This device consisted of an array of 5,904 adhesive pads, each atop the free end of a cantilever. As chicken embryo fibroblasts contacted each pad, its displacement was used to determine traction force at that location. The authors discovered that forward-directed traction magnitudes were
greatest at the tail of cells during migration. One disadvantage of this device is its ability to only report forces in one dimension, corresponding to the direction of cantilever displacement.

A two-dimensional discrete method for traction force calculation used microfabricated arrays of PDMS posts with differing geometry [52]. Various types of mammalian cells were seeded on top of this “bed of microneedles” and spread across multiple posts. The deflection of each post was calculated independently by assuming it behaved as a simple spring. By coating only selected posts with ECM, the authors were able to restrict cell spreading and demonstrate a positive correlation between spreading and traction force. This technique has since been adapted for measuring contraction force of rat cardiomyocytes and used to demonstrate that neonatal rat cardiomyocytes produce 30-fold less contraction force than their adult counterparts [53].

2.4.2 Continuum methods for traction force analysis

Contrary to discrete methods which rely on separate points, continuum methods for traction force measurement make use of a continuous elastic substrate as a force reporter. The first demonstration of a continuum method was in 1981, using substrates of silicone rubber [54]. These were formed by flaming the surface of silicone fluid, generating a thin polymerized film atop an unpolymerized fluid. Cells from chicken embryos were seeded onto these films and produced deep wrinkles easily observable under the microscope. Despite efforts to quantify wrinkle depth with microneedles, this method is generally accepted to be qualitative at best.
An improved continuum method for traction force measurement embeds fluorescent particles into the surface of flexible substrates, such as polyacrylamide hydrogels, and is termed traction force microscopy [55]. In the first application of this method, images of fluorescent particles were obtained over time during migration of fibroblasts and compared back to a reference image of the cell-free substrate. The authors found highest force magnitudes at the leading edge of cells, contradicting the earlier results of Galbraith and Sheetz, which they attributed to differences in cell source and substrate properties. Although traction force microscopy relies on positions of discrete particles within the substrate, it is considered a continuum method because complex computational analysis allows for assumption of behavior of the elastic substratum between the particles.

Traction force microscopy offers a combination of advantages which make it preferable over the formerly described methods. First, polyacrylamide hydrogel substrates are simple and inexpensive to produce. Their mechanical properties are easily tunable, enabling investigation of the effects of stiffness on traction forces. They are transparent and present no barriers to imaging. Traction force microscopy images are quantifiable with the aid of software such as LIBTRC, developed by Micah Dembo of Boston University. Finally, this method provides traction force information in two dimensions for cells seeded onto flat substrates. An exciting recent report demonstrated the feasibility of adapting traction force microscopy to cells encapsulated in three dimensional hydrogels, a situation more representative of the in vivo microenvironment [56].
3.6 Figures and tables

**Figure 2.1.** Components of the cellular microenvironment and possible fate outcomes that may result from their manipulation. Adapted from [1].
<table>
<thead>
<tr>
<th>Factor category</th>
<th>Factor description</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
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<td>3D environment</td>
<td>Cuboidal microwells with 100-500 µm side lengths</td>
<td>All sizes enhanced cardiogenesis relative to TCPS; 300 µm is optimal size</td>
<td>[4, 5]</td>
</tr>
<tr>
<td>3D environment</td>
<td>Micropatterned features of 200-800 µm diameters</td>
<td>Large endoderm-biased and small neural-biased EBs enhanced cardiogenesis</td>
<td>[6]</td>
</tr>
<tr>
<td>3D environment</td>
<td>3D microwells seeded with 100-4000 hESCs</td>
<td>Highest cardiogenesis in 1000 cell aggregates</td>
<td>[7]</td>
</tr>
<tr>
<td>3D environment</td>
<td>Concave PDMS microwells with 200-1000 µm diameters</td>
<td>Highest cardiogenesis in 1000 µm size</td>
<td>[8]</td>
</tr>
<tr>
<td>3D environment</td>
<td>Micropatterned features of 100-400 µm diameters</td>
<td>Highest cardiogenesis in 200 µm size</td>
<td>[9]</td>
</tr>
<tr>
<td>3D environment</td>
<td>Cardiac progenitors cultured in 3D electrospun scaffold</td>
<td>Increased proliferation</td>
<td>[29]</td>
</tr>
<tr>
<td>Co-culture</td>
<td>hESC-cardiomyocytes isolated or cultured with non-myocytes</td>
<td>Non-myocytes required for electrophysiological maturation</td>
<td>[11]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>BMP4 added to EBs in suspension</td>
<td>4-fold increase in beating frequency</td>
<td>[12]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>Sequential addition of Activin A, BMP4 to monolayer cultures</td>
<td>Cardiogenesis achieved in H7 hESCs</td>
<td>[13]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>BMP4, bFGF, Activin A, VEGF, and DKK1 added at varying concentrations/times</td>
<td>Cardiogenesis achieved in multiple cell lines with protocol modifications</td>
<td>[14, 15]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>&gt;45 variables optimized, including polyvinyl alcohol, BMP4, FGF2, and insulin concentrations/times</td>
<td>Universal protocol for cardiogenesis in 11 cell lines</td>
<td>[16]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>Activin A/BMP4 protocol plus Matrigel sandwich</td>
<td>Induced EMT and efficient cardiogenesis</td>
<td>[17]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>Activin A/BMP4 protocol plus BIO pre-treatment</td>
<td>Efficient cardiogenesis</td>
<td>[18, 19]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>Sequential addition of CHIR99021, IWP4 to modulate Wnt pathway</td>
<td>Efficient cardiogenesis in fully defined system</td>
<td>[19]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>Isoflurane addition to hESCs</td>
<td>Improved survival of cardiac progenitors</td>
<td>[27]</td>
</tr>
<tr>
<td>Soluble factors</td>
<td>Ghrerin addition to hESCs</td>
<td>Enhanced cardiogenesis</td>
<td>[28]</td>
</tr>
<tr>
<td>Mechanical forces</td>
<td>Laminar shear stress applied to mESCs</td>
<td>Histone modifications and upregulation of cardiac markers</td>
<td>[20]</td>
</tr>
<tr>
<td>Mechanical forces</td>
<td>Size-controlled EBs cultured in hypoxic conditions in bioreactor</td>
<td>Increased beating percentages</td>
<td>[21]</td>
</tr>
<tr>
<td>Mechanical forces</td>
<td>Rotary suspension culture of EBs</td>
<td>Increased cardiogenesis</td>
<td>[22]</td>
</tr>
<tr>
<td>Mechanical forces</td>
<td>Encapsulated ESCs cultured in spinner flasks</td>
<td>Increased cardiac gene expression</td>
<td>[23]</td>
</tr>
<tr>
<td>Electrical stimulation</td>
<td>Electric fields applied to mESC-EBs</td>
<td>Increasing field strength increased cardiogenesis</td>
<td>[24]</td>
</tr>
<tr>
<td>Electrical stimulation</td>
<td>Point-source electrical stimulation with controlled current</td>
<td>Stimulating day 7 EBs for 4 days at 30 µA was best for cardiogenesis</td>
<td>[26]</td>
</tr>
</tbody>
</table>

**Table 2.1.** Summary of the effects of microenvironmental factors on directing pluripotent stem cells to cardiomyocytes.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Substrate material</th>
<th>Stiffness effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs</td>
<td>Polyacrylamide</td>
<td>Soft (0.0-1 kPa): neurogenic;</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mid (8-17 kPa): myogenic;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stiff (25-40 kPa): osteogenic</td>
<td></td>
</tr>
<tr>
<td>hMSCs from umbilical cords</td>
<td>Polyacrylamide</td>
<td>Stiff (&gt;26 kPa): osteogenic</td>
<td>[38]</td>
</tr>
<tr>
<td>Rat NSCs</td>
<td>Polyacrylamide-PEG</td>
<td>Soft (100-500 Pa): neural;</td>
<td>[39, 40]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stiff (1-10 kPa): glial</td>
<td></td>
</tr>
<tr>
<td>Rat NSPCs</td>
<td>Methacrylamide chitosan</td>
<td>Soft (&lt;1 kPa): neural;</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stiff (&gt;7 kPa): oligodendrocyte</td>
<td></td>
</tr>
<tr>
<td>Chicken cardiac mesoderm</td>
<td>HA-PEG</td>
<td>Substrates tuned to stiffen over time;</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>increased cardiac maturity</td>
<td></td>
</tr>
<tr>
<td>Rat cardiospheres</td>
<td>Polyacrylamide</td>
<td>Mid (12-16 kPa) increased endothelial</td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>differentiation</td>
<td></td>
</tr>
<tr>
<td>mESCs</td>
<td>Polyacrylamide</td>
<td>0.6 kPa increased pluripotency over TCPS</td>
<td>[43]</td>
</tr>
<tr>
<td>mESCs</td>
<td>Polyacrylamide</td>
<td>Mid (7.5 kPa) decreased pluripotency,</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>increased mesendoderm</td>
<td></td>
</tr>
<tr>
<td>hESCs</td>
<td>PDMS micropost arrays</td>
<td>Stiff (1218.4 kPa) increased pluripotency</td>
<td>[45]</td>
</tr>
<tr>
<td>hESCs</td>
<td>Polyacrylamide conjugated</td>
<td>Stiff (10 kPa) increased pluripotency</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>with peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mESCs</td>
<td>PDMS</td>
<td>Stiff (2700 kPa) increased mesendoderm and osteogenic differentiation</td>
<td>[47]</td>
</tr>
</tbody>
</table>

Table 2.2. Summary of the effects of substrate stiffness on directing cell self-renewal or differentiation.
2.7 References


CHAPTER 3: MECHANICAL MODULATION IMPACTS DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS TO CARDIOMYOCYTES

3.1 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, studies have demonstrated the influence of substrate stiffness on differentiation of mesenchymal stem cells and neural stem cells [3, 4] and maintenance of mouse and human pluripotent stem cells [5-7]. Less is known about the effects of stiffness on differentiation of pluripotent stem cells to lineages of choice.

As heart failure is the leading cause of death in the United States, there is great interest in deriving cardiomyocytes from human pluripotent stem cells (hPSCs) to explore novel strategies for heart repair [8, 9]. Early methods for differentiation of human embryonic stem cells (hESCs) [10] and human induced pluripotent stem cells (hiPSCs) [11, 12] to cardiomyocytes employed embryoid bodies (EBs), spherical aggregates formed in suspension culture with serum [13, 14]. Recent methods involve application of small molecules or proteins to monolayers of hPSCs to efficiently direct differentiation to cardiomyocytes [15-18].

EB and monolayer-based differentiation to cardiomyocytes typically occur on rigid tissue culture polystyrene (TCPS) surfaces, which are not representative of physiological mechanics. Culture systems which incorporate elements of the dynamic mechanical microenvironment of
the heart benefit cardiogenesis. For example, applying mechanical strain to mimic cyclic contractions increased expression of early cardiac markers in mouse ESCs [19]. A hydrogel designed to stiffen 9-fold, representing the transition from mesoderm tissue to myocardium, enhanced maturation of chicken pre-cardiac cells [20]. In contrast, the absence of necessary forces is detrimental for cardiogenesis; blocking blood flow and its resultant shear stresses led to phenotypic defects in zebrafish hearts [21]. These examples illustrate that developing cardiomyocytes can sense mechanical cues, and their contextual presentation is significant.

Numerous studies have implicated the small GTPase RhoA and its downstream effector Rho-associated kinase (ROCK) in mechanotransduction influencing stem cell lineage commitment [22-25]. Cells seeded onto substrates with increasing stiffness, ligand density, or micropatterned feature area exhibit a parallel increase in RhoA/ROCK signaling activity. This stimulates phosphorylation of myosin light chain (MLC), increasing the association of myosin II and actin, thus increasing cytoskeletal tension [26]. Inhibition of this cascade can be achieved through addition of Y27632, a specific inhibitor of ROCK [27]. This provides an internal avenue to disrupt the cytoskeleton, while substrate stiffness alters cytoskeletal organization via external cues [1].

In this study, we used polyacrylamide hydrogels to present physiologically-relevant stiffnesses to EBs, pluripotent cells subjected to directed differentiation, and cardiac progenitor cells (CPCs), and we quantified effects of stiffness on cardiomyocyte purity in each culture context. We also used a ROCK inhibitor to disrupt cytoskeletal contractility of hPSCs preceding
cardiac differentiation on TCPS. Taken together, our results demonstrate that hPSCs are sensitive to mechanical modulation at early, but not late, stages of cardiac differentiation.

