Tissue-Engineered Micropatterned Platform to Mature Human PSC-derived Cardiomyocytes

By

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Abstract

Human pluripotent stem cell–derived cardiomyocytes (hPSC-CMs) have emerged as exciting new tools in the field of cardiac research that possess immense therapeutic potential and can serve as innovative pre-clinical platforms for drug development and disease modeling studies. However, these aspirations are limited by current culture methods in which hPSC-CMs resemble fetal human cardiomyocytes in terms of structure and function. Most prior efforts on improving hPSC-CM maturation have utilized cues stemming from the *in vivo* cardiac milieu. While these studies have resulted in modest hPSC-CM maturation improvements, individually they fail to fully recapitulate the adult cardiomyocyte phenotype. Thus, the field has gravitated towards the creation of more biomimetic microenvironments that have the ability to combine multiple signaling factors into one *in vitro* culture platform. Herein we provide a 2D *in vitro* substrate platform inspired by the myocardial microenvironment that improves hPSC-CM maturation while simultaneously allowing for quantitative measurements of mechanical and electrophysiological outputs.

Substrate stiffness and micropatterned ECM are two known hPSC-CM pro-maturation cues. We study the effects of these parameters on hPSC-CMs and cardiac fibroblast generated from human induced pluripotent stem cells (hiPSC-CFs). Similar to our prior result on glass substrates, hPSC-CMs patterned on 10kPa PDMS align their internal cytoskeletal network in accordance with the micropatterned lanes. We mimic a functional cardiac syncytium by connecting micropatterned lanes with micropatterned bridges seeded with hPSC-CMs. hPSC-CMs patterned on this 15° chevron pattern displays anisotropic electrical impulse propagation, as occurs in the native myocardium,

with speeds 2x faster in the direction of the lanes compared to the transverse direction. hPSC-CFs cultured on micropatterned lanes and the 15° chevron pattern remodel the underlying ECM and produce fibers of collagen and fibronectin parallel to the feature direction. When co-cultured together on this pattern, ECM is produced by hPSC-CFs and hPSC-CMs display improved calcium kinetics and contractile strain. The ability to test factors individually and concomitantly in one biomimetic platform will lend new insights and aid in the directed maturation of immature hPSC-CMs, ultimately furthering our basic understanding of cardiac biology and providing novel platforms for drug discovery and toxicity testing.

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Chapter 1: Background

Heart Disease

The number one cause of death in the developed world is cardiovascular disease (CVD). In 2015 CVD, which includes myocardial infarctions (heart attacks), strokes and hypertension, accounted for more than 17.9 million deaths and is expected to grow to more than 23.6 million by 2030 (Benjamin et al. 2018). In the United States, it is estimated 836,546 deaths occur annually due to CVD, which is nearly 1 of every 3 deaths. In addition to large-scale morbidity, this disease also imposes high social costs. CVD and stroke accounted for 14% of total health expenditures in 2013-2014 and this number is projected to increase to $749 billion in 2035 (Khavjou, Phelps, and Leib 2016). Furthermore, cardiovascular toxicity claims the highest incidence of adverse drug reactions in late-stage clinical development (Laverty et al. 2011). As the population continues to age, these numbers are only expected to increase unless the disease can be managed.

One of the main reasons CVD is so lethal is due to the limited regenerative capacity of the human heart. Looking at carbon-14 integration into genomic DNA of human cardiomyocytes, researchers found that 1% of cardiomyocytes undergo mitosis annually at the age of 25 and this turn over rate decreases to 0.45% by the age of 75 (Bergmann et al. 2009). More recent work used cell tracing methods to create a cell-by-cell map of dividing cells in a damaged heart and came to the sobering conclusion that no new cardiac muscle was generated (Kretzschmar et al. 2018). Instead, a myocardial infarction triggers an inflammatory response that results in myofibroblasts invading the

area and producing a non-contractile, collagen-dense scar tissue to replace the ischemic myocardium (Sun and Weber 2000). Unfortunately, both the mechanical (Holmes, Borg, and Covell 2005; Harvey and Leinwand 2011) and electrophysiological (Ursell et al. 1985) properties of the scar tissue differ from that of the surrounding myocardium which results in decreased cardiac function and can lead to further degradation of the heart. It is estimated 14% of people who have heart attacks will die from them, with an annual incidence of 720,000 new and 335,000 recurrent attacks in the US (Benjamin et al. 2018).

Cell Types of the Heart

While cardiomyocytes are responsible for the force-producing contractions of the heart, they are not the most abundant cell type and only account for 25% – 35% of all cells in the heart (Bergmann et al. 2015). The nonmyocyte population is predominately composed of three cell types: fibroblasts, endothelial cells and smooth muscle cells. While there is no debate these nonmyocytes exist, there is a lack of consensus on the composition of the cardiac nonmyocyte cell population. Previous reports estimated that cardiac fibroblasts occupy the highest fraction on non-myocytes (VLIEGEN et al. 1991; Deb and Ubil 2014) ,while others posit endothelial cells as the principal nonmyocyte constituents (Brutsaert 2003; Hsieh et al. 2006). Some of these discrepancies can be attributed to a lack of specific cell markers for certain cell types, particularly challenging is the cardiac fibroblast where not all the fibroblasts express the same markers equally such as DDR2, CD90, Sca-1, and Vimentin (Pinto et al. 2016). Although cardiomyocytes are often the focus of attention because their mechanical function is intrinsic to heart function, it does not imply the other cell types are insignificant. In fact, it

is highly likely all cell types play a crucial role in supporting cardiomyocyte function and maturation. Furthermore, we hypothesize that these cell-cell interactions will be critical in developing *in vitro* models that recapitulate the adult myocardium *in vivo* phenotype.

The Cardiomyocyte

The human heart is a fascinating organ with each of its chambers having different functions that all work synchronously to pump blood throughout the body and keep us alive. Not surprisingly then, the heart cells that reside in each chamber are unique and have different morphological and electrophysiological characteristics (Figure 1). Human cardiomyocytes can be split up into two categories: conducting cells and contractile cells. Sinoatrial (SA) and atrioventrcular (AV) nodal cells, also called pacemaker cells, are responsible for generating action potentials that spread through the atria and ventricles. Nodal cells are unique in that they possess the pacemaker current I_f which controls their rate of spontaneous activity/depolarization (Baruscotti, Bucchi, and DiFrancesco 2005). This current is relatively absent in atria and ventricles, the two major contractile cells of the heart, and thus they need exogenous stimulation to contract. Atrial cardiomyocytes have a lower inward rectifier K^+ current (I_{K1}), resulting in more depolarized resting membrane potential at -74 mV compared to -81 mV for ventricle cardiomyocytes (Piroddi et al. 2007; Grandi et al. 2011). Both atrial and ventricle cells contain striated myofibrils composed of repeating units of sarcomeres, the basic contractile unit necessary for cell contraction.

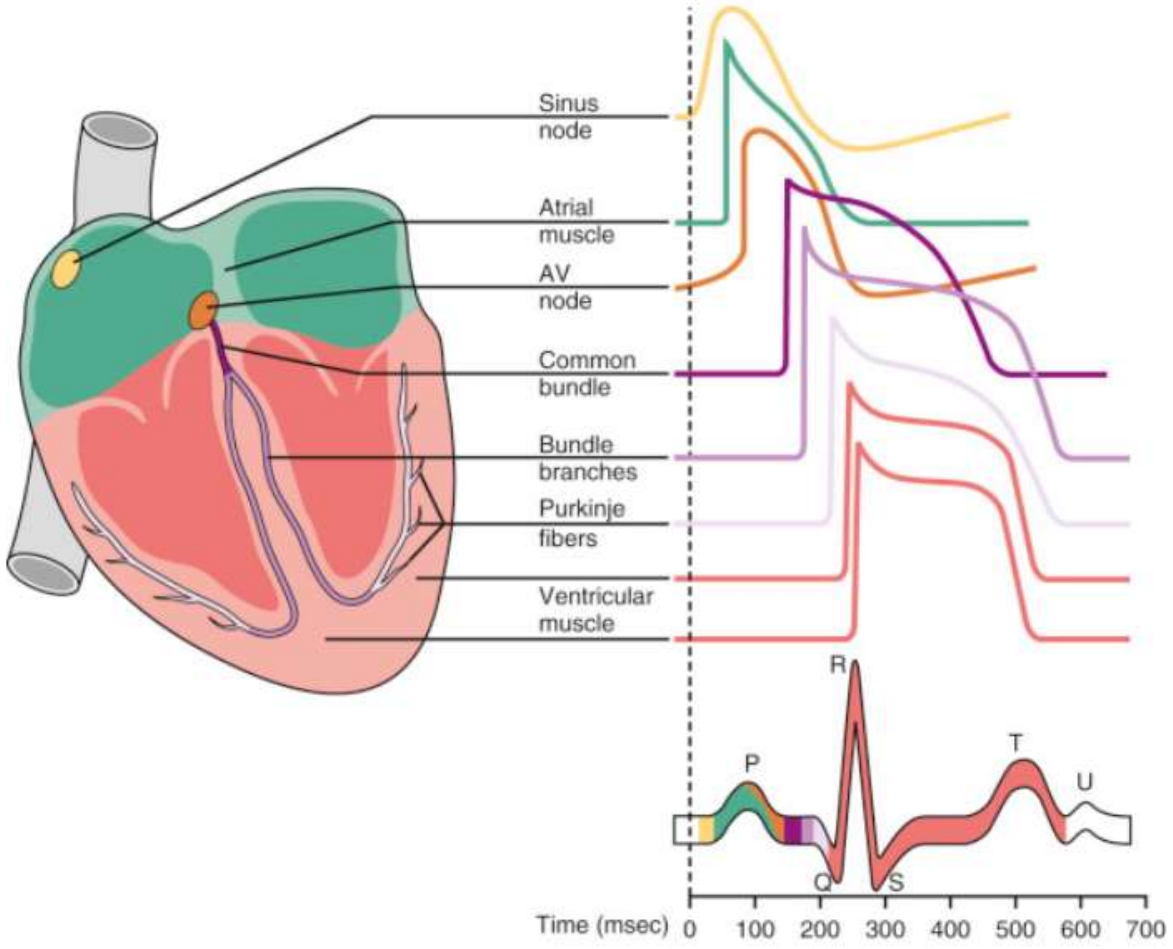

Figure 1: Electrical conductance system along with action potential curves for different regions of the heart. Pacemaking cells have slow, inward Na+ currents (I_f) causing the membrane potential to begin to spontaneously depolarize that, with the help of L-type and T-type Ca2+ channels, triggers an action potential. This electrical signal causes the atria to contract, travels down the AV node which slows down conductance and allows the ventricles to fill with blood. The signal then travels down the bundle of His and to the purkinje fibers located at the apex of the heart, causing ventricular contraction (Ellenbogen et al. 2017).

Sarcomeres are highly ordered structures that occupy the space between two Z-lines of a myofilament and their organization is critical for heat function (Figure 2A). In fact, a hallmark of both dilated and hypertrophic cardiomyopathies in humans is the presence of disorganized sarcomeres (Hughes 2004; Harvey and Leinwand 2011). For healthy, mature cardiomyocytes, the distance between Z-lines is 2.0-2.2 μm while in fetal cardiomyocytes it's 1.8 μm (Lundy et al. 2013). Each sarcomere is composed of actin

'thin' filaments and myosin 'thick' filaments. Actin is bound to the Z-line in the I-band region of the sarcomere, while myosin occupies space in the A-band, or the M-line which is the middle of the sarcomere. It is the interactions between myosin and actin that causes the cell to contract, but to fully understand these dynamics more information is needed about the molecular proteins involved.

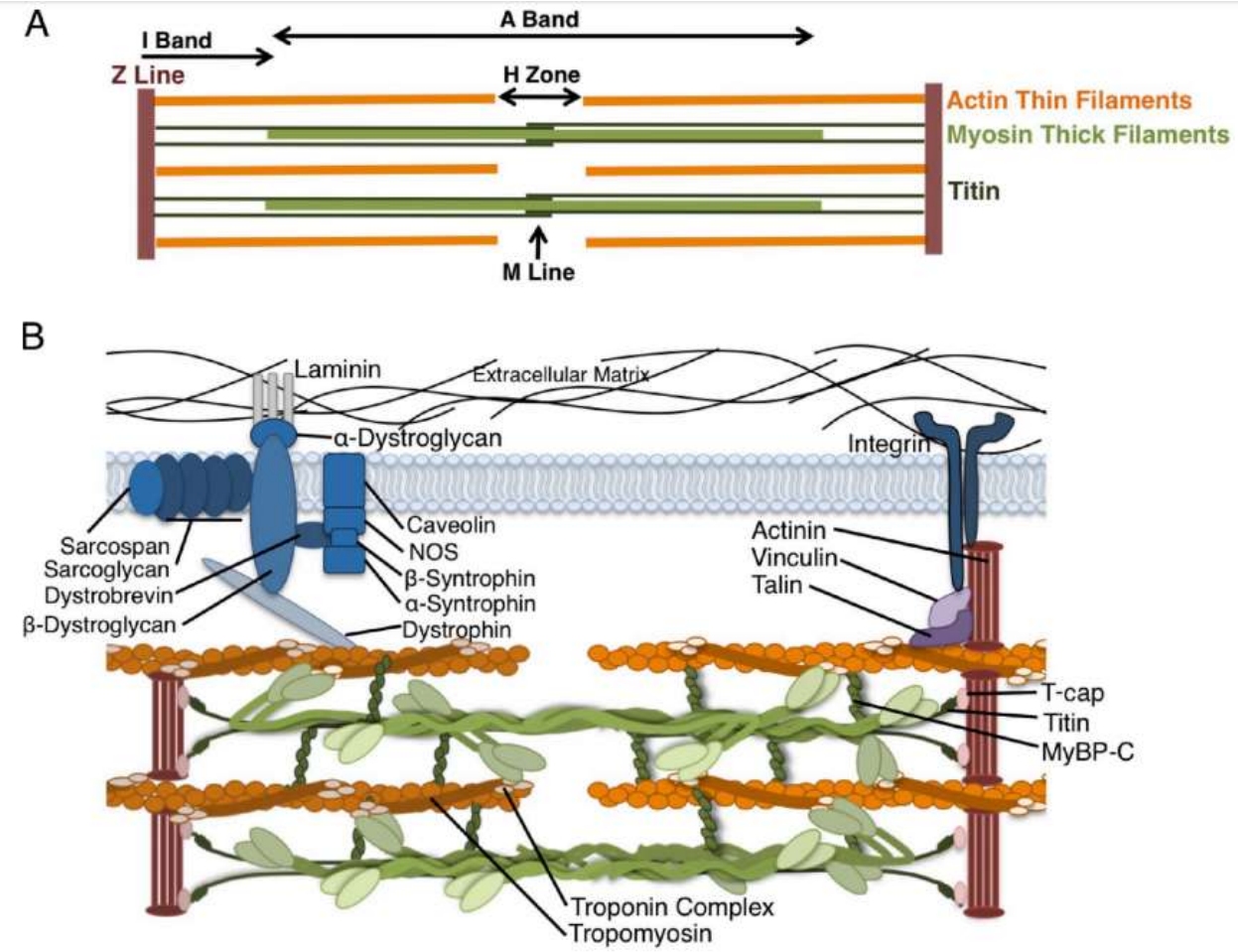

Figure 2: The anatomy of a cardiac sarcomere. (A) Diagram of basic regions within a sarcomere. Actin thin filaments are anchored to the Z-lines while titin serves as a bridge to connect myosin thick filaments to the Z-lines. (B) A more detailed schematic of the troponin and tropomyosin complex which serve as binding sites for myosin heads to attach and initiate a power stroke resulting in sarcomere shortening, or contraction of the cardiomyocyte. Force transduction and intracellular signaling are coordinated through the costamere, which along with integrins, link up the inside cytoskeletal network with the outside ECM world (Harvey and Leinwand 2011).

Cardiac myosin II is the molecular motor of the heart. It is a bipolar filament with a tail region and two globular head domains. One of the myosin heads binds to actin while the other binds to ATP which provides energy for muscle contraction (Yamauchi-Takihara et al. 1989). The thin filament actin is helical in nature and contains tropomyosin wrapped around it which prevents myosin from binding to actin. Attached to tropomyosin is troponin which has three subunits: cTnC binds calcium; cTnI binds to actin and inhibits contraction; and cTnT binds one complex to each tropomyosin molecule (White, Cohen, and Phillips Jr 1987). When cTnC is bound to calcium a conformational change occurs within its structure which in turn alters the structure of tropomyosin, revealing hidden myosin binding sites on the thin actin filament. Myosin heads bind to the newly uncovered binding spots on actin and initiate a power stroke which moves the actin filament inwards, thereby shortening the sarcomere. When an ATP molecule attaches to the myosin head it causes detachment from actin and primes myosin to bind with another actin binding site and initiate a second power stroke if calcium is present. If calcium is not present, the actin binding sites remain hidden and myosin is unable to attach to actin resulting in a relaxed muscle.

Since calcium results in muscle contraction, its concentrations need to be maintained within myocytes so they are not continuously contracting and spending valuable ATP. The sarcoplasmic reticulum (SR) is the organelle responsible for storing and releasing calcium. The process by which the SR releases calcium is known as calcium-induced calcium release (Fabiato 1983). Membrane depolarization during an action potential causes voltage-gated L-type calcium channels, located in invaginations of the cell membrane known as transverse tubules (T-tubules), to open which allows calcium to

flow into the cell. This influx of calcium binds to ryanodine receptors (RyRs) located on the SR which in turn releases more calcium into the cytosol which can bind to cTnC and produced muscle contractions as described above. Termination of muscle contraction occurs when calcium is pumped out of the cytosol and back into the SR.

Human pluripotent stem cell derived cardiomyocytes (hPSC-CMs)

For the first time researchers have access to an unlimited supply of human cardiomyocytes which has opened up new avenues of research. Unlike before when human cardiomyocytes were only available from primary tissue and short in supply, researchers can now differentiate cardiomyocytes from human pluripotent stem cells (hPSC-CMs), including both embryonic (hESC-CMs) and induced pluripotent (hiPSC-CMs) (J. Zhang et al. 2009a). These hPSC-CMs have wide ranging potential and can be used as replacement tissue for damaged myocardium (Caspi et al. 2007; Shiba et al. 2016) or as a cell source for cardiotoxicity drug screening and development (Jonsson et al. 2012) (Figure 3). They can also be effective disease models as it is now possible to re-create aspects of cardiovascular disease *in vitro* using patient sourced cardiomyocytes (Burridge et al. 2016). While early studies using hPSC-CMs are encouraging, we have only scratched the surface of their future potential.

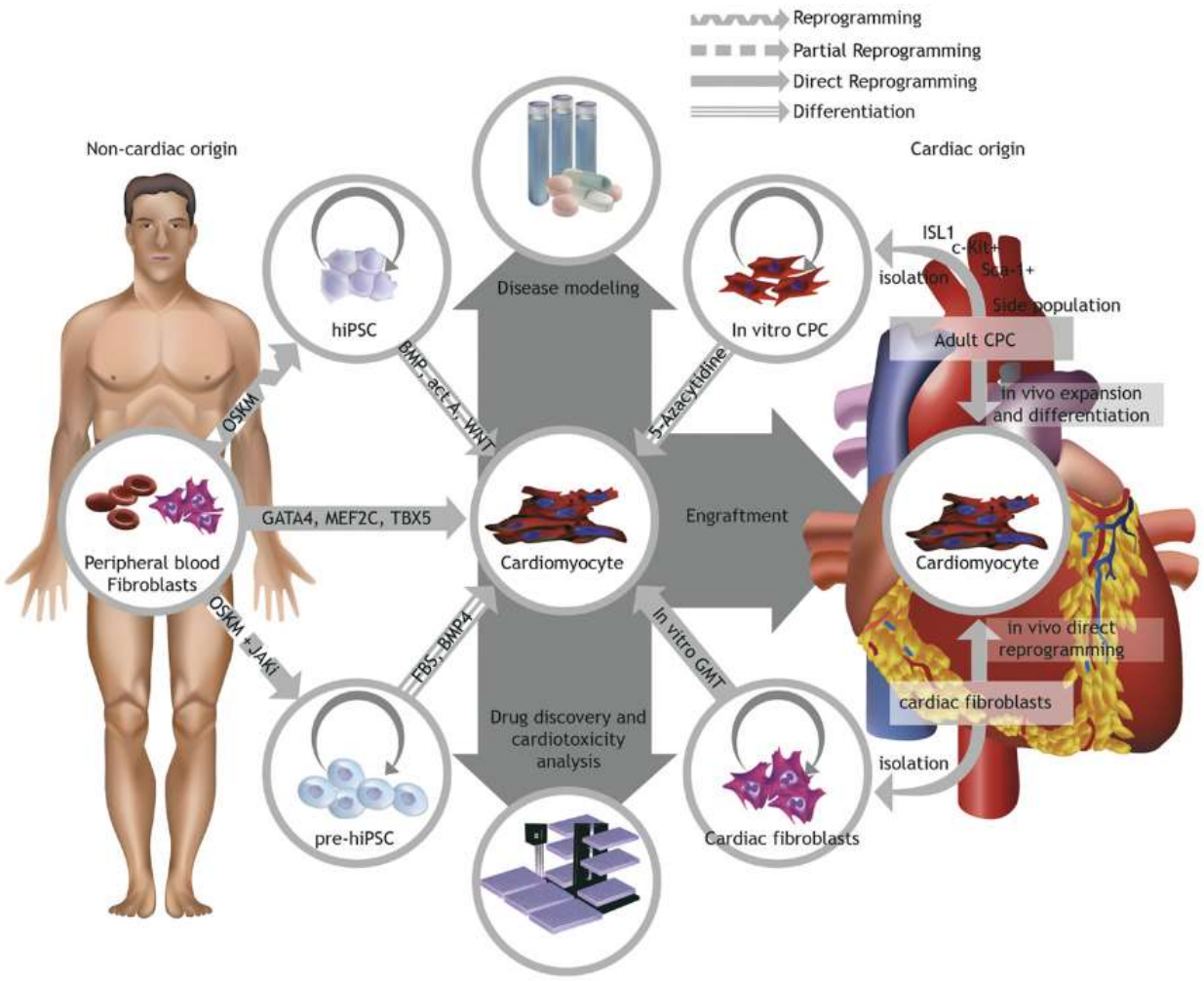

Figure 3: hPSC-CMs hold tremendous therapeutic potential and can serve as platforms for disease modeling, drug discovery and cardiac regeneration (Burridge et al. 2012).

The first hESCs were derived in 1998 (Thomson et al. 1998); meanwhile, the first spontaneously contracting cells, a unique characteristic of cardiomyocytes, were observed in embryoid bodies formed from hESCs *in vitro* (Itskovitz-Eldor et al. 2000). Since their initial discovery, researchers have worked on developing more efficient cardiomyocyte differentiation protocols by studying cardiomyogenesis in model organisms such as mouse (Kanno et al. 2004), *Xenopus* (Shi et al. 2000), chick (Hescheler et al. 1997) and drosophila (Frasch 1995). Some of the pathways and factors discovered in other animal species have shown to improve cardiomyocyte

differentiation from hPSCs, while others have no effect. For example, the application of Activin A and BMP4 to EBs (Kattman et al. 2011) and monolayers (Laflamme et al. 2007) were able to improve cardiomyocyte differentiation from hPSCs. On the other hand, when hESCs were cultured with dimethylsulfoxide (DMSO), retinoic acid and BMP2, known cardiogenic factors in mouse ESCs (Kehat et al. 2001), the number of beating areas did not increase compared to the control (Mummery et al. 2003). Now researchers have developed more efficient protocols for producing cardiomyocytes (Willems et al. 2011; Lian et al. 2012), which have reported efficiencies of up to 98%. In addition to high efficiency, chemically defined protocols also exist which will be necessary when translating research to the clinics (Burridge et al. 2014). In the cases where efficiency remains low, non-genetic methods of purification using lactate media exist that result in a 99% cardiomyocyte purity (Tohyama et al. 2013). These pure populations reduce the risk of tumor formation due to contamination of residual PSCs in cell transplantation therapies.