3.2 Materials and methods

3.2.1 hPSC 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/cm² in MEF medium. MEF medium consisted of DMEM supplemented with 10% heat-inactivated FBS and 1% 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/mL collagenase type IV (Life Technologies) in DMEM/F12 (Life Technologies) for 3 minutes 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 human recombinant bFGF (Waisman Biomanufacturing).

For directed differentiation experiments, TCPS 6-well plates were coated with 8.3 µg/cm² 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) and hiPSCs (19-9-11) were passaged every 4-5 days by exposure to Versene
Life Technologies) for 3 minutes at 37°C, followed by mechanical dissociation. hPSCs were maintained in mTeSR1 medium (STEMCELL Technologies).

3.2.2 Embryoid body formation

hESCs were passaged onto low-density MEFs (~13,000 cells/cm²) on day -5 and maintained in UM/F+. On day -4 through day -1, UM/F+ was supplemented with 2 µM 6-bromoindirubin-3’-oxime (BIO, Sigma). On day 0, hPSCs were exposed to 1 mg/mL dispase (Life Technologies) in DMEM/F12 for 10 minutes at 37°C. An equal volume of UM/F- was added, and colonies were removed from the plate with gentle pipetting and pooled into a conical tube. UM/F- consisted of DMEM/F12 culture medium supplemented with 20% KnockOut serum replacer, 1% MEM non-essential amino acid solution, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Colonies were rinsed with UM/F- three times, settling by gravity for 5 minutes between each rinse. Colonies were evenly distributed into ultra-low attachment 6-well plates (Corning) in UM/F-. The following day medium was exchanged with EB20, which consisted of DMEM/F12 culture medium supplemented with 20% fetal bovine serum (FBS, Life Technologies, lot tested for cardiogenic potential), 1% MEM non-essential amino acid solution, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Colonies transitioned into embryoid bodies (EBs) during maintenance in suspension with no medium change until day 5.

3.2.3 Polyacrylamide hydrogel substrate fabrication

Polyacrylamide (PA) substrates were fabricated as previously described [16]. Stock solutions of 10% acrylamide (Acros Organics) and 0.03-0.6% 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', -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 minutes, 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-sulfo succinimidyl-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 hours. Gels were exposed to UV light (OmniCure) at 365 nm, 90 mW/cm² for 2 minutes. The Sulfo-SANPAH addition, drying, and UV exposure steps were repeated once. Gels were transferred to individual wells of 12-well plates, hydrated in PBS, and exposed to germicidal UV light for 20 minutes to sterilize. Gels were coated with 0.6 µg/cm² fibronectin (Life Technologies) or 8.3 µg/cm² 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 [16].
3.2.4 Embryoid body culture on polyacrylamide hydrogels

Fibronectin-coated polyacrylamide hydrogels and TCPS control wells were washed with PBS, and a Teflon Raschig ring with 10.5 mm inner diameter (Sigma) was placed onto the center of each well to restrict EB attachment to the gels. 150 μL of fresh EB20 were added to each ring. Day 5 EBs were pooled into a conical tube, excess medium was aspirated, and 150 μL of old EB20 + EBs was added to each ring (about 25-50 EBs). The following day, 200 μL of EB20 were added to each ring. The following day, the rings were removed and medium was exchanged with EB20. Medium was exchanged daily with EB20 through day 10. Starting from day 11, medium was exchanged every other day with EB2. EB2 consisted of DMEM/F12 culture medium supplemented with 2% FBS (lot tested for cardiogenic potential), 1% MEM non-essential amino acid solution, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol. EB20 and EB2 were supplemented with 1% Antibiotic-Antimycotic fluid (A-A, Life Technologies). Beating EBs were counted using a 4x microscope objective on days 9, 12, 15, 20, and 30 of culture.

3.2.5 Directed differentiation of hPSCs to cardiomyocytes with GiWi protocol

A previously described protocol was employed for directed differentiation with slight modifications for hydrogel culture [17]. hPSCs were dissociated by exposure to Accutase (Innovative Cell Technologies) for 5 minutes at 37°C to generate single cells. Cells were seeded onto Matrigel-coated polyacrylamide hydrogels on day -3 at a density of 2.5 x 10^5 cells within a Teflon Raschig ring with 10.5 mm inner diameter to restrict cell attachment (seeding density ~2.9 x 10^5 cells/cm^2) in mTeSR1 medium supplemented with 5 μM Y27632 ROCK inhibitor (Tocris). Cells were maintained in mTeSR1 until reaching confluency. On day 0, medium was
exchanged with RPMI/B27 without insulin supplemented with 10-12 µM CHIR99021 (Selleck).  
24 hours later, medium was exchanged with RPMI/B27 without insulin. On day 3, medium was 
exchanged with RPMI/B27 without insulin supplemented with 5 µM IWP4 (Stemgent). On day 
5, medium was exchanged with RPMI/B27 without insulin. On day 7 and every 3 days 
following, medium was exchanged with RPMI/B27. In some experiments, media were 
supplemented with 1% A-A. This protocol is known as GiWi since it employs a Gsk3 inhibitor 
(CHIR99021) and a Wnt inhibitor (IWP4) for cardiac differentiation.

For directed differentiation on TCPS, hPSCs were seeded onto Matrigel-coated TCPS at a 
density of 1.0-1.25 x 10^5 cells/cm² in mTeSR1 supplemented with 5 µM Y27632. Four days 
later, differentiation was initiated as described above. When extended Y27632 pre-treatment 
was employed, mTeSR1 was supplemented with 5 µM Y27632 on appropriate days.

3.2.6 Cardiac progenitor cell culture on polyacrylamide hydrogels

Directed differentiation of hPSCs to cardiomyocytes was begun on TCPS as described 
above. On day 6 of differentiation, cardiac progenitor cells (CPCs) were dissociated by exposure 
to Accutase for 5 minutes at 37°C. Cells were resuspended in RPMI + 20% FBS + 1% A-A + 5 µM 
Y27632 and seeded onto TCPS or gels at a density of 1.0 x 10^5 cells/cm². Gels were seeded with 
a 100 µL drop of cell suspension and flooded with 1 mL/well medium 45 minutes later. The 
next day and every 3 days following, medium was exchanged with RPMI/B27 + 1% A-A.
3.2.7 Flow cytometry

Cells were washed twice with PBS and singularized with Accutase (< day 15 cells) or 0.25% trypsin-EDTA (>= day 15 cells, Life Technologies) for 5 minutes at 37°C. Cells were fixed with 1% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at 37°C and permeabilized with ice cold 90% methanol (Fisher) and stored at -20°C. 2.0 x 10^5 cells were aliquotted per sample, and samples were washed twice with FACS buffer. FACS buffer consisted of 0.5% bovine serum albumin (BSA, Sigma) and 0.1% Triton-X 100 (Fisher) in PBS. Samples were stained with primary antibodies overnight at 4°C, washed once with FACS buffer, and stained with secondary antibodies at 1:1000 for 30 minutes at room temperature. Antibodies used are listed in Table 3.1. Samples were washed once with FACS buffer and filtered through nylon mesh with 37 µm square openings (Small Parts) into flow tubes (Beckton, Dickinson, and Company, BD). Samples were analyzed on a FACSCalibur flow cytometer (BD) with Cell Quest software, with samples stained only with secondary antibody serving as negative controls. Data were analyzed using FlowJo software.

3.2.8 Immunocytochemistry and image analysis

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at room temperature. Cells were stored at 4°C in PBS. Cells were permeabilized with 0.4% Triton-X 100 (Fisher) in PBS for 1 hour at room temperature, and then blocked with 5% non-fat dry milk (Bio-Rad) in PBS for 1 hour at room temperature. Cells were stained with primary antibodies in 0.4% Triton X-100 in PBS overnight at 4°C. Antibodies used are listed in Table 3.1. Cells were washed 4 times in PBS, then stained with secondary antibodies at 1:1000
in 0.4% Triton X-100 in PBS for 1 hour at room temperature. Cells were washed 3 times in PBS, and nuclei were stained with Hoechst 33342 (Life Technologies) at 1:5000 in PBS for 5 minutes at room temperature. Prior to imaging, cells were inverted onto a glass coverslip containing one drop of SlowFade Gold antifade reagent (Life Technologies) and sandwiched with another glass coverslip on top. Images were collected on a Nikon A1R confocal microscope with a 20x objective or a Nikon Eclipse fluorescent microscope with a 4x objective.

To characterize the area and sum of H9 cTnT-GFP+ regions, cells were fixed and imaged as described above. Images were processed by employing the “analyze particles” feature in ImageJ. All regions greater than 250 µm² were included in our analysis.

3.2.9 Cell diameter measurements

hPSCs were seeded onto polyacrylamide hydrogels as described in Section 3.2.5 and grown to confluence. On day 0 (prior to the initiation of differentiation) or day 15 of differentiation, cells were singularized with 0.25% trypsin-EDTA for 5 minutes at 37°C and transferred to Vi-CELL sample cups (Beckman Coulter). Samples were analyzed using the Vi-CELL Cell Viability Analyzer (Beckman Coulter). Diameters between 6-22 µm were included in our analysis. We thank WiCell Research Institute for use of the Vi-CELL.

3.2.10 Statistical analysis

Statistical significance was determined using one-way or two-way ANOVA followed by post-hoc Tukey tests. Comparisons with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were determined to be significant. All error bars represent SEM.
3.3 Results

3.3.1 Differentiation of EBs to cardiomyocytes peaked at 49.4 kPa

Our initial experiments employed the classical embryoid body (EB) method for cardiac differentiation. We harvested hESC colonies with dispase treatment and cultured them in suspension in EB20 medium, which includes DMEM/F12 basal medium and 20% fetal bovine serum (FBS), for 5 days to form EBs. On day 5, EBs were seeded onto fibronectin-coated polyacrylamide hydrogels of varying elastic modulus. We visually monitored EBs throughout differentiation to quantify the percentage of EBs containing regions that spontaneously contracted, indicative of cardiomyocyte differentiation. This percentage of contracting EBs was greatest on 49.4 and 76.0 kPa hydrogels, reaching a maximum of ~12% beating EBs (Figure 3.1A). On day 30, we dissociated the EBs and performed flow cytometry for cardiac Troponin T (cTnT), a protein specific to cardiomyocytes. The percentage of cells expressing cTnT peaked on the 49.4 kPa hydrogel, suggesting that beating areas may be larger or enriched in cardiomyocytes on this stiffness as compared to the 76.0 kPa hydrogels (Figure 3.1B).

To characterize the morphology of cardiomyocyte regions in EBs cultured on hydrogels with different elastic moduli, we generated EBs from H9-hTnnTZ-pGZ-D2 cells, which express GFP under control of the cTnT promoter. On day 30, we fixed these cells and performed whole-well imaging to quantify the average area of cTnT-GFP+ regions and the average sum of cTnT-GFP+ regions for each well. Both area (Figure 3.2A) and sum (Figure 3.2B) of cTnT-GFP+ regions were greater on the 49.4 kPa hydrogel than on softer and stiffer hydrogels. Representative
images depicting this trend are presented in Figure 3.2C, where EBs were stained with α-actinin to show its co-localization with cTnT-GFP.

Basal medium has been shown to impact efficiency of EB formation, and RPMI 1640 is generally preferred over DMEM/F12 in modern cardiac directed differentiation protocols [15-18, 28]. Thus, we also generated EBs in RPMI 1640 + 20% FBS to test the dependence of our observations of an optimum stiffness for cardiomyocyte differentiation in EBs on basal medium. The percentage of beating EBs and the size of cTnT+ regions peaked on 49.4 kPa hydrogels when EBs were generated in RPMI 1640 in a similar fashion to DMEM/F12 (Figure 3.3).

3.3.2 Small molecule-induced directed differentiation to cardiomyocytes peaked at 49.4 kPa

As EBs are extremely heterogeneous and result in low purities of cardiomyocytes, we next explored the effects of stiffness in a more efficient directed differentiation system. We singularized hESCs or hiPSCs and seeded them onto Matrigel-coated polyacrylamide hydrogels at a high density, saturating the surfaces and resulting in consistent attachment efficiency across all stiffnesses (Figure 3.4). We cultured the cells for an additional 3 days in mTeSR1 pluripotency medium to achieve dense multilayer structures prior to initiating differentiation. We employed the GiWi small-molecule based cardiac differentiation protocol, activating Wnt signaling with the Gsk3 inhibitor CHIR99021 at day 0 and inhibiting Wnt signaling at day 3 with IWP4 [17]. Beating was first observed between days 7-9 and plateaued at day 12, and differentiation efficiency was quantified by flow cytometry as the fraction of cells expressing cTnT at day 15. In H9 hESCs, cTnT expression peaked on the 49.4 kPa hydrogel, significantly
higher than the 4.4 and 76.0 kPa hydrogels (Figure 3.5A). Immunocytochemistry of H9-derived cells revealed robust areas of cTnT+ cells on all stiffnesses (Figure 3.5B).

3.3.3 Effects of stiffness were evident early in directed differentiation

When performing flow cytometry on day 15 cells, we observed an increase in forward scatter, a relative measure of cell size, as substrate stiffness increased (representative histogram in Figure 3.6A). To quantify changes in cell size with stiffness, we used a Vi-CELL Cell Viability Analyzer, which calculates cell diameter through automated image analysis. A slight increase in cell size with stiffness was evident on day 0 in cells that were seeded onto hydrogels on day -3 (Figure 3.6B). By day 15, cell size on the 49.4 and 76.0 kPa hydrogels was significantly higher than the 4.4 kPa hydrogel.

As the effect of stiffness on cell size emerged early and amplified throughout differentiation, we asked whether stiffness primarily impacted cardiac differentiation at early or late stages. To explore kinetic events of early differentiation, we fixed cells at days 0 (pre-differentiation), 1, 3, and 5 of differentiation. Expression of the pluripotency marker Nanog decreased at a similar rate regardless of stiffness (Figure 3.7A), with essentially no pluripotent cells remaining by day 5 of this procedure. Expression of the mesendoderm marker brachyury peaked on day 1 on all stiffnesses with highest magnitudes achieved on the 49.4 kPa hydrogel (Figure 3.7B), consistent with the later peak in cTnT+ cardiomyocytes observed on this stiffness. This suggests that effects of stiffness on cardiomyocyte purity in the directed differentiation system are, to some extent, a manifestation of effects of stiffness on the transition from pluripotent cell to mesendoderm.
3.3.4 Substrate stiffness did not impact differentiation of cardiac progenitor cells to cardiomyocytes

We next investigated whether introducing stiffness cues later in cardiac differentiation could impact commitment of human pluripotent stem cell-derived cardiac progenitor cells (CPCs) to cardiomyocytes. To address this, we initiated hPSC differentiation on TCPS and split cells to Matrigel-coated hydrogels or TCPS at the Nkx2.5/Isl1+ stage (day 6) [17]. These multipotent cardiac progenitor cells have the capacity to become cardiomyocytes, smooth muscle, and endothelial cells [29, 30]. After several days of culture on hydrogels, but not TCPS, cells aggregated into beating, tissue-like structures (Figure 3.8A). Flow cytometry analysis at day 15 revealed that introducing stiffness cues at day 6 did not impact cardiomyocyte purity (Figure 3.8B). This demonstrates that commitment of pluripotent stem cell-derived CPCs to cardiomyocytes is not sensitive to mechanical modulation.

3.3.5 Priming hPSCs for differentiation with ROCK inhibitor impacted cardiomyocyte purity

To further examine effects of mechanics during the priming (pre-day 0) stage of hPSC differentiation to cardiomyocytes, we treated H9s on TCPS with 5 µM Y27632 ROCK inhibitor for different lengths of time prior to initiating differentiation. This small molecule is typically added on the day of seeding (day -4), as its reduction of actin-myosin contractility serves to prevent apoptosis in singularized hPSCs [31, 32]. Pre-treatment with Y27632 for an additional 1-3 days of culture significantly increased the percentage of cTnT+ cardiomyocytes on day 15, with the highest purity and consistency achieved after 3 days of pre-treatment (>92% cTnT+ in each well) (Figure 3.9). Taken together, these results demonstrate that hPSCs are sensitive to
mechanics during priming for and early stages of cardiac differentiation, and mechanical modulation can drastically alter their propensity to become cardiomyocytes.

3.4 Discussion

When making the decision to differentiate, hPSCs are faced with a combination of signals from their chemical and mechanical microenvironment [33]. While researchers have identified chemical factors to direct hPSC differentiation to cardiomyocytes, including Activin A/BMP4 and small molecule modulators of Wnt signaling [15, 17], less is known about the roles of mechanical factors. Developmental studies in zebrafish have demonstrated that forces from proximal blood flow are essential for cardiogenesis [21]. *In vitro* culture systems incorporating mechanical strain or shear stress increased cardiogenesis in pluripotent stem cells, suggesting the sensitivity of these cells to mechanical modulation and its significance in differentiation [19, 34]. Here we explored the role of mechanics on various stages of hPSC differentiation to cardiomyocytes, using polyacrylamide hydrogel substrates to modulate extracellular cues and a ROCK inhibitor to internally alter cellular tension. Our results demonstrate that hPSCs are sensitive to mechanical modulation during priming and early cardiac differentiation events, while mechanical modulation does not influence the later transition from cardiac progenitor cell to cardiomyocyte.

In both the EB and directed differentiation culture platforms, differentiation to cardiomyocytes was most efficient on 49.4 kPa hydrogels. Although EBs were seeded on day 5 of differentiation following a compulsory suspension culture phase while cells for directed differentiation were seeded on day -3, exposure to substrates with varying elastic moduli
occurred early in both of these protocols. Our lab has shown that Wnt activation, a precursor to brachyury expression, peaks on day 4 in the EB system, while brachyury expression peaks on day 1 in directed differentiation [17, 35]. Our results from both scenarios demonstrate that 49.4 kPa is a preferable stiffness for the transition from brachyury+ mesendoderm to cTnT+ cardiomyocytes in hPSCs. The optimum modulus for cardiogenesis that we identified, 18.4 to 49.4 kPa, is near the reported values for elastic moduli of adult rat heart tissue (11.9-46.2 kPa), perhaps indicating that physiologically relevant substrate stiffness enhances differentiation efficiency [3, 36]. It is possible that a true peak of cardiogenesis occurs at an intermediate modulus between 18.4 and 49.4 kPa, which we have not explored.

Since cells were seeded on day -3 in the directed differentiation platform, this enabled us to observe effects of stiffness during the transition from pluripotent cell to mesendoderm. Stiffness induced differences in cell size observable as early as day 0. Cell shape and size have been implicated in directing stem cell differentiation via alterations in cytoskeletal tension [22, 25]. In addition, our lab and others have demonstrated a link between cell size and progression through the cell cycle, which may alter differentiation propensity [37]. Stiffness also induced differences in brachyury expression at day 1 that paralleled eventual cTnT+ expression. These observations that early effects of stiffness manifest as differences in cardiomyocyte purity led us to suspect that mechanics can be used to prime hPSCs for cardiac differentiation. To further explore the priming stage, we pre-treated hPSCs on TCPS with Y27632 ROCK inhibitor to disrupt cytoskeletal contractility. Extended pre-treatment with Y27632 significantly increased cTnT+
expression, and this simple protocol modification could be incorporated by researchers aiming to increase their cardiomyocyte purities.

To examine later effects of mechanical modulation, we split cells at the Nkx2.5/Is11+ CPC stage from TCPS to hydrogels. These cells did not exhibit differences in cardiomyocyte purity with stiffness, suggesting that hPSCs are not sensitive to mechanics during the transition from CPC to cardiomyocyte. Although we have previously demonstrated that our cardiac differentiation protocol yields Nkx2.5/Is11+ cells on day 6 [17], it is worth noting that our split procedure did not involve elimination of heterogeneous cells. A more precise demonstration could incorporate a sorting step using Nkx2.5<sup>egFP/w</sup> cells to ensure a homogenous population of CPCs [30].

Moving forward, the true utility of physiologically relevant stiffnesses in cardiac differentiation may lie in their ability to bias phenotype of cardiomyocytes rather than purity. Current differentiation protocols on TCPS yield immature cardiomyocytes, a significant barrier for their usefulness in transplantation therapies [38]. Comparing the action potentials and calcium handling properties of cardiomyocytes generated on hydrogels versus TCPS to identify differences in functional maturity would be an interesting follow-up study to this work.

In summary, we used polyacrylamide hydrogels to demonstrate that hPSCs are sensitive to substrate stiffness during early, but not late, events in cardiac differentiation. 49.4 kPa was a preferable stiffness to enhance cardiogenesis in EBs and pluripotent cells. Intracellular mechanical manipulation through priming with Y27632 ROCK inhibitor generated cardiomyocytes with consistently high purity. This gives insight to the growing field of stem cell
mechanobiology and supports that hPSCs can sense and respond to their mechanical microenvironment with biased differentiation decisions.
Figure 3.1. Timecourse of development of beating regions and flow cytometry data for EB system in DMEM/F12 basal media. (A) H9 EBs were cultured in suspension for 5 days, then seeded onto polyacrylamide hydrogels. A baseline number of EBs attached was counted on day 8, and beating regions were counted on days 9, 12, 15, 20, and 30. % beating EBs for each day represents # beating on each day/# attached on day 8. 6 wells of each stiffness were averaged, representing 129-210 EBs. (B) Flow cytometric analysis of cTnT expression on day 30. EBs from 6 wells of each stiffness were pooled together and re-distributed into 3 tubes prior to fixing and staining for cTnT. The average of 3 tubes is shown for each stiffness to account for instrument error.
Figure 3.2. Quantification and representative images of cTnT+ regions in EBs derived from H9-hTnnTZ-pGZ-D2 cells. (A) Area and (B) sum of cTnT-GFP+ regions present on each stiffness. EBs were seeded onto hydrogels on day 5 and fixed on day 30. Whole-well images were obtained and analyzed using ImageJ to quantify GFP+ regions. Data from 2 independent experiments were averaged together (n=3 wells/stiffness in experiment 1, n=6 wells/stiffness in experiment 2). (C) Representative images of EBs on each stiffness on day 30. EBs were stained with α-actinin (red) to show its co-localization with cTnT-GFP (green). Scale bar = 50 µm.
Figure 3.3. Timecourse of development of beating regions and representative images for EB system in RPMI basal media. (A) H9 EBs were cultured in suspension for 5 days, then seeded onto polyacrylamide hydrogels. A baseline number of EBs attached was counted on day 8, and beating regions were counted on days 9, 12, 15, 20, and 30. % beating EBs for each day represents # beating on each day/# attached on day 8. 6 wells of each stiffness were averaged, representing 144-272 EBs. (B) Representative images of EBs on each stiffness on day 30. EBs were stained with cTnT (red). Scale bar = 50 µm.
Figure 3.4. Quantification of attachment efficiency on different stiffnesses. H9 cells were seeded onto hydrogels on day -3 with a seeding density of $2.5 \times 10^5$ cells/well. 24 hours later, cells were singularized and counted using a hemocytometer. 3 wells of each stiffness were counted. Attachment efficiency did not significantly differ with stiffness.
Figure 3.5. Flow cytometry data and representative images for H9 cells in directed differentiation system. (A) H9 cells were seeded onto hydrogels on day -3, subjected to directed differentiation with the GiWi protocol starting from day 0, and fixed on day 15. %cTnT+ on the 49.4 kPa hydrogel was significantly higher than %cTnT+ on the 4.4 and 76.0 kPa hydrogels. n=9 (3 wells each from 3 independent experiments). (B) Representative images of H9 cTnT+ cells (red) on each stiffness on day 15. Scale bar = 50 µm.
Figure 3.6. Cell diameter on different stiffnesses before and after directed differentiation. (A) 19-9-11 cells were seeded onto hydrogels on day -3, differentiated to cardiomyocytes starting from day 0, and harvested for flow cytometry on day 15. Representative histograms for 4.4 and 76.0 kPa demonstrate that forward scatter increased with stiffness. (B) 19-9-11 cells were seeded onto hydrogels on day -3, singularized on day 0 or day 15, and input to a Vi-CELL Cell Viability Analyzer. On day 0, cell diameter did not significantly differ with stiffness. On day 15, cell diameter on the 49.4 and 76.0 kPa hydrogels was significantly higher than cell diameter on the 4.4 kPa hydrogel. 3 wells of each stiffness were measured at each timepoint.
Figure 3.7. Kinetics during early directed differentiation on hydrogels. H9 cells were seeded onto hydrogels on day -3 and fixed for flow cytometry on days 0, 1, 3, and 5. (A) Nanog expression decreased similarly on all stiffnesses, and (B) brachyury expression peaked on day 1 and reached the highest magnitude on the 49.4 kPa hydrogel. For each marker, 3 wells/stiffness were analyzed on days 0, 1, and 3 and 2 wells/stiffness were analyzed on day 5.
Figure 3.8. Morphology and flow cytometry data for CPCs seeded onto hydrogels on day 6. (A) Differentiation was initiated in H9 cells on TCPS on day 0, and Nkx2.5/Isl1+ CPCs were seeded onto hydrogels or TCPS on day 6. Representative images from day 13 show that H9 CPCs self-aggregated into tissue-like structures on all hydrogel stiffnesses, but not TCPS. For comparison, an unsplit well on day 13 is shown. Scale bar = 100 µm. (B) Flow cytometric analysis of replated CPCs on day 15. %cTnT+ did not differ among hydrogel stiffnesses or TCPS. For comparison, the unsplit control is shown. 3 wells of each hydrogel stiffness, 3 wells of TCPS, and 2 unsplit wells were measured.
Figure 3.9. Flow cytometry data for H9 cells pre-treated with Y27632 ROCK inhibitor. Extended pre-treatment for 1-3 days significantly increased %cTnT+ relative to day -4 (serves as control, as Y27632 is always present on the day of seeding). For each condition, n=9 (3 wells from each of 3 independent experiments).
<table>
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<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
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<tr>
<td>Cardiac Troponin T (cTnT)</td>
<td>Lab Vision, mouse IgG1</td>
<td>1:200 (FC, IC)</td>
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<tr>
<td></td>
<td>Clone: 13-11</td>
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<tr>
<td>a-actinin</td>
<td>Sigma, mouse IgG1</td>
<td>1:500 (IC)</td>
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<tr>
<td></td>
<td>Clone: EA-53</td>
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<tr>
<td>Brachyury</td>
<td>R&amp;D Systems, polyclonal Ab, Goat IgG</td>
<td>1:200 (FC)</td>
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<tr>
<td></td>
<td>Clone: AF2085</td>
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<tr>
<td>Nanog</td>
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**Table 3.1.** Antibodies used for flow cytometry (FC) and immunocytochemistry (IC).
3.6 References