With the establishment of effective methods for generating pure populations of cardiomyocytes, the next challenge has been creating specific cardiomyocyte subtypes. As mentioned previously cardiomyocyte is an umbrella term that encompasses ventricular, atrial and nodal cells, each with their own characteristics. Access to enriched populations of cardiomyocyte subtypes is important for modeling diseases that affect specific regions of the heart, such as atrial fibrillation which is the most common cardiac arrhythmia and pharmacological treatment for this disease can have unwanted side-effects on ventricular cardiomyocytes which predispose the patient to sudden cardiac death (Eisen et al. 2016). To limit these adverse side-effects there needs to be

reliable chamber-specific hPSC differentiation protocols. To date, most protocols have modulated the Wnt/β-catenin signaling pathway which has resulted in mostly ventricular fated cardiomyocytes with small amounts of nodal and atrial cells present (Tran et al. 2009; Ren et al. 2011; Lian et al. 2012). More recent research has shown that retinoic acid (RA) signaling can drive mesodermal progenitors from PSCs towards atrial-like cardiomyocytes (Devalla et al. 2015; Lee et al. 2017). In addition, inhibition of neuregulin/ErbB signaling enhances the nodal phenotype in hESC-CMs (W.-Z. Zhu et al. 2010) while the addition of endothelin-1 to murine Nkx2.5^{+} cardiac progenitors promotes differentiation into pacemaking cells (X. Zhang et al. 2012). The ability to provide pure populations of cardiomyocyte subtypes will enable therapeutic strategies aimed at modeling chamber specific congenital heart defects and give more reliable insights compared to the use of mixed populations of cardiomyocytes.

hPSC-CM Maturation

With the creation of optimal differentiation protocols, one final hurdle preventing hPSC-CMs from reaching the ‘holy grail’ status remains their lack of maturity compared to adult cardiomyocytes *in vivo*. Although hPSC-CMs beat spontaneously, express sarcomeric proteins, ion channels and have comparable excitation-contraction coupling mechanisms as in the developing heart (R. Zhu et al. 2014), hPSC-CMs do not have the same morphological and functional characteristics as adult cardiomyocytes. To create accurate disease models that better predict responses to drug toxicity, it is important to have mature hPSC-CMs as they better reflect the adult cardiomyocyte physiology. For regenerative cardiac cell therapy, transplantation of immature cardiomyocytes into infarcted hearts may promote arrhythmia due to differences in their electrophysiological

properties (Shiba et al. 2012; Chong et al. 2014). To overcome these obstacles, new methods aimed at promoting hPSC-CM maturation need to be developed.

Below we focus on the definition of cardiomyocyte maturity and explain the characteristics of hPSC-CMs that make them different from *in vivo* adult cardiomyocytes. Afterwards, we discuss maturation strategies of the past and the current state of the art in the field of human cardiac tissue engineering. Only the maturation markers and methods pertinent to this research will be discussed. For a more extensive discussion, there are several excellent review papers (Veerman et al. 2015; Feric and Radisic 2016; Schroer et al. 2019).

hPSC-CM Morphology and Functional Properties

One of the most distinguishing features of a cardiomyocyte is its rod-shaped morphology with an average length-to-width ratio of 7:1, with lengths in the 100 μm range (Lieu et al. 2009). In contrast, hPSC-CMs grown *in vitro* are often pleomorphic in shape and smaller in size (Yang, Pabon, and Murry 2014; A. J. S. Ribeiro et al. 2015). An increase in cell area and volume results in increased membrane capacitance of ~150 pF in humans (Spach et al. 2004). In addition, the rod-shape of mature cardiomyocytes produces a highly organized internal cytoskeletal network consisting of myofibrils running parallel to the length of the cell body whereas there is no clear sarcomere organization in hPSC-CMs. It has been demonstrated that sarcomere organization is directly related to both Ca^{2+} transient kinetics and contractile stress (Feinberg et al. 2012; M. C. Ribeiro et al. 2015). Furthermore, in addition to an increase in the organization of sarcomeres, there is also an increase in the length of an individual

sarcomere. For mature cardiomyocytes, the average sarcomere length is 2.0-2.2 μm while in fetal cardiomyocytes it's 1.8 μm (A. J. S. Ribeiro et al. 2015).

Found at the distal ends of mature cardiomyocytes are intercalated discs which are composed of three different types of proteins: gap junctions, adherens junctions and desmosomes. Both adherens junctions and desmosomes are responsible for mechanical coupling between cardiomyocytes while gap junction proteins help aid in electrical coupling and permit the action potentials from one cardiomyocyte to propagate to the next. The most predominant cardiac gap junction protein is connexin-43. In adult cardiomyocytes, action potential propagation is anisotropic due to the abundance of connexin-43 located in the intercalated discs rather than at the lateral cardiomyocyte border (Jansen et al. 2015; Ongstad and Kohl 2016). In hPSC-CMs, connexin-43 is circumferentially expressed which leads to an isotropic action potential propagation. Speeds of conduction velocity for adult tissue range from 0.3-1 m/s while those of hPSC-CMs lag behind at 0.1 m/s (Feric and Radisic 2016).

hPSC-CM Maturation Strategies

Cardiomyocyte maturation *in vivo* is regulated by a myriad of factors that occur during the development of the human heart (Figure 4). Unsurprisingly, researchers have tried to mimic these cues *in vitro* in order to improve hPSC-CM maturity. Perhaps the most obvious cue needed for maturation is time. While hPSC-CMs can be generated within 15 days of differentiation, it normally requires ~ 6 years for human neonatal cardiomyocytes to reach their adult phenotype *in vivo* (Peters et al. 1994). Among the first to test prolonged *in vitro* culture was Lundy and colleagues in which they compared

early-stage (20–40 days of *in vitro* differentiation and culture) and late-stage hPSC-CMs (80–120 days) and showed improvements in morphological changes such as multinucleation, increase in sarcomere length, cell size, and elongated shape (Lundy et al. 2013). Another study cultured hPSC-CMs for 360 days and observed the presence of M-bands, albeit not in all cells tested (Kamakura et al. 2013). While these studies are impressive, the adult cardiomyocyte phenotype was never acquired, nor is prolonged culture economical.

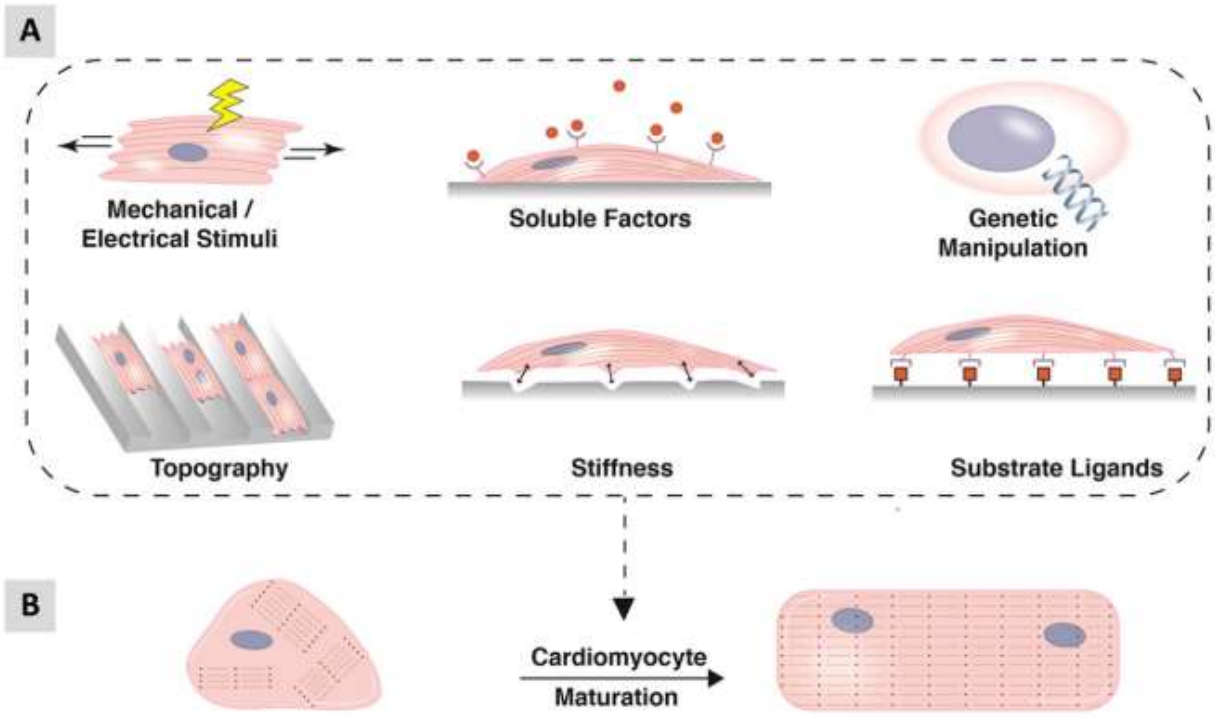

Figure 4: Cardiomyocytes in vivo are exposed to a myriad of biochemical and biophysical cues within the myocardial niche. With time and more research, it is becoming evident all these factors influence the cardiomyocyte phenotype and are necessary to mature both cardiomyocytes *in vivo* and hPSC-CMs *in vitro*. (Denning et al. 2016)

Another potential strategy to help promote maturation is culturing hPSC-CMs on a physiological relevant stiffness. Traditional culture involves platting cells on either plastic or glass which have a stiffness in the GPa range (Ashby, Shercliff, and Cebon 2018). In

contrast, the human heart is a dynamic, compliant structure composed of ECM proteins with a healthy elastic modulus ranging from 10-20 kPa and diseased myocardium ~ 50 kPa (Berry et al. 2006). Jacot et al. cultured neonatal rat ventricular myocytes on polyacrylamide gels of varying stiffness and found cardiomyocytes cultured on 10 kPa became elongated, produced the greatest contractile stress, largest calcium transients and sarcoplasmic calcium stores compared to more compliant and stiffer substrates (Jacot, McCulloch, and Omens 2008). Similarly, cardiomyocytes cultured on stiffer fibrotic-mimicking substrates (34kPa) generated less force, lack striated myofibrils and stop beating (A. J. Engler et al. 2008). In addition to cardiomyocytes, a seminal paper by Engler demonstrated the differentiation of mesenchymal stem cells was biased by the stiffness of the underlying substrate (Adam J. Engler et al. 2006). The profound role of substrate stiffness in influencing cell fate and behavior warrants its inclusion as one critical design element when making hPSC-CM maturation platforms.

As already discussed, cell morphology is a key characteristic that distinguishes mature and immature cardiomyocytes. To induce cell alignment and create order from chaos, researchers have forced cardiomyocytes to align using microfabrication techniques. Several studies utilizing rat and mice cardiomyocytes have revealed that two-dimensional micropatterned lanes are capable of improving cell alignment and myofibril organization (McDevitt et al. 2002; Cimetta et al. 2009). Furthermore, the alignment of myofibrils in neonatal rat ventricular cardiomyocytes on micropatterned substrates lead to an increase in the peak stress generated (Feinberg et al. 2012). Salick et al demonstrated a relationship between the micropatterned lane width and cellular alignment within a pure population of hPSC-CMs (Salick et al. 2014). More recently,

Ribeiro combined micropatterning with substrate stiffness and demonstrated single hPSC-CMs cultured on 7:1 micropatterned islands produced the highest mechanical output while increased stiffness and applied overstretch induced myofibril defects (M. C. Ribeiro et al. 2015). Other microfabrication techniques that have produced similar alignment include PEG-and-groove combinations (Motlagh et al. 2003), nanotopographies (D.-H. Kim et al. 2010) and “wrinkled” polyethylene (PE) shrink-films (A. Chen et al. 2011). In addition, our previous work demonstrated the feasibility of mechanical analysis of aligned cardiac tissue on soft PDMS substrates (Notbohm et al. 2019). These results demonstrate cell-matrix interactions influence the morphology and functional phenotype of the attached cells.

In vivo, primary cardiomyocytes in the atria and ventricles are stimulated by nodal cardiomyocytes, which determine pace and contractility. To mimic this excitation–contraction coupling, researchers have used exogenously applied electrical signals. Radisic and colleagues used electrical field stimulation on neonatal rat ventricular myocytes which induced elongated morphology concurrent with increased sarcomere volume and numbers of mitochondria, intercalated discs, gap junctions and contractility compared to non-stimulated cells (M. Radisic et al. 2004). Similar results have been reported in 3D tissues (Nunes et al. 2013; Eng et al. 2016). All of the aforementioned studies tested one pacing frequency during the duration of culture. A recent study modulated the pacing frequency from 2 Hz to 6 Hz by an increase of 0.33 Hz per day, followed by one week at 2 Hz in what they called intensity training (Ronaldson-Bouchard et al. 2018). The results of this study provide one of the best improvements in maturation for hPSC-CMs with adult-like gene expression profiles, well-organized

ultrastructure and the presence of transverse tubules. Still, functional properties such as resting membrane potential (−70.0 mV) and conduction velocity (25.0 cm/s) were lower than the adult phenotype.