CHAPTER 4: EFFECTS OF SUBSTRATE MECHANICS ON CONTRACTILITY
OF CARDIOMYOCYTES GENERATED FROM HUMAN PLURIPOTENT
STEM CELLS

4.1. Introduction

Cardiovascular disease is the leading cause of mortality in the United States, resulting in 1 of every 2.9 deaths in 2006 [1]. Current mitigation methods are plagued by low effectiveness, and heart transplants are limited by the number of available donor hearts [2, 3]. Engineered heart tissue constructs offer the promise of novel cell-based therapeutic options to restore heart function, but because adult cardiomyocytes have a limited proliferative capacity [4], a source of cardiomyocytes is required for development and implementation of such applications. Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) [5] and human induced pluripotent stem cells (hiPSCs) [6, 7] offer the potential to produce an unlimited number of cardiomyocytes for tissue engineering and other applications, due to their capacity for unlimited self-renewal and multilineage differentiation. hPSCs have been shown to differentiate to cardiomyocytes, originally via formation of embryoid bodies [8, 9], and more recently, via directed differentiation [10, 11]. These studies utilized molecular techniques to assess cardiomyocyte phenotype, such as examining the temporal expression of the cardiac progenitor markers KDR and PDGFR-α [11] or the cardiac transcription factors Nkx2.5 and GATA4 and definitive cardiac markers such as MLC2a, MLC2v, α-MHC, MF20, α-actinin, cTnT, and cTnl [8, 9]. Immunocytochemistry for the aforementioned definitive markers,
which are structural proteins, is also commonly employed to visualize sarcomere definition.

The functionality of these cardiomyocytes is typically qualitatively assessed by the presence of spontaneous contraction, and can be further characterized using electrophysiology to determine subtype (nodal, atrial, or ventricular) [12].

Response to changes in contractile demand is another critical indicator of functionality. The healthy heart linearly increases its contractile output in response to increased blood flow through a phenomenon known as the Frank-Starling Law [13]. Failure to exhibit this response is a characteristic of mitral stenosis and atrial fibrillation diseased phenotypes [14]. Although communication between neighboring cells undoubtedly plays a role, we hypothesized that singularized cardiomyocytes may maintain this functional response. In order to test this hypothesis, a culture system which can allow for simultaneous modulation of contractile demand and measure of contractile output is required. Polyacrylamide (PA) hydrogels are a well-studied, easily tunable system for studying the effects of substrate stiffness on cell processes [15, 16]. In our case, increasing substrate stiffness is used to increase contractile demand by providing greater resistance to contraction. Addition of fluorescent beads into the pre-polymer allows for optical tracking of the displacement of the PA hydrogels. Through computational analysis, bead displacements can be resolved into cell tractions in a technique known as traction force microscopy [17]. This method has been previously used to quantify contractility of quail, rat, and hESC-derived cardiomyocytes [18-21], but the physiological responses of hESC- and hiPSC-derived cardiomyocytes to changes in substrate mechanical properties have not yet been demonstrated. Increasing our understanding of the functional
properties of hPSC-derived cardiomyocytes is a critical step to enable their use in further applications, such as in vitro drug screening, developmental studies, and engineered heart tissue constructs.

We hypothesized that hPSC-derived cardiomyocytes would increase their contractile output in response to increased substrate stiffness. To test this, we seeded neonatal rat and hPSC-derived cardiomyocytes onto PA hydrogels with a range of stiffnesses and measured their contraction stress using traction force microscopy. We then quantified cell size and shape to identify links between morphology and contraction stress. Finally, we examined changes in hPSC-derived cardiomyocyte contraction stress as a result of drug treatment and increased time in culture.

4.2. Materials and Methods

4.2.1 hPSC maintenance

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/cm² in MEF medium. MEF medium consisted of DMEM supplemented with 10% heat-inactivated FBS and 1% MEM non-essential amino acid solution (all components from Life Technologies). hESCs (H9) and hiPSCs (19-9-11) were passaged onto the feeder layers every 5-6 days by exposure to 1 mg/mL collagenase type IV (Life Technologies) in DMEM/F12 (Life Technologies) for 3 minutes at 37°C, followed by mechanical dissociation and centrifugation for 5 minutes at 1000 RPM. hPSCs were maintained in 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 (H9) or 100 ng/mL (19-9-11) human recombinant bFGF (WiCell). At least 1 passage prior to differentiation, hPSCs were seeded onto 8.3 µg/cm² growth factor reduced Matrigel (BD Biosciences) and maintained in mTeSR1 medium (STEMCELL Technologies). Matrigel coating was performed 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 for 1-24 hours at 37°C. hPSCs maintained on Matrigel were passaged every 5-6 days by exposure to Versene (Life Technologies) for 3 minutes at 37°C, followed by mechanical dissociation.

4.2.2 Differentiation of hPSCs to cardiomyocytes with GiAB protocol and characterization via flow cytometry

On day -5, hPSCs were dissociated by exposure to Accutase (Life Technologies) for 5 minutes at 37°C to generate single cells. Cells were seeded onto 8.3 µg/cm² Matrigel at a density of 100,000 cells/cm² in mTeSR1 medium supplemented with 5 µM Y-27632 ROCK inhibitor (Stemgent). Cells were maintained in mTeSR1 medium for 2 days. On day -3 through day -1, mTeSR1 was supplemented with 1 µM 6-bromoindirubin-3'-oxime (BIO, Sigma). On day 0, medium was exchanged with RPMI/B27 without insulin (Life Technologies) supplemented with 100 ng/mL Activin A (R&D Systems) and 1% KnockOut serum replacer. 24 hours later, medium was exchanged with RPMI/B27 without insulin supplemented with 5 ng/mL BMP4 (R&D Systems). On day 5, medium was exchanged with RPMI/B27 without insulin. On day 7 and every 3 days following, medium was exchanged with RPMI/B27 (Life Technologies). This
protocol is known as GiAB because it employs a Gsk3 inhibitor, Activin A, and BMP4 for cardiac differentiation.

To characterize cardiomyocyte purity via flow cytometry, cells were dissociated into single cells by exposure to 0.25% trypsin-EDTA (Life Technologies) for 5 minutes at 37°C on day 15 of differentiation. Cells were fixed with 1% paraformaldehyde (Electron Microscopy Sciences) in PBS (Life Technologies) for 20 minutes at room temperature. Cells were stained with Troponin T, cardiac isoform Ab-1 mouse monoclonal primary antibody (Thermo Scientific) at 1:200 followed by Alexa Fluor 488 goat anti-mouse secondary antibody (Life Technologies) at 1:1000 in PBS plus 0.1% Triton X-100 (Sigma) and 0.5% BSA (Sigma). Data were collected on a FACSCalibur flow cytometer (Beckton Dickinson) and analyzed using FlowJo.

1-2 weeks prior to seeding cardiomyocytes onto polyacrylamide hydrogels, cardiomyocytes were dissociated by exposure to 0.25% trypsin-EDTA (Life Technologies) for 5 minutes at 37°C, centrifuged for 3 minutes at 1000 RPM, and seeded onto TCPS coated with 0.6 µg/cm² fibronectin (Life Technologies) at a density of 50,000 cells/cm² in EB20 medium. Fibronectin coating was performed by resuspending 30 µg fibronectin in 6 mL PBS, adding 1 mL to each well of a 6-well plate, and incubating overnight at 37°C. EB20 medium consisted of DMEM/F12 culture medium supplemented with 20% FBS (Life Technologies), 1% MEM non-essential amino acid solution, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol. The next day and every 3 days following, medium was exchanged with RPMI/B27.
4.2.3 Neonatal rat cardiomyocyte isolation

Based on an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at the Medical College of Wisconsin, cardiomyocytes were isolated from 1-2 day old neonatal Sprague Dawley rats. Ventricular portions of isolated rat hearts were cut into small pieces (~2mm cubes) and digested with a single (15 min) and repetitive (10 min) incubations with 0.25% trypsin (Life Technologies) and collagenase type IA (75-100 U/ml, Sigma) respectively. We normally obtain ~1 million viable cardiomyocytes from a single heart.

4.2.4 Polyacrylamide hydrogel substrate fabrication

Polyacrylamide (PA) substrates were fabricated using a method adapted from previous studies [15, 22]. Stock solutions of 10% acrylamide (Acros Organics) and 0.03-0.6% 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 minutes, 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 1.27 cm diameters were generated from the polymer slabs using a punch cutter (McMaster-Carr). The gels then had two potential fates. For gels designated for traction force microscopy, a 10 µL drop of appropriate pre-polymer with the addition of 0.72 µm diameter green fluorescent beads in aqueous solution (Fisher) at 1:50 was added to the surface of each gel, and this polymerized under a Rain-X coated glass coverslip (Fisher) for 75 minutes. Polymerization was halted by addition of 50 mM HEPES buffer, the glass coverslips were removed, and gels were immersed in HEPES buffer for 1-3 days. This two-step polymerization fixed the beads in a single focal plane for improved image quality [22]. Gels designated for immunocytochemistry were immediately immersed in HEPES buffer for 1-3 days and did not undergo the fluorescent bead layer polymerization step to prevent bead bleed-through into other fluorescent channels from impacting image quality.

To functionalize the gels for protein adhesion, 25 µL of 1 mM N-sulfosuccinimidyl-6-[4'-azido-2' -nitrophynlamino] (Sulfo-SANPAH, Pierce) in HEPES buffer was dried onto all gel surfaces in a 60°C oven for 1.5 hours. Gels were exposed to UV light (OmniCure) at 365 nm, 90 mW/cm² for 2 minutes. The Sulfo-SANPAH addition, drying, and UV exposure steps were repeated once. Gels were transferred to individual wells of 12-well plates, hydrated in PBS, and exposed to germicidal UV light for 20 minutes to sterilize. Gels were coated with 0.6 µg/cm² fibronectin at 37°C overnight. If not used the next day, gels were transferred to 4°C and stored for up to two days.
4.2.5 Polyacrylamide hydrogel substrate characterization

Pre-polymer of each bis-acrylamide concentration was prepared as described in the previous section, 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 minutes 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, which correlated to a strain rate of approximately 0.0025 sec⁻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 \textit{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 below 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 1.25x 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.
4.2.6 Seeding cardiomyocytes onto polyacrylamide hydrogels

hPSC-derived cardiomyocytes were dissociated by exposure to Accumax (Sigma) for 5 minutes at 37°C, centrifuged for 3 minutes at 1000 RPM, and resuspended in EB20 medium + 1% Antibiotic-Antimycotic (Life Technologies) at a density of 500 cells/µL. Neonatal rat cardiomyocytes were centrifuged for 15 minutes at 650 RPM and resuspended in DMEM + 10% FBS + 1% Antibiotic-Antimycotic at a density of 500 cells/µL. Each hydrogel was washed with PBS and seeded with a 50 µL drop of cell suspension (25,000 cells/gel; seeding density of ~19,700 cells/cm²) and placed in a 37°C incubator. 45 minutes later, each well was flooded with 1 mL of appropriate culture medium.