While cardiomyocytes are the first cell type that come to mind when discussing the heart, other cell types are present and it is therefore logical to ask if these cells contribute to the cardiomyocyte maturation process. One of the first studies to directly interrogate this question came from Kim and coworkers in which they isolated pure populations of hESC-CMs from EBs by lentivirus-engineered Puromycin resistance (C. Kim et al. 2009). When cultured with non-myocytes, hPSC-CMs had a faster upstroke velocity, more hyperpolarized maximum diastolic potential and higher action potential amplitude compared to when cultured alone. Another study utilized metabolic selection to purify hPSC-CMs and reported higher peak force production at a final cell ratio of 1:1 fibroblast : cardiomyocyte (Tiburcy et al. 2017). While these studies created defined culture conditions, by far the most common use of co-culture systems involve a heterogeneous population of cells including fibroblasts or mesenchymal cells which are necessary for matrix compaction and remodeling of 3D matrices (Nunes et al. 2013; Boudaoud et al. 2014; Tiburcy et al. 2017; Ronaldson-Bouchard et al. 2018). Although such 3D culture techniques are capable of better replicating the three-dimensional cell-cell interactions found *in vivo*, deconvolving differences in heterogeneous cell populations is more challenging and can lead to challenges with reproducibility.

hPSC-CM Platforms for Drug Discovery and Cytotoxicity Studies

With enhanced hPSC-CM maturity, their utilization for drug discovery has increased. As mentioned early, cardiovascular toxicity claims the highest incidence of adverse drug reactions in late-stage clinical development, often resulting in failed clinical trials and millions of dollars wasted (Laverty et al. 2011). Prior to human clinical trials and animal testing, new drugs must first undergo an *in vitro* human *ether-à-go-go*-related gene (hERG) assay to determine cardiotoxic effects (Food and Drug Administration, HHS 2005). hERG encodes a subunit of the rapid delayed rectifier K^+ channel which plays an essential role in the repolarization phase of the cardiac action potential and mutations in this gene have been linked to long QT syndrome (LQTS), ventricular arrhythmia and sudden cardiac death (Gutman et al. 2003; Mitcheson 2008). Some of the common cells used for the hERG assay include HL-1 cells which come from a mouse atrial cardiomyocyte tumor lineage and endogenously express hERG (Claycomb et al. 1998), or human embryonic kidney (HEX) 293 which can exogenously express hERG (Lastraioli et al. 2004). While these cell lines are useful in their ability to assess hERG channel block, other cardiac ion channels not present in these cells are overlooked and thus understanding of the drug pharmacokinetics is limited (Li et al. 2017). hPSC-CMs offer a potential replacement cell line for the *in vitro* hERG assay to determine cardiotoxic effects. In order to screen the new drugs appropriately, platforms used to grow hPSC-CMs need to be amenable to electrical and mechanical outputs, the two main functional outputs of cardiomyocytes.

To date, most studies that have used hiPSC-CMs to screen for cardiotoxicity or employ in drug development have examined the impact of these compounds on the electro-

physiological properties of the cell (Schroer et al. 2019). Electrical outputs in two-dimensional (2D) can be measured using microelectrode arrays (MEA) (Ulmer et al. 2018), patch clamping (Caspi et al. 2009), impedance measurements (M. K. Jonsson et al. 2009) or by optical mapping studies involving voltage or Ca^{2+} sensitive dyes (Herron, Lee, and Jalife 2012). Equally important as electrical function is the mechanical function of cardiomyocytes, including both chronotropic and inotropic responses. The rate of cardiomyocyte contraction, or chronotropic response, can easily be counted manually or with automated open source software (Huebsch et al. 2015). The strength of hPSC-CM contraction, or inotropic response, requires more sophisticated methods. The most commonly used approach is the edge-detection method, which measures changes in myocyte length (J. Ren and Wold 2001). Other methods that require more expertise include traction force microscopy (TFM) which measures force production of cells on the surrounding substrate (Munevar, Wang, and Dembo 2001) and digital image correlation which measures displacements and from that strains can be calculated (Notbohm et al. 2019).

Perhaps the simplest platform for drug screening applications is to culture hPSC-CMs as an unpatterned monolayer, albeit at the loss of structural organization.
Herron et al. demonstrated improved maturation of hPSC-CMs when the cells were cultured in a monolayer on PDMS substrates rather than on conventional 2D cell culture formats such as glass coverslips (Herron et al. 2016). Conduction velocities for hPSC-CMs monolayers on PDMS substrates were 43 cm/sec while those on glass were 28 cm/sec; however, both conduction velocity profiles were isotropic compared to anisotropic conduction that occurs in the *in vivo* myocardium. Nguyen and colleagues

cultured a monolayer of hPSC-CMs on a MEA chamber and observed an increase in beat rate in the presences of the β-adrenergic agonist isoproterenol but failed to mention an inotropic response (Nguyen et al. 2014). To create aligned cardiac cells, researchers have printed single hPSC-CMs on micropatterned islands on soft polyacrylamide substrates and showed both a positive chronotropic and inotropic response when stimulated with isoproterenol (A. J. S. Ribeiro et al. 2017); however, while being high-throughput, single cell systems fail to recapitulate a multi-cellular tissue and thus conduction velocities cannot be assessed. Aligned cardiac tissues have been generated using nanotopologies (D.-H. Kim et al. 2010) and microcontact printing (Petersen et al. 2018) which have resulted in anisotropic conduction velocity compared to isotropic monolayers; however, these tissues were not stimulated with pharmacological agents. There is currently a void in the literature detailing the mechanical and electrical effects of pharmacological agents on aligned cardiac tissues in 2D.

It is generally accepted that a three-dimensional (3D) architecture can provide a more physiologically-relevant microenvironment for cells. The simplest approach for culturing cardiomyocytes in 3D is to grow them in spheroids either by forced aggregation or by hanging drop method (Nguyen et al. 2014; Beauchamp et al. 2015). 3D cardiac spheroids responded in the expected pharmacological manner when inhibited with myosin ATPase or stimulated by β-adrenergic modulation (Beauchamp et al. 2015). More recent research has showed that doxorubicin, a renowned cardiotoxic and profibrotic agent, triggered apoptosis and disrupted vascular networks in vascularized cardiac spheroids (Figtree et al. 2017). While spheroids provide a 3D environment that

better recapitulates *in vivo* conditions, cardiomyocyte alignment is not achieved and often resembles 2D monolayers. In order to induce alignment, by far the most common 3D approach is to culture cardiomyocytes in engineered cardiac tissues (ECTs) or engineered heart tissues (EHTs). By culturing hPSC-CMs in a gel suspended around silicon posts, cell alignment is achieved during gel compaction and force can be measured by post deflection. Several researchers have shown improvements in hPSC-CM maturation and correct responses to pharmacological interventions (Nunes et al. 2013; Ronaldson-Bouchard et al. 2018). More recently, it has been demonstrated that atrial EHTs and ventricular EHTs can be generated from hPSC-CMs with distinct electrophysiology and drug responses (Zhao et al. 2019). While EHTs have resulted in the highest hPSC-CM maturity to date, the large number of cells required for these constructs make them not feasible for high throughput screening. Furthermore, live cell imaging techniques of sarcomeres within single hPSC-CMs are not amenable when culturing in 3D (Toepfer et al. 2019).

Conclusion

As the field has progressed, it has become evident that spatial, mechanical and biochemical cues found *in vivo* are all important for hPSC-CM maturation. While individually each of these cues help nudge hPSC-CMs to a more mature phenotype, it is perhaps not surprising that the best improvements have come from combinatorial approaches. The following chapters describe a bottom-up approach for engineering hPSC-cardiac tissues suitable for drug discovery and toxicity studies (Fig. 5). Starting with a tunable elastomer substrate, we use micropatterning to induce cell alignment leading to anisotropic conduction velocity within a pure population of hPSC-CMs. Next,

electrical stimulation is added with improvements in electrophysiological outputs. Finally, cardiac fibroblasts are introduced to mimic cell-cell interactions and create a co-culture model with improvements in hPSC-CM electrical and mechanical output. The ability to test these factors individually and concomitantly in one biomimetic platform will lend new insights and aid in the directed maturation of immature hPSC-CMs, ultimately furthering our basic understanding of cardiac biology and providing novel platforms for drug discovery and toxicity testing.

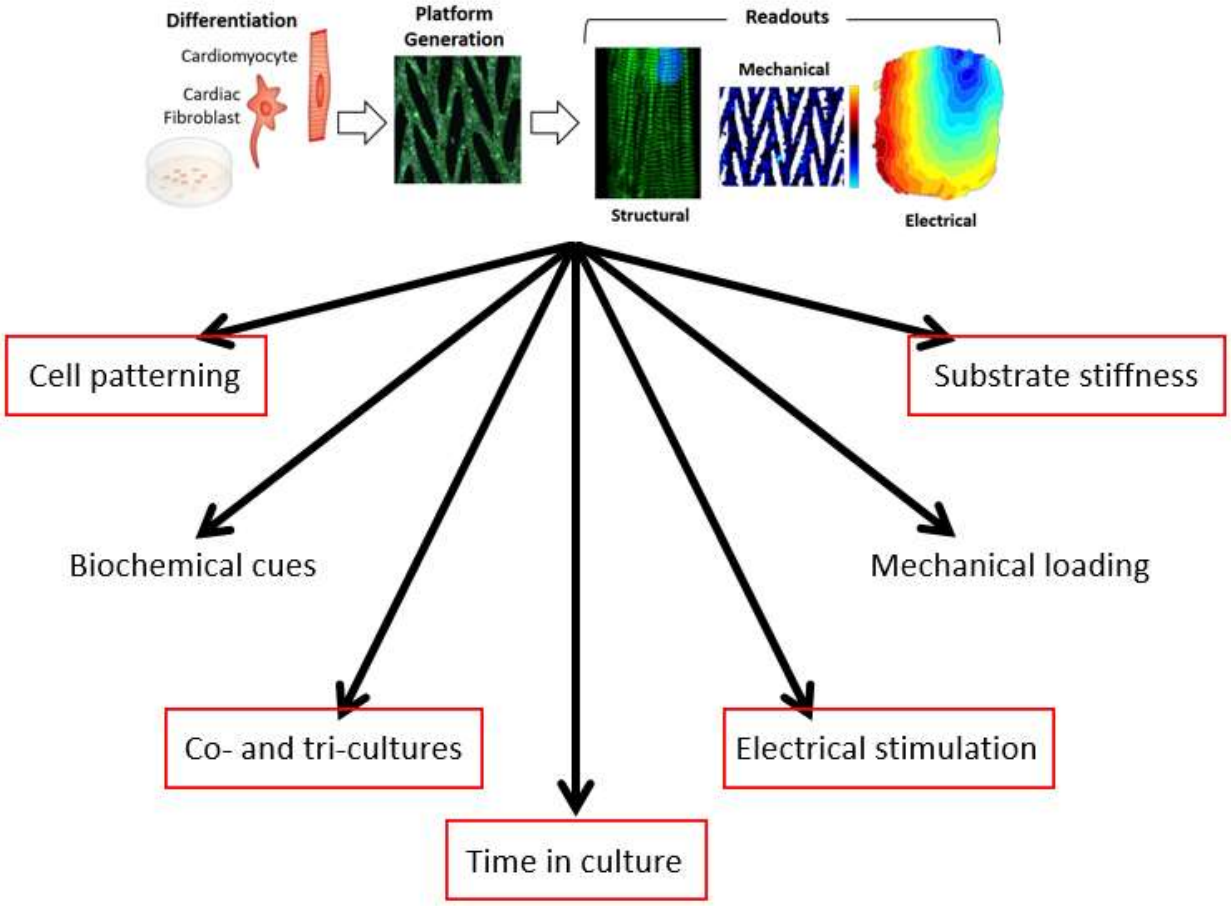

Figure 5: 2D micropatterned platform for drug discovery and cardiac toxicity testing. Arrows indicate methods to be incorporated into the micropatterned platform to improve the functional properties of hPSC-CMs. Red boxes indicate areas of investigation in the work below.