4.2.7 Contraction stress measurements and isoprenaline treatment

Approximately 24 hours after seeding, contracting cells were imaged to obtain contraction stress data. Imaging was performed within +/- 2 days of any timepoint (for example, “day 30” indicates that measurements were taken between days 28-32 of differentiation). Prior to imaging, each hydrogel was inverted onto a glass-bottomed 35 mm dish (MatTek). Cells were maintained at 37°C during imaging by placement on a heated ring connected to a temperature controller (Fryer). Contracting cells were imaged using a Nikon A1R confocal microscope with a 20x objective. A bidirectional scan speed of 30 frames per second (fps) was used with line averaging of 8 frames to reduce noise, resulting in a capture speed of 3.75 fps. Each cell was imaged for 5 seconds to capture at least 1 contraction cycle.
In some experiments, 9 µM isoprenaline (Sigma) was dosed into the culture medium 5 minutes prior to the start of imaging. As this caused the beating rate to greatly increase, averaging only 4 frames was required to fully capture cell movement, resulting in a capture speed of 7.5 fps. Imaging was completed within 30 minutes of isoprenaline addition.

To quantify contraction stress, one frame corresponding to the maximum point of the contraction cycle, where the cell was at its smallest (the “strain” frame), and one frame corresponding to the minimum point of the contraction cycle, where the cell was at rest (the “null” frame), were identified for each contracting cell. These frames were split into transmitted light and green fluorescent channels and exported using Nikon NIS-Elements software. LIBTRC version 2.4 software, developed by Prof. Micah Dembo of Boston University, was used to determine contraction stress of each cell. The calculations involved have been described in detail elsewhere [17]. Briefly, bead displacements between the “strain” and “null” frames were calculated and loaded into a template file, along with pixel position data for the cell outline in the “strain” frame obtained from ImageJ and numerical values describing the substrate mechanical properties, fluorescent bead characteristics, and image properties. A mesh within the cell outline was created, and the most likely contraction vectors between the “strain” and “null” frames were calculated and converted to contraction stresses. An average of the absolute value of contraction stresses over area generated by each cell at the maximum point of its contraction cycle is reported (avg contraction stress), as calculated in LIBTRC by the following equation:

$$AV \frac{E}{A} T = \frac{1}{A_{er1}} \int_{cr1} |T| da$$
Where \( T \) = contraction stress, \( A_{cr1} \) = total cell area, and \( a \) = area of a subsection associated with a particular contraction stress value. When maximum (max) contraction stress is reported, this value is the upper limit of the range of contraction stresses generated by each cell at the maximum point of its contraction cycle. Data sets with <150 bead displacement vectors were rejected as insufficient to accurately determine contraction stress.

4.2.8 Immunocytochemistry and morphology characterization

24 hours after seeding, cells were fixed with 16% paraformaldehyde (Electron Microscopy Sciences), diluted to 4% in PBS, for 15 minutes at room temperature. Cells were stored at 4°C in PBS for up to 1 week prior to the initiation of immunocytochemistry. Cells were permeabilized with 0.4% Triton-X 100 (Sigma) in PBS for 1 hour at room temperature, and then blocked with 5% non-fat dry milk (Bio-Rad) in PBS for 1 hour at room temperature. Cells were stained with mouse monoclonal anti-\( \alpha \)-actinin primary antibody (Sigma) at 1:500 in 0.4% Triton X-100 in PBS overnight at 4°C. Cells were washed 4 times in PBS for 15 minutes each time. Cells were stained with Alexa Fluor 555 goat anti-mouse secondary antibody (Life Technologies) at 1:1000 in 0.4% Triton X-100 in PBS for 1 hour at room temperature. Cells were washed 3 times in PBS for 15 minutes each time, and nuclei were stained with Hoechst 33342 (Life Technologies) at 1:5000 in PBS for 5 minutes at room temperature. Prior to imaging, cells were inverted onto a glass coverslip containing one drop of SlowFade Gold antifade reagent (Life Technologies) and sandwiched with another glass coverslip on top. Images were collected on a Nikon A1R confocal microscope with a 20x or 60x water immersion objective.
Images were processed with CellProfiler software using a customized pipeline to obtain cell area and eccentricity. Data were sorted based on cell area, and the top 10% and bottom 10% were excluded to eliminate cell debris and clumps.

4.2.9 Statistical analysis

Statistical significance was determined using one-way or two-way ANOVA followed by post-hoc Bonferroni tests, or linear regression where appropriate. Comparisons with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)) were determined to be significant. All error bars represent SEM.

4.3. Results

4.3.1 Elastic moduli of polyacrylamide hydrogels were physiologically relevant

The elastic modulus, or stiffness, of each polyacrylamide hydrogel composition was determined through tensile testing. The concentration of acrylamide was held constant at 10% and the concentration of bis-acrylamide crosslinker was varied from 0.03-0.6%. Elastic modulus varied linearly with bis-acrylamide concentration over this composition range (Figure 4.1).

The moduli of the PA hydrogels used in this study, 4.4 kPa to 99.7 kPa, are physiologically relevant for mimicking the bulk stiffness of cardiac tissue in vivo. The elastic moduli of neonatal rat heart tissue were measured to be between 4.0 and 11.4 kPa, and elastic moduli of adult rat heart tissue were found to range from 11.9 to 46.2 kPa [20]. After myocardial infarction, tissue stiffness can increase threefold due to remodeling events and
changes in the extracellular matrix composition [24]. Thus, these PA hydrogel substrates encompass a range of moduli representative of developing, mature, and diseased heart tissue.

4.3.2 Directed differentiation of hPSCs yielded pure populations of cardiomyocytes

To differentiate hPSCs to cardiomyocytes, we implemented a monolayer-based directed differentiation protocol [10, 11] with minor modifications. This protocol cultures undifferentiated cells in medium containing 6-bromoindirubin-3'-oxime (BIO) Gsk3 inhibitor prior to induction of differentiation by Activin A and BMP4 (GiAB protocol). Figure 4.2A demonstrates that H9 hESCs differentiated via this protocol underwent efficient differentiation to cardiomyocytes, with >90% of the population expressing cardiac Troponin T (cTnT) at 15 days post-induction of differentiation. Cells differentiated via this protocol were seeded onto fibronectin-coated PA hydrogels (Figure 4.2B).

4.3.3 Contraction stress of cardiomyocytes increased with substrate stiffness

Contracting cells seeded on the hydrogels were imaged with a confocal microscope, permitting simultaneous capture of the transmitted light and green fluorescent channels (Figure 4.2C). Frames corresponding to the maximum and minimum points in the contraction cycle were identified. The transmitted light image corresponding to the maximum contraction point was used to define the contracted cell boundary. The green fluorescent images at the maximum and minimum points were compared using LIBTRC traction force microscopy software, and bead displacements as a result of cell contraction were determined. Along with inputs of numerical values describing the substrate mechanical properties, fluorescent bead
characteristics, and image properties (Table 4.1), this information was used to obtain a contraction stress map of each contracting cell (Figure 4.2D). This map shows both the range and localizations of contraction stresses generated by each cell to produce the observed bead movement. When we refer to “maximum (max) contraction stress”, this denotes the upper limit of the range of contraction stresses generated by each cell. In most cases, in order to simplify comparisons between different cells and substrates, an average of the absolute values of contraction stresses over area is reported for each cell, referred to as “average (avg) contraction stress”. As an example, contraction stresses generated by the cell in Figure 4.2D ranged from 5.10E+01 to 7.25E+03 Pa (0.051 to 7.25 mN/mm$^2$), and its average contraction stress over area, was 2.60E+03 Pa (2.60 mN/mm$^2$).

To obtain a baseline for cardiomyocyte contractility on our PA hydrogels, we first measured contraction stresses of individual neonatal rat cardiomyocytes. The average contraction stress of these cells increased with substrate stiffness, and means for the stiffnesses exhibited statistically significant differences using one-way ANOVA (overall $p = 0.0018$) (Figure 4.3A). A similar stiffness-dependent profile was observed when comparing maximum contraction stress of neonatal rat cardiomyocytes (Figure 4.3B). Next, we measured the contraction stresses of D30 (30 days post-differentiation) H9 hESC-derived cardiomyocytes and D30 19-9-11 hiPSC-derived cardiomyocytes. In both types of human pluripotent stem cell-derived cardiomyocytes, average contraction stress increased with substrate stiffness, and means had statistically significant differences using one-way ANOVA (H9 overall $p < 0.0001$ and 19-9-11 overall $p < 0.0001$) (Figure 4.3A). D30 H9- and D30 19-9-11-derived cardiomyocytes
generated statistically significantly higher magnitudes of average contraction stress than rat cardiomyocytes on the 99.7 kPa stiffness ($p < 0.001$ for D30 H9 vs. rat and $p < 0.05$ for D30 19-9-11 vs. rat). We also compared the maximum contraction stresses generated by D30 H9- and D30 19-9-11-derived cardiomyocytes. Stiffness-dependent profiles were similar to those of average contraction stress, and statistically significant differences between cell lines were observed only on the 99.7 kPa gel ($p < 0.001$ for D30 H9 vs. rat and $p < 0.05$ for D30 H9 vs. D30 19-9-11) (Figure 4.3B).

Contraction stress also increased with substrate stiffness in 19-9-11-derived cardiomyocytes subjected to modifications in the derivation protocol. When BIO was omitted from the culture medium and Matrigel was added on days -3 and 0, the Matrigel protocol resulted in significantly higher levels of average contraction stress relative to the BIO protocol on the two stiffest substrates (Figure 4.4A). For 19-9-11 cells differentiated with the GiWi protocol, described in Section 3.2.5 of this document, average contraction stress was not significantly different on any stiffness relative to cells differentiated with the GiAB protocol (Figure 4.4B).

To examine the effect of PA hydrogel culture time, we measured the contraction stresses of neonatal rat cardiomyocytes at 1 day and 3 days after seeding. Average contraction stress increased with stiffness at both time points, and was not significantly different on any stiffness at 3 days relative to 1 day (Figure 4.4C).

To test whether cells in clusters act cooperatively to generate more contraction stress than single cells, we plated incompletely dissociated H9-derived cardiomyocytes on PA
hydrogels, resulting in a mixture of single cells and clumps containing approximately 2-20 cells. We found that cell clumps contracted in a coordinated manner, suggesting electrical coupling, but did not produce more average contraction stress than single cells ($p = 0.10$) (Figure 4.5).

4.3.4 *Bead displacement and beating rate did not increase with substrate stiffness*

Next we examined the extent to which the cardiomyocytes displaced fluorescent beads embedded in the PA hydrogels, as this is a critical input for determining contraction stress. Bead displacement was detected on all stiffnesses used in this study, with average displacement magnitudes per cell ranging from 0.2-0.35 µm (Figure 4.3C). These average magnitudes per cell represent both near-field beads, which were within or close to the cell boundary and were displaced greater distances than average upon contraction, and far-field beads, which were displaced only slight distances upon contraction. For all cell lines, substrate stiffness significantly affected bead displacement using one-way ANOVA (overall $p < 0.0001$, $p = 0.0001$, $p = 0.0003$ for rat, D30 H9, and D30 19-9-11, respectively); however, linear regression revealed a statistically significant decreasing trend in bead displacement with stiffness for rat and D30 H9 and a non-statistically significant increasing trend for D30 19-9-11.