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Chapter 2: 2D Micropatterned Platform for Cardiomyocyte Culture

Some of the results presented in this chapter appear in:

J. Notbohm, B.N. Napiwocki, W.J. deLange, A. Stempien, A. Saraswathibhatla, R.J. Craven, M.R. Salick, J.C. Ralphe, W.C. Crone, “Two-Dimensional Culture Systems to Enable Mechanics-Based Assays for Stem Cell-Derived Cardiomyocytes”, to appear in *Experimental Mechanics,* 2019. DOI: 10.1007/s11340-019-00473-8

Additionally, portions of this chapter will appear in a publication that is currently in preparation and is anticipated to include the following co-authors:

B.N. Napiwocki, A. Stempien, R. Kruepke, P. Stan, W.C. Crone
Jianhua Zhang, Timothy J. Kamp
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INTRODUCTION

Cardiovascular toxicity claims the highest incidence of adverse drug reactions in late-stage clinical development, often resulting in failed clinical trials and millions of dollars wasted (Laverty et al. 2011). As such, Food and Drug Admiration ICH S7B guideline stipulates all drugs must first undergo an *in vitro* human *ether-à-go-go*-related gene (hERG) assay to evaluate cardiovascular safety (Food and Drug Administration, HHS 2005). Due to a lack of available human primary cardiac tissue, common cells used for the hERG assay include HL-1 cells which come from a mouse atrial cardiomyocyte tumor lineage and endogenously express hERG (Claycomb et al. 1998), or human embryonic kidney (HEX 293) and Chinese hamster ovary (CHO) which can both exogenously express hERG (Haraguchi et al. 2015). While these cell lines are useful in their ability to assess hERG channel block, other cardiac ion channels not present in these cells are overlooked and thus our understanding of the drug pharmacokinetics is

limited (Li et al. 2017). Human pluripotent stem cell–derived cardiomyocytes (hPSC-CMs) offer a potential replacement cell line for the hERG assay and other pre-clinical *in vitro* assays; however, cell culture platforms need to be developed that are amenable to electrical and mechanical outputs, the two main functional outputs of cardiomyocytes necessary for drug assessment.

To date the most common approach for obtaining hPSC-CM electrical and mechanical function is to culture them in 3D gels suspended between pillars (Nunes et al. 2013; Zhao et al. 2019). While this approach has yielded impressive levels of maturation (Ronaldson-Bouchard et al. 2018), the large number of cells required for such platforms are not scalable for high-throughput drug screening analysis. Furthermore, live cell imaging techniques of sarcomeres within single hPSC-CMs are not amenable when culturing in 3D (Toepfer et al. 2019). A more scalable approach that requires smaller numbers of hPSC-CMs is to culture them in 2D. The most common approach for culturing hPSC-CMs in 2D is to culture them in monolayers on glass or plastic (Zhang et al. 2009; Lee et al. 2012; Bizy et al. 2013). While these monolayers are electrically coupled, the internal cytoarchitecture remains unaligned and isotropic impulse propagations are observed. Additionally, the rigid substrate prevents measurements of an inotropic drug response, or force of cardiomyocyte contraction. Therefore, researches are now developing new culture platforms made from soft biomaterials that are physiologically-relevant in stiffness. While allowing for quantification of contractile mechanics, soft substrates also improve sarcomere organization and contractile force (Jacot, McCulloch, and Omens 2008; Ribeiro et al. 2015), as well as impact

cardiomyocyte action potentials (Boothe et al. 2016) and calcium transients (Herron et al. 2016).

In this study, our goal was to engineer a 2D *in vitro* substrate platform that includes patterned ECM and physiological substrate stiffness that is amenable to both mechanical and electrical analysis. The result is the formation of cardiac tissue constructs that beat synchronously with cell morphology and anisotropic behavior closer to that seen in adult cardiomyocytes *in vivo*. We investigated the electrophysiological properties of the patterned cardiac tissues and show that they have anisotropic electrical impulse propagation, as occurs in the native myocardium, with speeds 2x faster in the direction of the lanes compared to the transverse direction. Lastly, we interrogate the mechanical function of the pattern constructs and demonstrate the utility of this platform in recording the strength of cardiomyocyte contractions.

METHODS

iPSC Cardiomyocyte Differentiation

A human induced pluripotent stem cell (hiPSC) line iPS-DF19-9-11T (male) (WiCell) was differentiated into cardiomyocytes using the GiWi protocol (Wrighton et al. 2014). As illustrated in Figure 1A, iPSC were seeded on Day -5 in a 6-well plate at a density of two million cells/well and cultured with mTeSR media (WiCell). Media was changed every day until the cells were at or over 100% confluent, typically 5 days after the initial seed. On Day 0, mesoderm induction was initiated by changing the media to RPMI 1640 medium (Thermo Fisher) supplemented with B-27 Minus Insulin (Thermo Fisher), referred to as R/B- media, 12μM of the GSK inhibitor CHIR99021 (Biogems) and

1µg/mL Insulin (Sigma). On Day 1, the media was aspirated and changed to R/B- media. On Day 3, the media was changed to R/B- media containing 7.5µM of the Wnt inhibitor IWP2 (Biogems). On Day 5, the media was changed back to R/B- media. On Day 7, and every 2 days thereafter, the media used was RPMI 1640 medium (Thermo Fisher) supplemented with B-27 Sereum-Free (Thermo Fisher). Beating cells were typically observed between Day 9-12 of differentiation. On Day 15, hiPSC-CMs were frozen down in Fetal Bovine Serum (FBS, Invitrogen) containing 10% DMSO (Sigma) and stored in Liquid Nitrogen.

PDMS Substrate Preparation

Two types of commercially available polydimethylsiloxane (PDMS), Sylgard 184 elastomer and Sylgard 527 gel (Dow Corning), were blended together to create PDMS substrates with tunable mechanical properties. Briefly, Sylgard 184 was made per manufacturer's directions by mixing 10 parts base to 1 part curing agent. Sylgard 527 was prepared per manufacturer's directions by mixing equal weights of part A and part B. Both Sylgard 184 and 527 were mixed for 5 minutes with a glass stir rod. After each blend was properly mixed, they were then combined in different mass ratios of Sylgard 184:527 and again mixed for 5 minutes. The mass ratios used were based on previously published data (Palchesko et al. 2012). For this study, PDMS substrates with a Young's modulus of 5, 10 and 50kPa were made. Once mixed, the PDMS was poured into 100 mm diameter petri dishes and cured overnight at 60°C. To overcome challenges associated with handling low modulus PDMS substrates, the PDMS was cured on top of a foundation layer of Sylgard 184 with a final thickness of 2mm. The

following day the PDMS substrates were cut into 1cm squares with a razor blade and UV sterilized prior to the transfer of extracellular matrix.

Substrate Material Characterization by Uniaxial Tensile Testing

The stiffness of the different PDMS blends was confirmed independently by tensile testing. Each PDMS blend was poured into a Teflon mold of standard tensile test sample dimensions (with a total length of 35 mm, a gauge length of 8.0 mm, a gauge width of 1.5 mm and a thickness of 2 mm) and cured overnight at 60°C (Johnson et al. 2004). After samples were fabricated for tensile testing, glass beads of 30–50 μm diameter (Polysciences, Inc.) were applied to the surface to allow for optical strain measurement during the test. Uniaxial tensile testing was performed on the samples using an Instron 5548 MicroTester mechanical testing machine with a 10N load cell. Samples were fully submerged in phosphate-buffered saline (PBS) and tested in an environmental chamber held at 37°C to match native cell culture conditions. Time-lapse images were captured by microscopy to observe the displacement of the beads during the tensile test. During testing, the gage length region of the sample was imaged at 24 fps using QCapture Pro and a Q Imaging MicroPublisher 5.0 RTV Camera. Using a Matlab code developed by the Crone Lab, the strains were calculated by measuring the vertical (axial) distance between pairs of beads during the test (Salick 2014). Testing was conducted at 1 mm/min, which correlates to a strain rate of approximately 0.0025 sec^{-1}. At least 6 measurements from 2 different sample preparations were analyzed per condition. The elastic modulus for each sample is the slope of the linear regression of the stress-strain curves (Fig. 1).

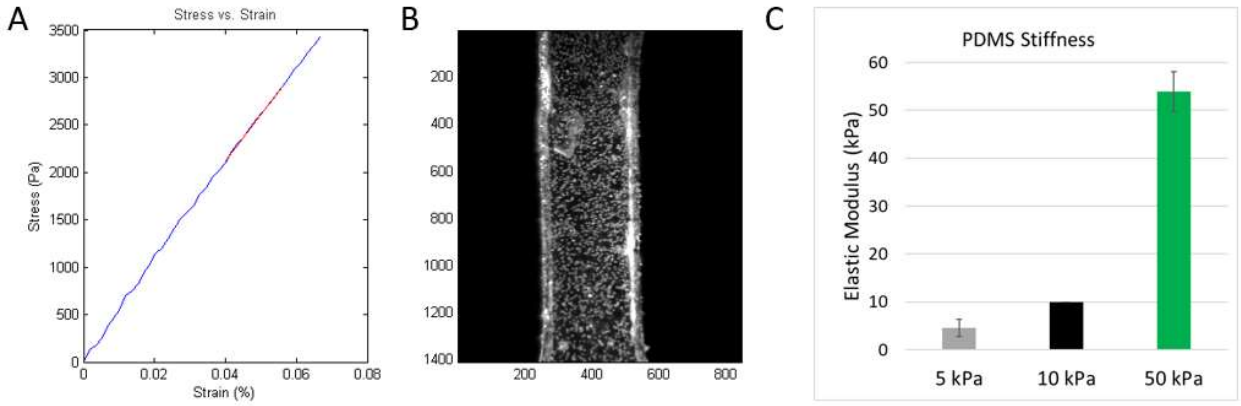

Figure 1: Uniaxial tensile testing of 1:10 (184:527) PDMS blend with an elastic modulus of 50kPa. (A) The stress-strain curve developed from (B) uniaxial tensile testing of a sample with beads tracked by software. (C) Elastic modulus for the three PDMS blends tested: 4.5 ± 1.7 kPa (0:1), 10kPa (1:50) and 53.8 ± 4.1 kPa (1:10). n = 6.

ECM Transfer to Patterned and Monolayer Substrates

Designs used in this study were made in AutoCAD. The 15° chevron pattern consisted of 30 μm lanes spaced apart by 100 μm and connected by 30 μm bridges placed at an angle of 15° with respect to the micropatterned lanes. A silicon wafer patterned with photoresist was produced from the AutoCad file (FlowJEM, Toronto, ON, Canada). Patterned ECM for the platform was created from reusable microcontact printing stamps that were fabricated on the patterned silicon wafer. PDMS (Sylgard 184) was poured on top of the patterned silicon wafer and cured at 60°C overnight to produce a reverse replica of the patterned silicon wafer. After removal from the silicon wafer, the PDMS was cut into individual stamps which were coated with ECM proteins at 37°C overnight (83 μg/mL Matrigel).

The patterns were microcontact printed on the soft PDMS substrates utilizing a sacrificial polyvinyl alcohol (PVA) film (Yu et al. 2012; Napiwocki et al. 2017; Notbohm et al. 2019). Briefly, 0.5 g of PVA (Sigma Aldrich) was dissolved in 10 mL of deionized water and dried overnight in a petri dish. The following day the sacrificial PVA film was

cut into rectangles slightly larger than the PDMS stamps. Using microcontact printing, the ECM-coated PDMS stamps were placed on top of the rectangular PVA films. A glass slide and 50 g weight were added to apply an even pressure distribution and ensure better pattern transfer from the PDMS stamp to the PVA film. After one hour, the PVA film was removed from the PDMS stamp and then brought into conformal contact with the soft substrate for 30 min. Afterward, the substrate was washed to dissolve the sacrificial PVA film, leaving behind the patterned proteins which were then seeded with cells. A 1cm diameter PVC tube was placed on top of the soft PDMS substrates and 83 μg/mL Matrigel (WiCell) was added inside the PVC tube to create the unpatterned control monolayers which were subsequently seeded with cells.

iPSC-CM Thawing, Purifying and Seeding onto Soft PDMS Substrates

Figure 7A is a schematic of the experimental timeline used in this study. Frozen Day 15 hiPSC-CMs were thawed onto Matrigel coated 6 well plates at a density of 2.5 million cells/well and fed with EB20 media: DMEM/F12 (Invitrogen), 20% FBS (Invitrogen), NEAA (Invitrogen), GlutaMax (Lifetech) and 2-Mercaptoethanol (Sigma). On Day 17, the media was switched to lactate media to begin CM purification (Tohyama et al. 2013). The lactate media consisted of RPMI-glucose (Life Technologies), B27-complete (Life Technologies) and 5mM lactate (Sigma). The cells were exposed to lactate for 10 days (Day 27) and then the media was changed to EB2 media, same formulation as above expect the FBS concentration is changed from 20% to 2%. The purified hiPSC-CMs are again fed with EB2 media on Day 29. This method produces CM purities of greater than 95% after lactate purification as confirmed via flow cytometry (Fig. 2). On Day 30, purified hiPSC-CMs are dissociated and singularized with TyrpLE 10x (Lifetech) and

seeded onto both patterned and monolayer substrates using EB20 media, referred to as Day 0. For patterned substrates, hiPSC-CM seeding density must be high enough to ensure complete pattern coverage by the cells, while low enough to allow for CM hypertrophy. The patterned substrates were seeded at a density of 2,668 CMs/mm^2 (95,000CMs/35.605mm^2) while monolayers were seeded at a density of 1,527 CMs/mm^2 (120,000CMs/78.54mm^2). To control cell attachment, a 1cm PVC tube was placed on top of the PDMS substrates and the cell suspension was added inside the tube. The PVC tube was removed the following day. Although the initial monolayer density was lower than the pattern density, the seeding density at end of culture on Day 18 are equivalent by a count of nuclei/area of available ECM (P = 0.67; Fig. 3). After seeding, the media was changed to EB2 media and exchanged every two days during the length of culture.

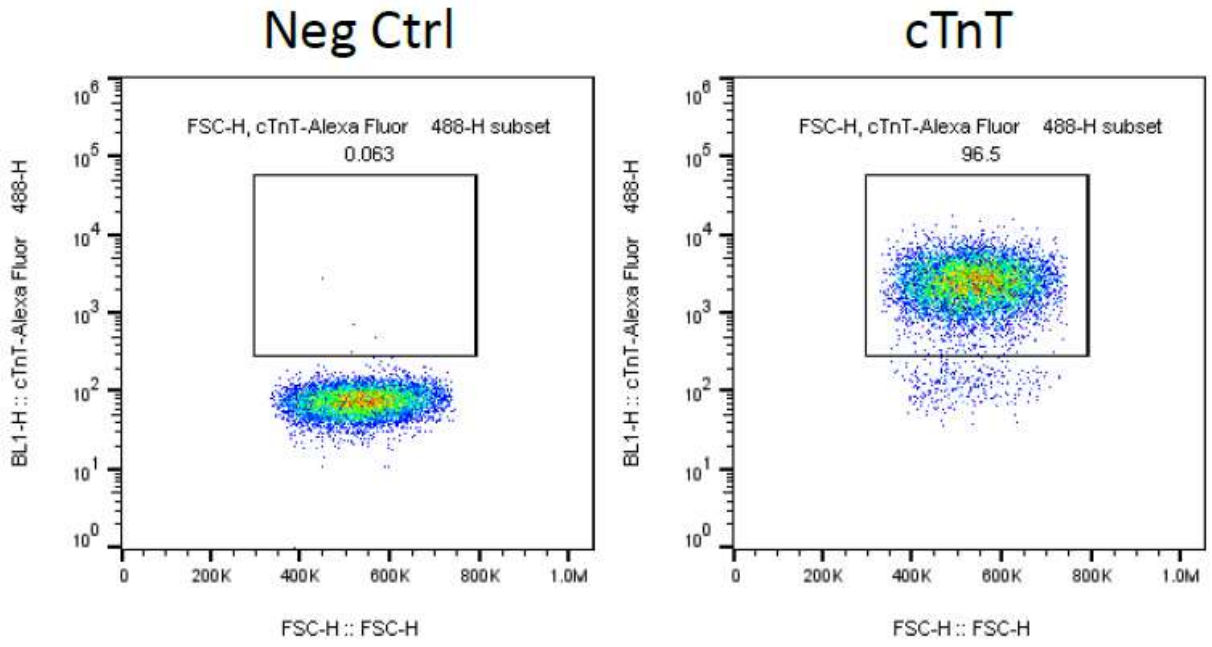

Figure 2: Representative flow cytometry results of Day 30 hPSC-CMs after lactate purification. Data courtesy of Jianhua Zhang.

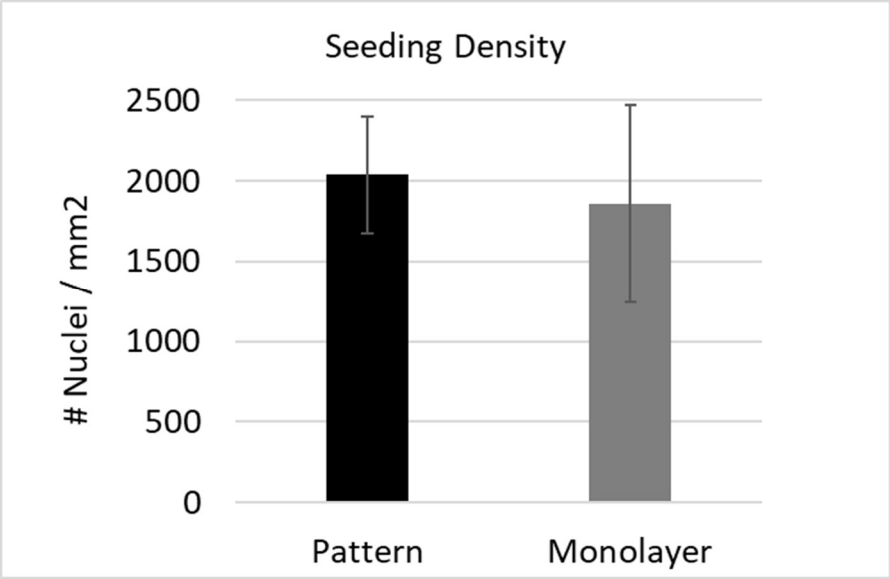

Figure 3: Day 18 seeding density for pattern and monolayer constructs. No statistical difference was observed in seeding densities (P = 0.67). Pattern and monolayer n = 4.

Imaging and Immunofluorescence

Day 18 samples from each of the six conditions were washed once with PBS and fixed in a 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) for 15-min at room temperature. Afterwards, cells were washed with PBS and treated with 0.1% Triton (Sigma) for 10 min at room temperature to permeabilize the cell membrane. The cells were then washed with PBS and treated for 30-60 min with a blocking solution consisting of PBS, 2% FBS, 0.1% Triton, 11.2 mg/mL glycine, and 50 mg/mL BSA. Primary antibodies were diluted in blocking solution and left on the sample overnight at 4°C. The next day the cells were washed with PBS and the secondary antibodies were applied in blocking solution for 1 hour. Afterwards the samples were washed and phalloidin conjugated to tetramethylrhodamine B isothiocyanate (TRITC) (Sigma) was applied at a 50 mg/mL concentration to label actin filaments and DAPI was applied at a 1:1000 dilution to label nuclei. Cells were washed with PBS and transferred to coverslips, where they were mounted using ProLong Gold Antifade (Life Technologies).

Primary antibodies used include Alpha-Actinin (1:250 dilution, Abcam), N-Cadherin (1:250 dilution, BD Biosciences). Secondary antibodies include Alexa Fluor (H+L) 488 goat anti-mouse and Alexa Fluor 647 (H+L) goat anti-mouse. Samples were imaged on a Nikon A1RSi Confocal Microscope with an attached Photometrics CoolSNAP HQ2 camera.

Electrical Impulse Propagation via Optical Mapping

Di Lang performed optical mapping for electrophysiologic characterization. Pattern cell preparations were stained with 5uM Fluo4-AM for 15 min followed by a washout with Tyrode solution for another 15min before imaging. Fluorescence recording then was collected using Nikon microscope at 20X objective (Ex: 483±15 nm; Em: 535±20 nm). Signals was recorded with high-resolution CMOS camera (100X100 pixels; 1000 frames/s) and then was analyzed using Matlab. Experiments were applied at room temperature and 30 degree. Calcium transient duration was measured and propagation activation map was reconstructed based on the signals.

Contractility and Strain Analysis

Alana Stempien performed strain analysis on bright field videos of contracting cells. These were acquired at various time points during culture using Nikon-D elements software and a Nikon DS-Qi1Mc camera with a frame rate of 18.9 fps. Cells were maintained at 37°C using a Tokai Hit environmental chamber. As described in her thesis (Stempien 2018) mechanics analysis was done using previously developed open source software with additional modifications. The open source digital image correlation software, Fast Iterative Digital Image Correlation (FIDIC), can be found at

https://github.com/FranckLab/FIDIC. A script to call the FIDIC functions and displacement to strain calculations was provided by Jacob Notbohm [1]. Subsequent analysis software, was created for this study to process multiple experimental outputs.

Figure 4 is a schematic workflow of contractile strain analysis. X and Y displacements were used to calculate normal and shear strains by taking the gradient of the displacement data. Principal strains were then calculated. A mask was used to eliminate any potential noise due to areas void of cells. The second principal strain was averaged for each frame of the video and the peak value over time was used as a comparative measure of the strain for that sample.

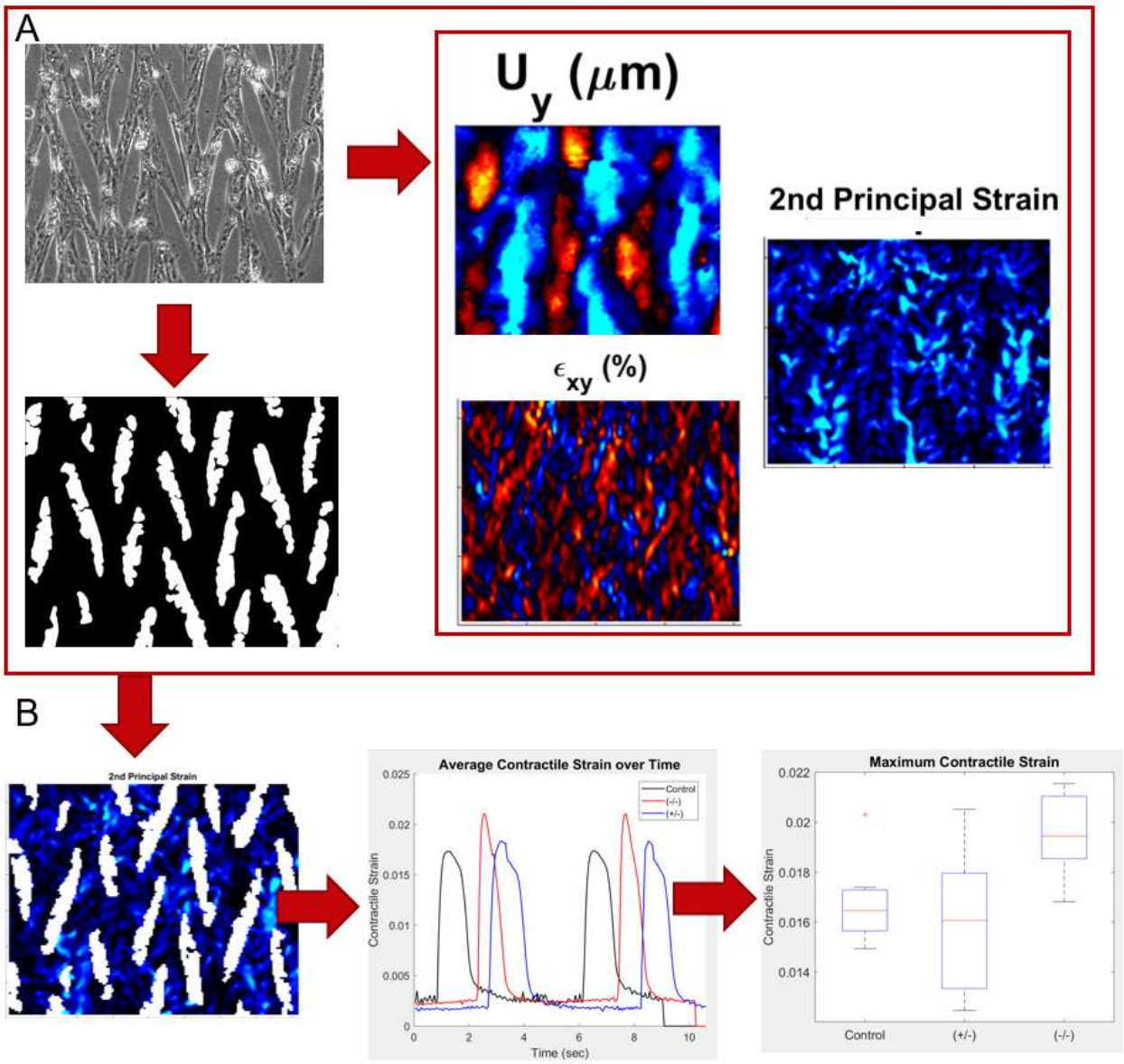

Figure 4: Schematic workflow of contractile strain analysis. (A) From bright field videos of a hPSC-CM contraction displacements and strains are calculated, examples of which are shown here. A mask is used to cover areas not occupied by cells. (B) The second principal strain was calculated for each location containing cells and then averaged for each frame of the video and the peak value over time, identified as the maximum contractile strain, was used as a comparative measure of the strain for that sample.