We then calculated the beating rate for contracting cells on all stiffnesses, and we found that substrate stiffness did not significantly affect beating rate (Figure 4.3D). Mean beating rates of rat, D30 H9, and D30 19-9-11-derived cardiomyocytes on all stiffnesses were not significantly different using one-way ANOVA for each cell line (overall $p = 0.92$, $p = 0.18$, $p = 0.07$ for rat, D30 H9, and D30 19-9-11, respectively).
4.3.5 Substrate stiffness affected cardiomyocyte area

We next examined the morphology of neonatal rat and hPSC-derived cardiomyocytes on all stiffnesses in an effort to identify correlations between contraction stress and morphology. We used immunocytochemistry to stain cells for α-actinin, a protein which localizes at the Z-band of sarcomeres. We imaged cells at low magnification (20x) to capture many cells in a frame and quantified their morphology using CellProfiler software, and at high magnification (60x) to visualize the sarcomere structure of individual cells. We quantified cell area and eccentricity, or degree of elongation (0 = circular, 1 = fully elongated), to represent cell size and shape. For D30 19-9-11-derived cardiomyocytes, substrate stiffness significantly affected mean cell area (overall $p < 0.0001$ via one-way ANOVA). Cell area was greatest on the 49.4 kPa PA hydrogel, and this represents a statistically significant difference from areas on the 18.4 and 61.6 kPa hydrogels (Figure 4.6A). Cell eccentricity averaged ~0.6 on all substrate stiffnesses, and did not differ significantly with stiffness (overall $p = 0.12$ via one-way ANOVA) (Figure 4.6B). Well-defined sarcomeres were observed on all substrate stiffnesses (Figure 4.6C).

Similar observations for morphology and sarcomere organization were made using neonatal rat, D30 H9-derived, and D60 H9-derived cardiomyocytes (Figures 4.7, 4.8, and 4.9), with key differences being a higher eccentricity value (~0.8) for neonatal rat cardiomyocytes on all stiffnesses, and an emerging dependence of eccentricity on stiffness for D60 H9-derived cardiomyocytes.
4.3.6 hPSC-derived cardiomyocytes responded appropriately to isoprenaline

Another key indicator of cardiomyocyte functionality is that contractility should change appropriately in response to pharmaceutical agents. Isoprenaline, a β₁-adrenoceptor agonist, has been demonstrated to increase both beating rate and force of cardiomyocytes [25, 26]. We dosed 9 µM isoprenaline into the culture medium of hPSC-derived cardiomyocytes on PA hydrogels prior to imaging. Upon isoprenaline treatment, the mean value of average contraction stress did not change significantly on any stiffness (overall \( p = 0.40 \) for effect of treatment via two-way ANOVA), although it was slightly higher than that of untreated cells on the 18.4, 49.4, and 76.0 kPa substrates (Figure 4.10A). The beating rate was higher on all substrates and was significantly affected by isoprenaline treatment (overall \( p < 0.0001 \) for effect of treatment via two-way ANOVA), with a statistically significant increase on the 4.4 and 49.4 kPa substrates in the treated versus untreated populations (Figure 4.10B).

These results were verified with neonatal rat cardiomyocytes, with a non-statistically significant impact of isoprenaline treatment on contraction stress and a significant impact on beating rate on the 4.4, 76.0, and 99.7 kPa substrates (Figure 4.11).

4.3.7 Contraction stress of hPSC-derived cardiomyocytes remained stable with time

It has been demonstrated that hPSC-derived cardiomyocytes increase their functional maturity with time through the metrics of ion channel expression and electrophysiology [27], but effects of culture time on contraction stress generation are unknown. To investigate this, we measured contraction stresses of H9-derived cardiomyocytes at both day 30 (D30) and day
60 (D60) of differentiation. Time in culture was not found to significantly affect average or maximum contraction stress at these two time points via two-way ANOVA (overall $p = 0.29$ and overall $p = 0.14$ for avg and max, respectively) (Figure 4.12A and 4.12B).

Similar observations were made when comparing average and maximum contraction stresses of D30 and D60 19-9-11-derived cardiomyocytes (Figure 4.13A and 4.13B), with no significant difference observed over time on any stiffness (overall $p = 0.20$ (avg) and overall $p = 0.97$ (max) for effect of time via two-way ANOVA).

Stiffness did not significantly affect beating rates of D60 H9-derived cardiomyocytes (overall $p = 0.13$), but a statistically significant increase in beating rate occurred on the 4.4 and 18.4 kPa substrates between D30 and D60 (Figure 4.12C). A similar observation was made in D60 19-9-11-derived cardiomyocytes, with a non-significant impact of stiffness on beating rate at D60; however, statistically significant decreases in beating rate were detected on the 18.4 and 49.4 kPa substrates between D30 and D60 (Figure 4.13C).

4.4. Discussion

These results demonstrate that contraction stress of cardiomyocytes generated from hPSCs increases with substrate stiffness. We further show that beating rate and morphology are not linked to this functional response. Finally, we demonstrate that hPSC-derived cardiomyocytes respond to isoprenaline, and that contraction stress remains stable during maintenance in culture. By demonstrating similarities in the functional responses of native and hPSC-derived cardiomyocytes to substrate stiffness and isoprenaline treatment, we hope to
motivate the use of hPSC-derived cardiomyocytes in further applications such as drug discovery and regenerative medicine.

The increase of contraction stress with substrate stiffness is predicted by the Frank-Starling Law, which states that the heart will increase its contractile output in response to increased demand [13]. Although this law pertains to the intact organ, we demonstrated that singularized cardiomyocytes also exhibit this functional response by increasing their contraction stress on substrates where increased stiffness provides greater resistance to contraction. The effect of contraction stress increasing with substrate stiffness has been previously demonstrated in rat cardiac tissue monolayers [20] and digested chicken heart tissue [28]. In contrast, other studies demonstrated that contraction stress of neonatal rat cardiomyocytes [19] and contractile work of quail cardiomyocytes [18] peaked on 10 kPa substrates, but differences in cell source, extracellular matrix, measurement timing, and/or electrical stimulation existed between these studies and ours and could account for changes in contractile behavior. Importantly, the magnitudes we measured for average contraction stress at maximum contractile cycle points are in agreement with previous studies of primary human and rat cardiomyocytes. Hasenfuss et al. reported a peak twitch tension for human myocardium of 44 +/- 11.7 mN/mm², and similarly 56.4 +/- 4.4 mN/mm² for rat myocardium [29]. Lin et al. measured a maximal contraction stress of adult rat cardiomyocytes of 23.7 +/- 8.6 mN/mm² [30]. We demonstrated that average contraction stress increases with stiffness both at 1 day and 3 days after seeding onto PA hydrogels in neonatal rat cardiomyocytes, but long-term contractile properties of individualized cells are not known. Others have demonstrated that the
percentage of beating cardiomyocytes *in vitro* is inversely proportional to substrate stiffness and declines with time on stiff substrates [18, 19, 31]. *In vivo*, fibrosis of heart tissue after myocardial infarction is a contributing factor to heart failure [32]. These are indications that cardiomyocytes cultured for extended times on stiff substrates may have difficulty maintaining a healthy contractile phenotype.

Comparison of average contraction stresses of single cells and small clumps of 2-20 cells failed to reveal a relationship between contraction stress and clump size. The cells in our clumps were randomly oriented, often with multiple axes of contraction. Topographic control, such as nanogrooved surfaces, may encourage alignment and anisotropic contraction of cardiomyocyte aggregates [33].

Cardiomyocytes generated similar extents of bead displacement on all substrates, suggesting that cells are able to generate similar levels of strain across the range of substrate stiffnesses evaluated in this study. A contributing factor to observed variations in bead displacement could be differences in bead distribution across the surfaces, which results in different ratios of near-field versus far-field beads in each data set. To better control marker position, one could micropattern dots onto hydrogels instead of embedding beads, similar to the approach used by Balaban et al. [34].

In neonatal rat, D30 H9-, and D30 19-9-11-derived cardiomyocytes, substrate stiffness did not significantly affect beating rate. For all cell types and stiffnesses, the average beating rate varied between about 20-35 beats/minute. This is much slower than the average human embryonic heart rate of 80-196 beats/minute [35], the average resting adult heart rate of 60-
100 beats/minute, and reported beating rates of 50-70 beats/minute for hPSC-derived embryoid bodies [9]. Electrical pacing of hPSC-derived cardiomyocytes may be used to obtain physiologically-relevant beating rates [12].

We found that, for cardiomyocytes differentiated from all hPSC lines, spread area peaked at an intermediate stiffness (either 49.4 kPa or 61.6 kPa). An increase in cell area with stiffness up to 34 kPa was observed in a previous study in embryonic quail cardiomyocytes cultured on PA hydrogels for 4 or 24 hours [18], which is consistent with our observations. In another study with neonatal rat cardiomyocytes on PA hydrogels of 1-50 kPa, stiffness did not affect the area of cells fixed 7 days after seeding [19].

There are several common descriptors of cardiomyocyte shape, including eccentricity, circularity index, and aspect ratio, all of which serve to describe elongation of the cells. In vivo, neonatal rat cardiomyocytes have an elongated shape and an aspect (length-to-width) ratio of 7:1, translating to an eccentricity value approaching 1. In vitro, however, cardiomyocytes undergo a shape change as they adapt to 2D culture and their eccentricity lowers [36, 37]. We did not observe a change in eccentricity with substrate stiffness in rat, D30 H9-, and D30 19-9-11-derived cardiomyocytes, thus providing another indication that all substrates used in this study provide an environment similarly conducive for beating. In D60 H9-derived cardiomyocytes, eccentricity exhibited a statistically significant increase with stiffness, but this shape change did not manifest in greater contraction stress generation compared to D30. Neonatal rat cardiomyocytes exhibited an eccentricity of about 0.8 on all stiffnesses, while hPSC-derived cardiomyocytes possessed an eccentricity of about 0.6. This is consistent with the
expectation that cells cultivated *in vivo* should have a higher eccentricity value than those cultivated *in vitro*.

Well-defined sarcomeres were observed on all stiffnesses, suggesting that degree of sarcomere organization was not linked to contraction stress production. The representative images demonstrate that, although we quantified general trends in cell size and shape, cell morphology was very heterogeneous across the population. Additionally, most of the hPSC-derived cardiomyocytes possessed multiple myofibril axes. Although cardiomyocyte size and shape do not appear to directly impact contraction stress on the substrates evaluated in this study, they may contribute to the large variability in our contraction stress measurements. Others have demonstrated that cell shape impacts traction stress distribution and magnitudes in NIH 3T3 cells and smooth muscle cells [38, 39]. To control cardiomyocyte size and shape, Bray et al. used microcontact printing of 2500 µm² fibronectin islands and demonstrated that cells on the native aspect ratio of 7:1 exhibit directional anisotropy [37]. Adapting a similar fibronectin micropatterning approach may reduce variability in morphology of hPSC-derived cardiomyocytes, and by extension decrease variability in contraction stress.

A potential application of hPSC-derived cardiomyocytes is identification of drugs which are toxic to cardiomyocytes or impact their contractility. We treated hPSC-derived cardiomyocytes with isoprenaline to demonstrate that these cells respond appropriately to this drug by increasing their beating rate. Significant effects of isoprenaline treatment on average contraction stress were not observed in this study. We measured contraction stress values from two separate populations, one untreated and one isoprenaline treated. Considering the
heterogeneity of our contraction stress measurements, it may be necessary to take untreated and treated measurements from the same population to accurately quantify the contractile response of a single cell. This could be accomplished using a perfusion system, where drugs could be introduced into the medium during measurement without disturbing the position of the substrate and attached cells.

Others have demonstrated that hESC-derived cardiomyocytes mature over time periods up to 110 days in culture, as evidenced by their ultrastructure and withdrawal from the cell cycle [40] as well as their ion channel expression and electrophysiology [27]; however, these cells do not reach a maturation state characteristic of adult cardiomyocytes. We measured contraction stress of hPSC-derived cardiomyocytes at 1 and 2 months post-differentiation (D30 and D60), likely corresponding to an intermediate stage of maturation, and discovered that average and maximum contraction stresses remained stable over this period. Analyses of hPSC-derived cardiomyocyte shape and beating rate at D30 and D60, however, revealed that some properties of these cells are dynamic with respect to time. As new metrics are discovered for measuring hPSC-derived cardiomyocyte maturation, we hope to link contraction stress generation to structural or functional changes which occur over time. Understanding the development of functional responses of these cells will be critical if we hope to use them as a model system for adult cardiomyocyte behavior.