RESULTS

hPSC-CM Myofibril Alignment and Substrate Platform Design

Mature alignment of myofibrils in a population of purified hPSC-CMs was achieved with culture on Matrigel-patterned lanes of varying widths on three different PDMS substrate stiffnesses, with a Young's modulus of 5, 10 and 50kPa. The dependence of myofibril organization on lane width observed with glass substrates (Salick et al. 2014) was replicated with the PDMS substrates (Fig. 5). Namely, smaller lane widths yielded aligned myofibrils while larger lane widths displayed myofibril disarray. Although this range of Young's modulus for the substrate is physiologically relevant with respect to human tissue, 10 kPa is close to that of healthy myocardium and produced well-defined sarcomeres (A. J. Engler et al. 2008). When comparing the hPSC-CM actin cytoskeleton across the different elastic moduli, myofibril buckling was present only on the 5kPa PDMS substrate, which has also been observed on 6kPa polyacrylamide hydrogels (Fig. 6) (Ribeiro et al. 2015).Strain analysis showed hPSC-CMs produced more strain on a 10 kPa compared to 50 kPa PDMS substrate (Notbohm et al. 2019); therefore, a substrate stiffness of 10kPa was selected for use in all subsequent experiments.

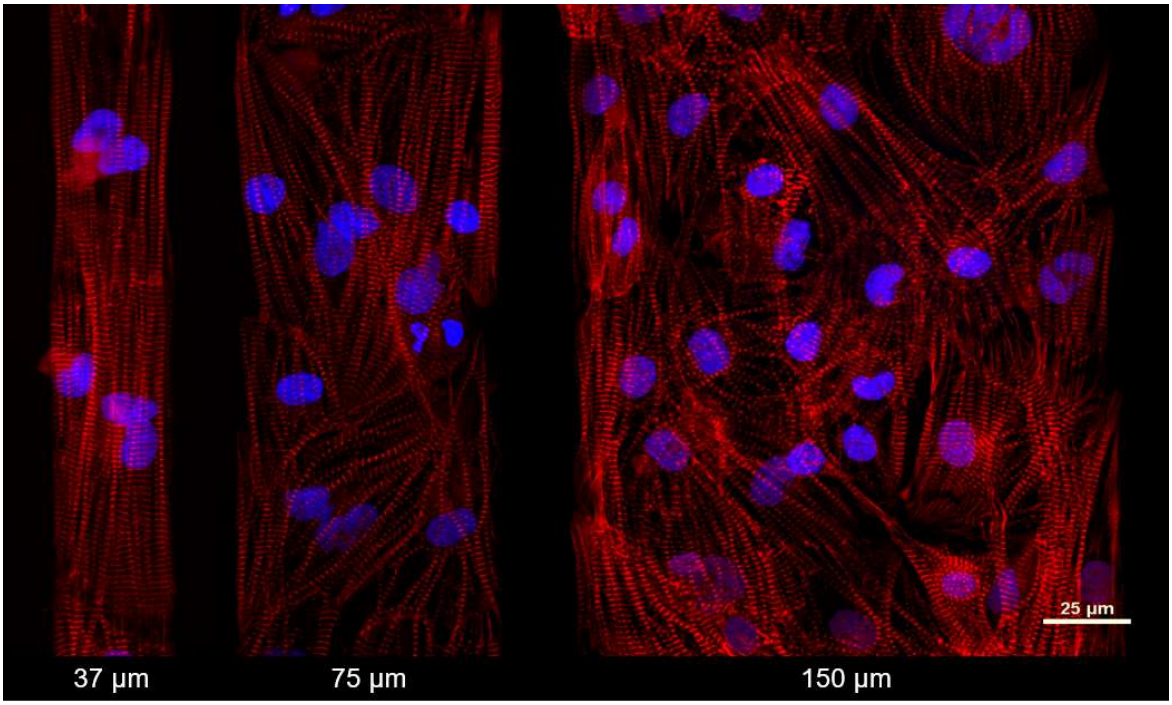

Figure 5: Alignment of hiPSC-CMs myofibrils on different micropatterned lane widths on a 10kPa PDMS substrate (Notbohm et al. 2019). Red = Actin, Blue = DAPI. Scale bar = 25 µm.

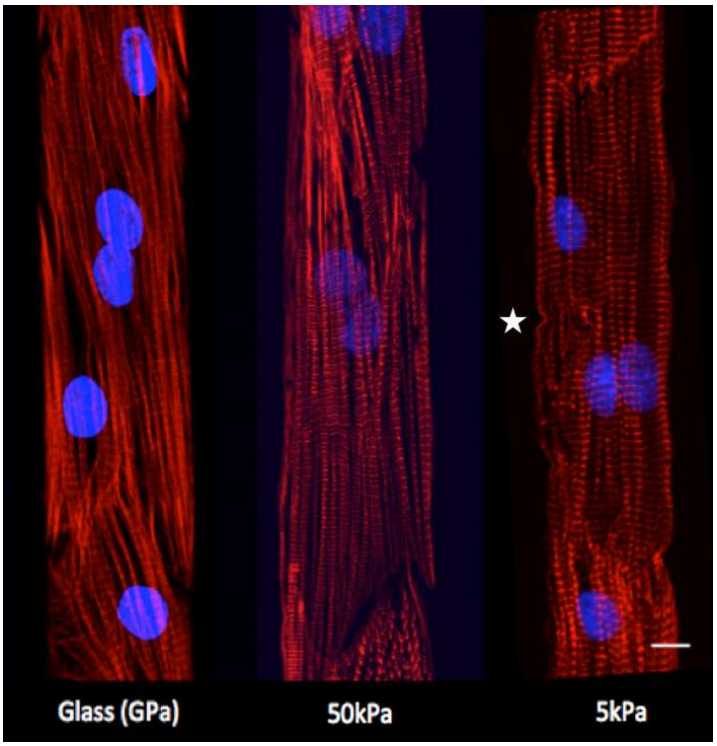

Figure 6: hPSC-CMs grown on different Young's moduli all with a lane width of 36 µm (Notbohm et al. 2019). Star denotes myofibril buckling. F-actin (red) and DAPI (blue). Scale bar = 10 µm.

To create the patterned hPSC-CM constructs, purified Day 30 hPSC-CMs were seeded onto a Matrigel-patterned 10kPa PDMS substrate and cultured for 18 days (referred to as Day 18 CMs) (Fig. 7A). A lane width of 30 μm was selected because it yields 1-2 CMs in the width of the features and maintains alignment of myofibrils. The 30 μm lanes were spaced apart by 100 μm. To induce synchronous contraction among lanes, adjacent lanes were connected with 30 μm wide bridges at an angle of 15° with respect to the lane direction (Fig. 7B – D) (Notbohm et al. 2019). Other bridge angles were considered including 90° perpendicular to the lane direction; however, the cell-cell attachments in these mesh patterns failed at the intersections and the patterns deteriorated with time in culture (Fig. 8). hPSC-CMs seeded on the 15° chevron patterns attached to the Matrigel-patterned areas and synchronous contraction among the tissue construct was observed 1-2 days after seeding and was sustained throughout the 48 days of culture (Fig. 7D).

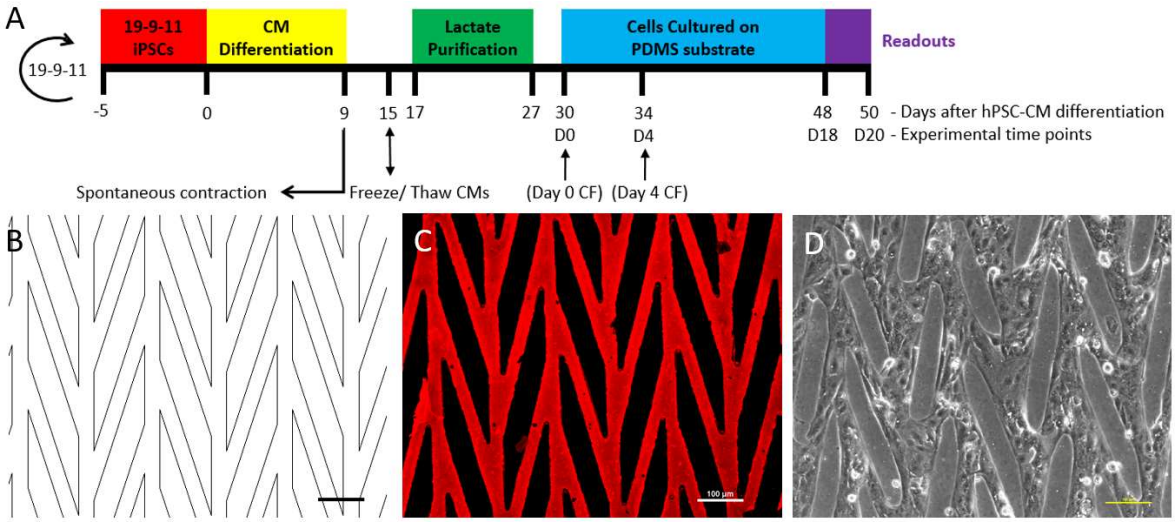

Figure 7: (A) Schematic of the experimental timeline. (B) A 15° chevron pattern design in AutoCAD. (C) Matrigel pattern transferred to a 10kPa PDMS substrate and stained for Laminin. (D) Bright field image of Day 18 hPSC-CM construct with the 15° chevron pattern. Scale bars = 100 μm.

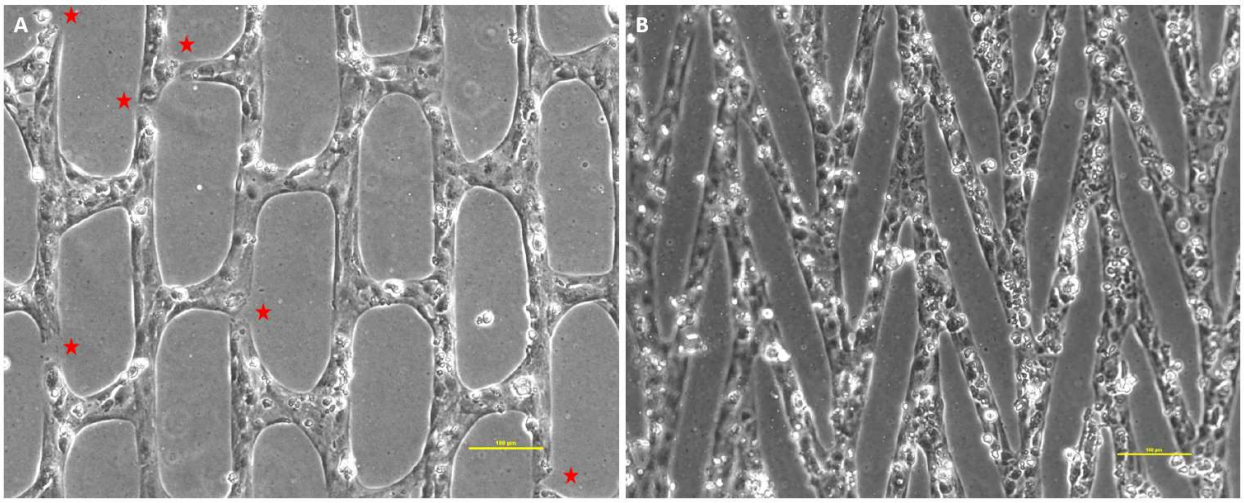

Figure 8: Bright field images hPSC-CM constructs with (A) 90° and (B) 15° chevron patterns on 10kPa PDMS. Stars in the 90° pattern indicate areas of pattern

hPSC-CM Alignment in Pattern and Monolayer Constructs

Prior to co-culture with hPSC-CFs, we further characterized hPSC-CMs seeded on 10kPa PDMS substrates in the 15°chevron pattern and compared them to unpatterned monolayer controls (Fig. 9). In addition to being able to quantify ECM fiber orientation, SGFT was specifically designed to characterize myofibrils in both well aligned and disarrayed samples using images obtained after staining with α-actinin to highlight the sarcomere structures. As can be seen in the SGFT histogram output, myofibrils in the Day 18 monolayer hiPSC-CMs were orientated in all directions. In comparison, two peaks can be seen in the SGFT histogram output for the Day 18 15°chevron pattern corresponding to micropatterned lane and bridge features (Fig. 9D). Overall, myofibrils in the pattern construct had a more mature aligned phenotype with 61% of the myofibrils aligned within ± 10° of the primary direction compared to 18% for hiPSC-CMs in the monolayer control. Furthermore, using N-Cadherin to denote cell boundaries, we found that Day 18 hiPSC-CMs in the patterned constructs were elongated in the pattern

direction and had an average length-to-width ratio of 4:1 while Day 18 hiPSC-CMs in the monolayer controls were 2:1 (Fig. 9F).