In summary, this study demonstrates that hPSC-derived cardiomyocytes on stiff substrates generate more contraction stress than those on soft substrates. Beating rate and morphology were not directly linked to substrate-dependent effects on contractility. When
treated with isoprenaline, hPSC-derived cardiomyocytes exhibited increased beating rate. The baseline contraction stress of these cells remained stable for up to 2 months in culture. Considering their appropriate contractile responses, hPSC-derived cardiomyocytes have the potential to be an integral component of engineered heart tissue constructs or *in vitro* drug studies, among other applications.
4.5 Figures and tables

Figure 4.1. Tensile testing data for polyacrylamide hydrogels shows a linear relationship between elastic modulus and crosslinker concentration. The concentration of acrylamide monomer was held constant at 10% and the concentration of bis-acrylamide crosslinker was varied between 0.03-0.6%. Substrate stiffness increased linearly with bis-acrylamide concentration over the tested range of compositions. Linear regression demonstrated a significantly non-zero slope ($m = 159.4 \pm 4.6, p < 0.0001$). $n = 6-13$ for each hydrogel composition and error bars represent SEM.
Figure 4.2. Methods and sample data for obtaining contraction stress measurements. (A) Flow cytometry data shows a typical H9-derived cardiomyocyte population of >90% purity on day 15 of differentiation, demonstrated by the percentage of cells expressing cardiac Troponin T (cTnT). (B) Schematic of polyacrylamide hydrogel cross-section after surface treatment and cell seeding. (C) Merged image shows a contracting D30 19-9-11-derived cardiomyocyte and green fluorescent beads embedded in the 76.0 kPa substrate beneath the cell. The cell was at its maximum point in the contraction cycle. Scale bar = 20 µm. (D) Contraction stress map for the cell pictured in (C) shows the range and localizations of contraction stresses.
Figure 4.3. Contraction stress of cardiomyocytes increased with substrate stiffness; bead displacement and beating rate did not increase with stiffness. (A) Average contraction stress of neonatal rat, D30 H9-derived, and D30 19-9-11-derived cardiomyocytes increased with substrate stiffness. Each data point represents an average of the absolute values of contraction stresses over area for a single cell at the maximum point of its contraction cycle. Mean contraction stress values were significantly affected by stiffness via one-way ANOVA (overall $p = 0.0018$ for rat and overall $p < 0.0001$ for D30 H9 and D30 19-9-11). *** ($p < 0.001$) and * ($p < 0.05$) indicate statistically significant differences relative to neonatal rat cardiomyocytes on the same stiffness. (B) Maximum contraction stress of neonatal rat, D30 H9-derived, and D30 19-9-11-derived cardiomyocytes increased with substrate stiffness. Each data point represents the upper limit of the range of contraction stresses generated by a single cell at the maximum point of its contraction cycle. *** ($p < 0.0001$) and † ($p < 0.05$) indicate statistically significant differences relative to neonatal rat and D30 19-9-11-derived cardiomyocytes, respectively, on the same stiffness. (C) Displacement of fluorescent beads occurred to a similar extent on all stiffnesses. Each data point represents an average of the absolute values of bead displacement for a single cell, including both near- and far-field beads. For all cell lines, substrate stiffness significantly affected bead displacement using one-way ANOVA (overall $p < 0.0001$, $p = 0.0001$, $p = 0.0003$ for rat, D30 H9, and D30 19-9-11 respectively), with an overall significant decreasing (rat and D30 H9) or non-significant increasing (D30 19-9-11) trend via linear regression. (D) Beating rate was similar on all stiffnesses. Substrate stiffness did not significantly affect beating rate via one-way ANOVA for each cell line (overall $p = 0.92$, $p = 0.18$, $p = 0.07$ for rat, D30 H9, and D30 19-9-11, respectively). For (A)-(D), $n = 7$-11 cells for each stiffness and error bars represent SEM.
Figure 4.4. Contraction stress of cardiomyocytes subject to protocol modifications increased with substrate stiffness. (A) Effect of replacing BIO with Matrigel in the Activin A/BMP4 protocol on contraction stress of D30 19-9-11-derived cardiomyocytes. BIO cells were derived with addition of 1 µM BIO on days -3 to -1 (GiAB) while Matrigel cells were derived with addition of 8.3 µg/cm² of growth factor-reduced Matrigel on day -3 and day 0 (MatrigelAB). All other elements of the differentiation protocol were held constant. In both cases, average contraction stress increased with substrate stiffness. On the 76.0 and 99.7 kPa substrates, the Matrigel protocol resulted in significantly higher contraction stress relative to the BIO protocol (overall $p = 0.0003$ for effect of protocol via two-way ANOVA). *** ($p < 0.001$) and * ($p < 0.05$) indicate statistically significant differences. n = 7-11 cells per stiffness for BIO and 4-9 for Matrigel. (B) Effect of growth-factor based (GiAB) versus small-molecule based (GiWi) protocols on contraction stress of D30 19-9-11-derived cardiomyocytes. GiWi cells were derived as described in Section 3.2.5. Effect of protocol was not significant on any stiffness via two-way ANOVA (overall $p = 0.61$). n = 7-11 cells per stiffness for GiAB and 12-19 for GiWi. (C) Effect of time cultured on PA hydrogels on contraction stress of neonatal rat cardiomyocytes. Measurements were taken at 1 day and 3 days after seeding. In both cases, average contraction stress increased with substrate stiffness. The effect of time cultured on PA hydrogels was not significant on any stiffness via two-way ANOVA (overall $p = 0.52$). n = 7-10 cells per stiffness for 1 day and 5-9 for 3 days. For all panels, error bars represent SEM.
Figure 4.5. Cell clump size did not impact contraction stress generation. D30 H9-derived cardiomyocytes were seeded onto 18.4 kPa hydrogels after 3 minutes of Accumax treatment and gentle pipetting to intentionally generate clumps. Cell clumps did not act cooperatively to generate more contraction stress, as linear regression demonstrated a non-significant relationship between clump size and average contraction stress ($p = 0.10$).
Figure 4.6. Morphological characterization of D30 19-9-11-derived cardiomyocytes on polyacrylamide hydrogels. Cells were seeded onto the hydrogels at 1 month post-differentiation, fixed 24 hours later, and immunostained for α-actinin. Morphology was characterized using CellProfiler software. (A) Substrate stiffness significantly affected cell area (overall \( p < 0.0001 \) via one-way ANOVA). Cell area peaked on the 49.4 kPa hydrogel. *** \( (p < 0.001) \) and * \( (p < 0.05) \) indicate statistically significant differences. (B) Substrate stiffness did not significantly affect eccentricity (overall \( p = 0.12 \) via one-way ANOVA). For (A) and (B), \( n = 81-112 \) cells per stiffness and error bars represent SEM. (C) Representative images show sarcomere organization on all stiffnesses. α-actinin is shown in red, and nuclei (stained with Hoechst) are shown in blue. Scale bar = 10 µm.
Figure 4.7. Morphological characterization of neonatal rat cardiomyocytes on polyacrylamide hydrogels. Cells were harvested and seeded onto hydrogels, fixed 24 hours later, and immunostained for α-actinin. Morphology was characterized using CellProfiler software. (A) Substrate stiffness significantly affected cell area (overall $p < 0.0001$ via one-way ANOVA). Cell area peaked on the 49.4 kPa hydrogel. ** ($p < 0.01$) indicates statistically significant differences. (B) Substrate stiffness did not significantly affect eccentricity (overall $p = 0.12$ via one-way ANOVA). For (A) and (B), $n = 60-104$ cells per stiffness and error bars represent SEM. (C) Representative images show sarcomere organization on all stiffnesses. α-actinin is shown in red, and nuclei (stained with Hoechst) are shown in blue. Scale bar = 10 µm.
Figure 4.8. Morphological characterization of D30 H9-derived cardiomyocytes on polyacrylamide hydrogels. Cells were seeded onto the hydrogels at 1 month post-differentiation, fixed 24 hours later, and immunostained for α-actinin. Morphology was characterized using CellProfiler software. (A) Substrate stiffness significantly affected cell area (overall $p < 0.0001$ via one-way ANOVA). Cell area peaked on the 61.6 kPa hydrogel. *** ($p < 0.001$) and * ($p < 0.05$) indicate statistically significant differences. (B) Substrate stiffness did not significantly affect eccentricity (overall $p = 0.80$ via one-way ANOVA). For (A) and (B), $n = 65-98$ cells per stiffness and error bars represent SEM. (C) Representative images show sarcomere organization on all stiffnesses. α-actinin is shown in red, and nuclei (stained with Hoechst) are shown in blue. Scale bar = 10 µm.
Figure 4.9. Morphological characterization of D60 H9-derived cardiomyocytes on polyacrylamide hydrogels. Cells were seeded onto the hydrogels at 2 months post-differentiation, fixed 24 hours later, and immunostained for α-actinin. Morphology was characterized using CellProfiler software. (A) Substrate stiffness significantly affected cell area (overall $p < 0.0001$ via one-way ANOVA). Cell area peaked on the 49.4 kPa hydrogel. (B) Substrate stiffness significantly affected eccentricity (overall $p < 0.0001$ via one-way ANOVA). *** ($p < 0.001$), ** ($p < 0.01$), and * ($p < 0.05$) indicate statistically significant differences. For (A) and (B), $n = 60-117$ cells per stiffness and error bars represent SEM.
Figure 4.10. Isoprenaline treatment increased beating rate but not contraction stress of D30 H9-derived cardiomyocytes. Cells were seeded onto polyacrylamide hydrogels at 1 month post-differentiation and imaged 24 hours later to obtain contraction stress and beating rate. One population was untreated, and the other was treated with 9 µM isoprenaline for 5 minutes prior to imaging. (A) Average contraction stress was not significantly affected by isoprenaline treatment (overall $p = 0.40$ for effect of treatment via two-way ANOVA). $n = 8$-9 cells per stiffness for untreated and 5-7 cells per stiffness for isoprenaline treated. (B) Beating rate significantly increased upon isoprenaline treatment (overall $p < 0.0001$ for effect of treatment via two-way ANOVA). ** ($p < 0.01$) and * ($p < 0.05$) indicate statistically significant differences. $n = 9$-11 cells per stiffness for untreated and 6-9 cells per stiffness for isoprenaline treated. For (A) and (B), error bars represent SEM.
Figure 4.11. Isoprenaline treatment increased beating rate but not contraction stress of neonatal rat cardiomyocytes. Cells were harvested, seeded onto polyacrylamide hydrogels, and imaged 24 hours later to obtain contraction stress and beating rate. One population was untreated, and the other was treated with 9 µM isoprenaline for 5 minutes prior to imaging. (A) Average contraction stress was not significantly affected by isoprenaline treatment (overall $p = 0.08$ for effect of treatment via two-way ANOVA). $n = 7-10$ cells per stiffness for untreated and 3-10 cells per stiffness for isoprenaline treated. (B) Beating rate was significantly affected by isoprenaline treatment (overall $p < 0.0001$ for effect of treatment via two-way ANOVA). ** ($p < 0.01$) and * ($p < 0.05$) indicate statistically significant differences. $n = 10-12$ cells per stiffness for untreated and 7-10 cells per stiffness for isoprenaline treated. For (A) and (B), error bars represent SEM.
Figure 4.12. Contraction stress remained stable with time past differentiation, while beating rate changed on soft substrates. H9-derived cardiomyocytes were seeded onto polyacrylamide hydrogels at 30 and 60 days post-differentiation and imaged 24 hours later to obtain contraction stress and beating rate. (A) Average contraction stress increased with substrate stiffness, but was not significantly different on any stiffness at D30 and D60 (overall $p = 0.29$ for effect of time via two-way ANOVA). (B) Maximum contraction stress increased with substrate stiffness, but was not significantly different on any stiffness at D30 and D60 (overall $p = 0.14$ for effect of time via two-way ANOVA). For (A) and (B), $n = 8$-$9$ cells per stiffness for D30 and 9$-$13$ cells per stiffness for D60. (C) Stiffness did not significantly affect beating rates of D60 H9-derived cardiomyocytes (overall $p = 0.13$ via one-way ANOVA), but beating rate increased on soft substrates between D30 and D60. * ($p < 0.05$) indicates statistically significant differences. $n = 8$-$11$ cells per stiffness for D30 and 10$-$13$ cells per stiffness for D60. For (A)-(C), error bars represent SEM.
Figure 4.13. Contraction stress remained stable with time past differentiation, while beating rate changed on soft substrates. 19-9-11-derived cardiomyocytes were seeded onto polyacrylamide hydrogels at 30 and 60 days post-differentiation and imaged 24 hours later to obtain contraction stress and beating rate. (A) Average contraction stress increased with substrate stiffness, but was not significantly different on any stiffness at D30 and D60 (overall $p = 0.20$ for effect of time via two-way ANOVA). (B) Maximum contraction stress increased with substrate stiffness, but was not significantly different on any stiffness at D30 and D60 (overall $p = 0.97$ for effect of time via two-way ANOVA). For (A) and (B), $n = 7-11$ cells per stiffness for D30 and 7-10 cells per stiffness for D60. (C) Stiffness did not significantly affect beating rates of D60 19-9-11-derived cardiomyocytes (overall $p = 0.21$ via one-way ANOVA), but beating rate decreased on the 18.4 and 49.4 kPa substrates between D30 and D60. * $(p < 0.05)$ indicates statistically significant differences. $n = 9-11$ cells per stiffness for D30 and 8-10 cells per stiffness for D60. For (A)-(C), error bars represent SEM.
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<td>POSRA</td>
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<td>Assumed value for all PA compositions</td>
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<td>Thickness of PDMS spacers used to generate PA hydrogels</td>
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Table 4.1. Parameter values input to the LIBTRC template file and used to obtain contraction stress data. For parameters not listed in this table, the default values were used. Reference: Dembo, M. (2010) The LIBTRC User’s Guide for Version 2.4.
4.6 References


5.1 Summary of findings

Human pluripotent stem cell (hPSC)-derived cardiomyocytes have potential applications in drug discovery and regenerative medicine. Their adoption depends on the ability to derive pure populations of cardiomyocytes from hPSCs and demonstrate their appropriate contractile functionality. This research explored each of these topics, using polyacrylamide hydrogel substrates to probe the effects of mechanics on differentiation of hPSCs to cardiomyocytes, and as a force reporter system to quantify contractility.

Although much has been elucidated about chemical factors which drive hPSC differentiation to cardiomyocytes, less is known about the effect of mechanics. We used polyacrylamide hydrogels with varying elastic moduli to study the impact of mechanical modulation during hPSC differentiation to cardiomyocytes. We found that, in embryoid body-based and directed differentiation, cardiomyocyte purity peaked on 49.4 kPa hydrogels. When hPSC-derived cardiac progenitor cells were split to hydrogels, no difference in cardiomyocyte purity was observed. This suggested that hPSCs are sensitive to mechanical modulation during priming and early specification events in cardiogenesis, while the terminal differentiation of cardiac progenitor cells to cardiomyocytes is unaffected by mechanical modulation. When hPSCs were treated with Y27632, an inhibitor of cytoskeletal tension, prior to undergoing cardiac differentiation, cardiomyocyte purities were significantly increased. This further
illustrates the sensitivity of hPSCs to mechanical modulation during priming, and advises a protocol modification that can be employed to increase cardiomyocyte purity.

To encourage the use of hPSC-derived cardiomyocytes in therapeutic applications, we must characterize not only their phenotype but their contractile functionality. We developed a quantitative assay for cardiomyocyte contraction stress using traction force microscopy techniques. We cultured hPSC-derived and neonatal rat cardiomyocytes on polyacrylamide hydrogels embedded with fluorescent beads and imaged bead displacements beneath contracting cells. We converted bead displacements to contraction stresses, and demonstrated that contraction stress of each cardiomyocyte type increased with stiffness. To investigate the response of hPSC-derived cardiomyocytes to a cardioactive drug, we treated cells with isoprenaline and observed the expected increase in beating force and rate. Finally, we quantified contraction stress of hPSC-derived cardiomyocytes after 1 and 2 months in culture and saw no significant changes. This demonstrates that hPSC-derived cardiomyocytes have expected responses to substrate mechanics and to isoprenaline, and the assay we described may be useful to quantify the effects of novel cardioactive drugs.

5.2 Future recommendations

Although the efforts summarized above have led to greater understanding of the role of mechanics in differentiation of hPSC-derived cardiomyocytes and their contractile functionality, they have also uncovered areas for further exploration. This section will identify four such areas and suggest opportunities for future work.
5.2.1 *Examine effects of strain on yield and maturation of hPSC-derived cardiomyocytes*

In Chapters 2 and 3, it was established that various components of the mechanical microenvironment can impact cardiogenesis of hPSCs. We hypothesize that applying mechanical strain, simulating the dynamics of a beating heart, may prove beneficial for obtaining or maturing cardiomyocytes from hPSCs. Our lab has previously studied the effects of strain on pluripotency using a commercially available FlexCell Tension System [1, 2]. In this system, cells are cultured on flexible silicone membranes which are deformed using vacuum pressure to apply cyclic strain at a given magnitude, frequency, and waveform. When a similar system was used to apply strain to mouse ESC-derived EBs, strained EBs showed upregulation of cardiac genes relative to unstrained control EBs [3].

The EB system is not ideal for this study, as preliminary fate decisions occur during the suspension phase of EB culture, and EB protocols typically result in low purity cultures of cardiomyocytes. For future studies, we suggest using our GiWi small molecule-based directed differentiation protocol [4]. Various timepoints of applying mechanical strain during this protocol are of interest: 1) during the undifferentiated hPSC expansion phase to “prime” cells for differentiation, 2) during differentiation (days 0-15), where strain may influence commitment to mesendoderm, cardiac progenitor cells, or cardiomyocytes, and 3) to post-day 15 cardiomyocyte cultures to influence maturation of these cells. Given the current state of the field, the third option has the highest impact potential. Although high purity populations of cardiomyocytes can now be generated from hPSCs, these cells retain an embryonic-like phenotype, even after extended periods of culture [5]. This hinders the usefulness of these
cells to serve as a model for adult cardiomyocytes and poses dangers for transplantation applications [6]. We propose applying mechanical strain to cardiomyocytes and assaying for hallmarks of cardiomyocyte maturation. These include loss of smooth muscle actin (SMA) expression, loss of proliferative capacity (Ki67 or BrdU incorporation), transition from myosin light chain 2 atrial (MLC2a) to myosin light chain 2 ventricular (MLC2v), and changes in action potential characteristics [4, 7]. If strain proves beneficial for cardiomyocyte maturation, it could be applied to cultures designated for drug studies or transplantation, where phenotype is of great importance.

Moving forward, we suggest seeking alternatives for the FlexCell system. This system requires that cells be cultured in a 6-well format, and only one strain magnitude and frequency can be applied per experiment. This demands an investment of large quantities of cells, medium, and time to investigate multiple strain conditions. Our collaborators in the Pruitt lab at Stanford University have developed an alternative system that may be more advantageous for these studies [8]. This system has a 96-well format and a baseplate design with different post diameters underlying the cell culture membrane, allowing for 5 strain magnitudes to be tested in a single experiment.

5.2.2 Characterize the mechanism of mechanotransduction in hPSCs

In Chapter 3, we suggested that mechanotransduction in hPSCs may occur through activation of the small GTPase RhoA. We cultured hPSCs on polyacrylamide hydrogels with varying stiffness, presumably altering activation of RhoA [9, 10], and saw effects on their differentiation to cardiomyocytes. We also treated hPSCs on TCPS with Y27632, an inhibitor of
the RhoA effector ROCK, prior to differentiation and saw significant increases in cardiomyocyte purity. To tie these observations together, we suggest characterizing activation of RhoA signaling in day 0 cells cultured in each of these two systems (after 3 days on hydrogels, or 4 days on TCPS with different extents of Y27632 treatment). This could be achieved through Western blots for critical proteins in this cascade including total RhoA vs. activated RhoA (RhoA-GTP, isolated through immunoprecipitation) and total MLC2 vs. active MLC2 (phosphorylated at Thr18 and/or Ser19) [11, 12]. We hypothesize that activation levels will show similarities on the 49.4 kPa hydrogel and in the Y27632 d-4, -3, -2 conditions, where cardiomyocyte purity peaked in each system. We also propose combining these two approaches for mechanical modulation, and treating hPSCs cultured on hydrogels with Y27632 each day prior to day 0. If RhoA signaling through ROCK is critical for mechanotransduction, its extended inhibition should eradicate differences in cardiomyocyte purity seen on different stiffnesses.

In addition to phosphorylating ROCK and MLC2, RhoA regulates other proteins that have been implicated in mechanotransduction. These include Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), which translocate to the nucleus and activate transcription of Hippo pathway genes in MSCs cultured on stiff substrates [13]. Additional examples are focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK), whose phosphorylation upon cyclic stress in rat cardiomyocytes is regulated by RhoA [14]. Thus, examining localization of YAP/TAZ and phosphorylation of FAK and ERK in hPSCs cultured on different stiffnesses may also be instructive. The value of identifying the
mechanism of mechanotransduction is its potential perturbations, which may be used to modulate differentiation and further increase purity and consistency.

5.2.3 Improve polyacrylamide substrates used for force measurements

In Chapter 4, we described the development of an assay to quantify contraction strength of hPSC-derived cardiomyocytes. Although current substrate fabrication techniques are adequate, they are not optimal and may contribute to high levels of variation in our data. We suggest two modifications for substrate improvement.

Current methods employ a random distribution of fluorescent beads throughout the top layer of polyacrylamide. Imperfections in surface topography lead to areas with clumped beads or lack of beads, and either situation is not ideal for computational analysis of bead displacement in LIBTRC. We propose an alternative micropatterning approach to yield consistent arrays of spots on these hydrogels. One method described by Balaban et al. embeds fluorescent, photoresist dots into the surface of an elastomer [15]. Another approach is to incorporate a fluorophore into the top layer of polyacrylamide, resulting in an entirely fluorescent top layer, and use a mask to photobleach areas excepting an array of dots (Figure 5.1). A preliminary experiment in our lab, where fluorescently-labeled acrylamide monomers were successfully incorporated into polyacrylamide, suggested this approach is promising.

Another issue with our current substrates is the heterogeneity in cardiomyocyte shape that results from unrestricted cell attachment. Although we observed cells with the elongated shape and directional anisoptropy in contraction characteristic of native cardiomyocytes, we
also observed circular cells with directional isotropy. To overcome this, we propose using microcontact printing to stamp islands of fibronectin onto polyacrylamide, which can be achieved using a protein-inked PDMS stamp, instead of coating the whole surface with fibronectin [16]. These islands should have a length-to-width ratio of 7:1 to mimic the native shape of cardiomyocytes and encourage directional anisotropy [17].

5.2.4 Characterize contractility of diseased hiPSC-derived cardiomyocytes

In our proof-of-concept examples with our contraction stress assay in Chapter 4, we used cardiomyocytes generated from standard hESC and hiPSC lines. One exciting property of hiPSCs is the ability to derive these cells from patients with genetic diseases, and differentiate them to a cell type of interest which exhibits the disease phenotype. To date, cardiomyocytes have been derived from hiPSCs from patients with the cardiovascular diseases long-QT syndrome and LEOPARD syndrome [18, 19]. Each of these diseases is caused by a specific mutation which manifests in cardiomyocytes as prolonged action potentials (long-QT) or hypertrophic phenotype (LEOPARD). We propose using our contraction stress assay to characterize the contractility of these diseased hiPSC-derived cardiomyocytes. The aim of this would be to detect differences in contraction stress in diseased cardiomyocytes relative to cardiomyocytes derived from healthy hiPSCs, increasing our understanding of these diseased phenotypes. We could then apply cardioactive drugs to the diseased cardiomyocytes and look for restoration of a healthy contractile phenotype. This approach would be useful for testing the effects of potentially beneficial new drugs, or identifying the effectiveness of a given drug for a specific patient.
This study would be best performed by measuring contractility of a given cell, then applying drugs to the same cell and registering its contractile changes. In our current measurement technique, contracting cells are sandwiched between glass and polyacrylamide and are not easily accessible. Culture system modifications, such as slight elevation of polyacrylamide gels with spacers and incorporating microfluidics to replace fresh medium with drug-containing medium during imaging, could make this approach feasible.

5.3 Conclusions

In this work, we investigated the impact of mechanics on differentiation and contractility of cardiomyocytes generated from hPSCs. Using polyacrylamide hydrogel substrates as a model system, we found that a physiologically relevant stiffness enhanced differentiation of embryoid bodies and hPSCs to cardiomyocytes, but not the terminal differentiation of cardiac progenitor cells to cardiomyocytes. Adding a thin layer of fluorescent beads atop our polyacrylamide hydrogels transformed them into force reporters, from which we learned that contractility of hPSC-derived and native cardiomyocytes increased with substrate stiffness. Our contributions to the field include identifying a window of mechanosensitivity during early differentiation of hPSCs to cardiomyocytes, and developing a quantitative assay for cardiomyocyte contractility. Continued exploration of the differentiation, maturation, and functionality of hPSC-derived cardiomyocytes is essential to accelerate their life-saving transition from bench to bedside.
Figure 5.1. Schematic demonstrating current and suggested methods for generating traction force microscopy substrates. In the top panel (current method), fluorescent beads are randomly distributed throughout the top layer of polyacrylamide, yielding areas with clumped beads or lack of beads. In the bottom panel (suggested method), an entirely fluorescent substrate is generated and a mask is used to photobleach a uniform array of fluorescent dots.
5.5 References