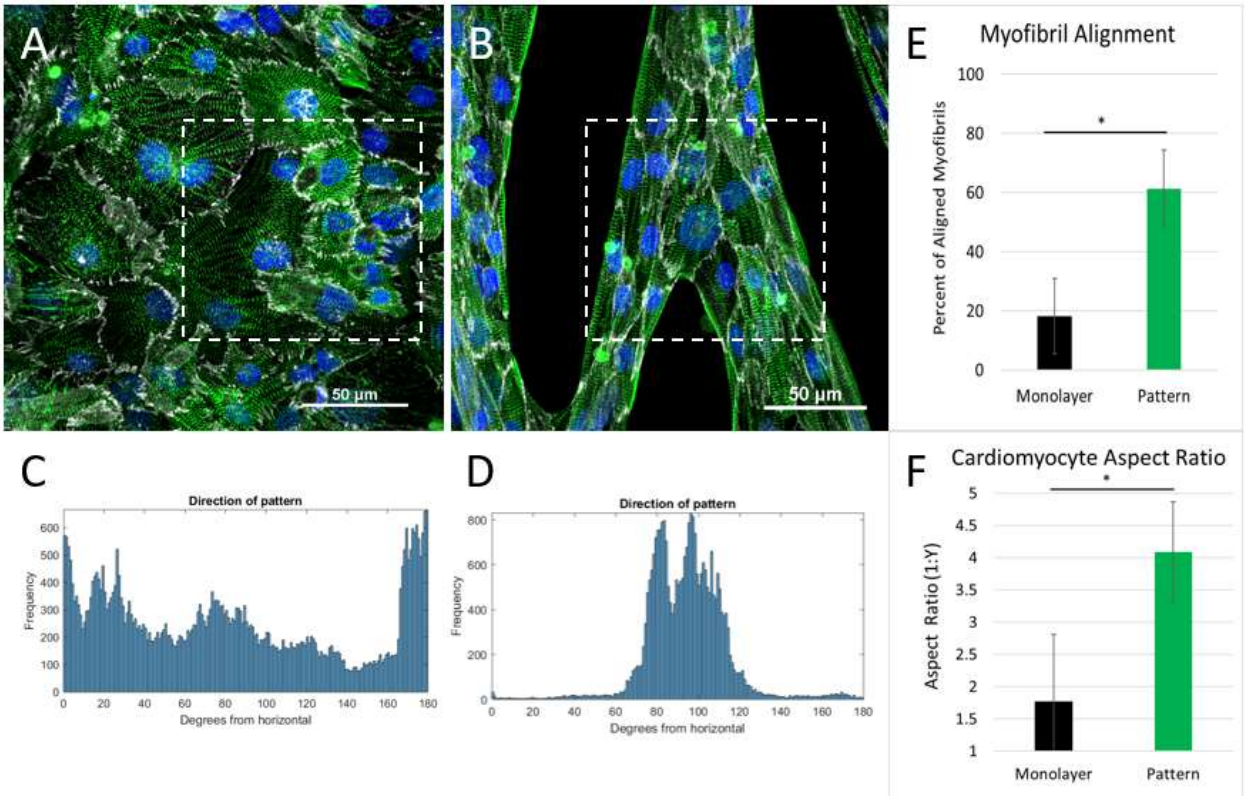

Figure 9: (A) Monolayer control and (B) 15°chevron pattern with hiPSC-CMs cultured for 18 days and stained for Green = Alpha-actinin, White = N-Cadherin, Blue = DAPI. Scale bars = 50 µm. White dashed boxes in A and B represent SGFT myofibril alignment histogram output for (C) monolayer control and (D) 15°chevron pattern. (E) Myofibril alignment and (F) CM aspect ratio compared for patterned construct and monolayer control. (E) Percent of myofibrils aligned within 10 degrees of the primary axis. *$p < 0.05$, unpaired t-test. Myofibril alignment n = 6, cardiomyocyte aspect ratio n = 4.

Functional Properties of hPSC-CM Patterned and Monolayer Constructs

Functional properties of patterned and monolayer hPSC-CM constructs were assessed to determine their maturation status. Electrical properties and the direction of Ca^{2+} flow in the hPSC-CM was determined using optical mapping methods. Optical mapping results demonstrated isotropic electrical impulse propagation in the monolayer

constructs, while patterned constructs displayed anisotropic conduction which also occurs in the native myocardium (Fig . 10A-B). Comparing the longitudinal conduction velocity (CV_L) to the transverse conduction velocity (CV_T) for the pattern hPSC-CMs revealed an anisotropic CV_L/CV_T ratio of ~2 (Fig. 10D). In addition to directionality, the conduction velocity speed for the patterned hPSC-CMs (19 ± 7 cm/sec) was significantly faster than the monolayers (13 ± 4 cm/sec) ($P < 0.05$; Fig. 10C).

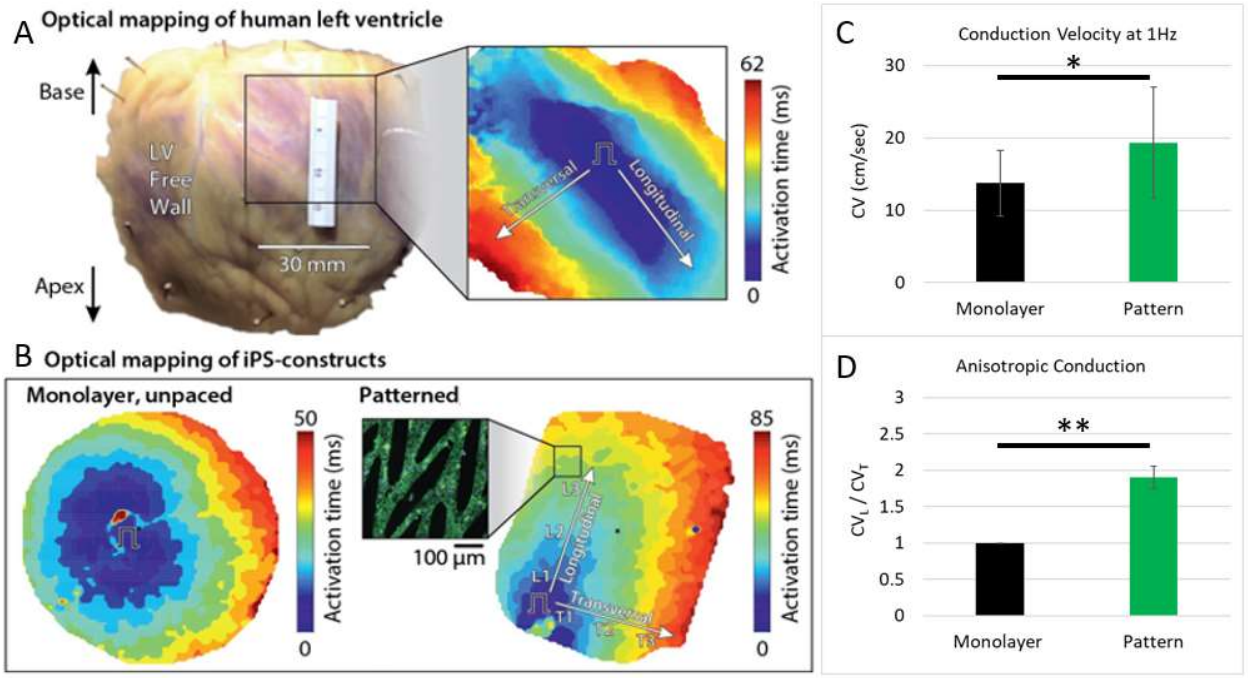

Figure 10: Optical mapping of the (A) human left ventricle and (B) monolayer and patterned constructs. Conduction velocities for monolayer and patterned constructs when paced at 1Hz.(D) Anisotropic conduction determined by comparison of the longitudinal and transverse conduction velocity speeds. . Optical mapping data courtesy of Alexey Glukhov and Di Lang. *$p < 0.05$, **$p < 0.0001$ unpaired t-test. Monolayer n = 14, pattern n = 10.

Maximum contractile strain was used to characterize mechanical output. On 10 kPa PDMS substrates, the contractile strain for both the monolayer and patterned constructs was ~ 2% on Day 6 and remained the same when tested again on Days 12 and 18(Fig. 11).

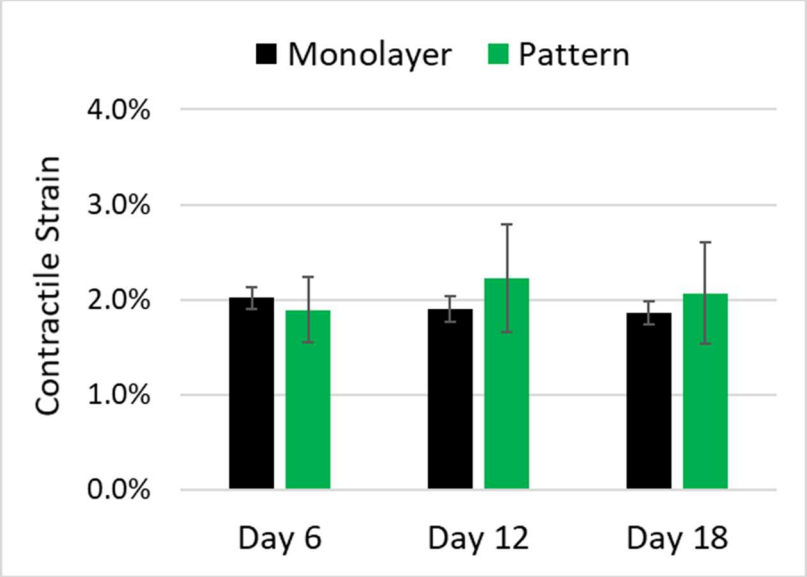

Figure 11: 2nd principal strain was used to compute contractile strain for monolayer and patterned hPSC-CMs. No statistical difference between monolayer or patterned hPSC-CMs for the three time points tested. Monolayer n = 15, pattern n = 19. Strain analysis courtesy of Alana Stempien.

DISCUSSION

In order to enhance the translational prospects of hPSC-CMs, platforms need to be developed that capture the electrical and functional behavior of hPSC-CMs in response to drugs. Herein, we provide a novel 2D *in vitro* cell culture platform that combines substrate stiffness with microcontact printing and enables functional assembly of cardiac tissues. The low number (~100,000 per construct) of hPSC-CMs makes this platform more scalable for high throughput drug screening. In addition, this platform is amenable to microscopy and provides multiparametric readouts of physiological responses including mechanical and electrical, the two main functional characteristics of CMs.

Unlike traditional culture platforms such as glass and tissue culture plastic which have a stiffness in the GPa range (Ashby, Shercliff, and Cebon 2018), the PDMS used in this study had an elastic modulus ranging from 5 – 50 kPa mimicking the elasticity of both healthy and diseased myocardium (Palchesko et al. 2012; Berry et al. 2006). It also necessary to distinguish the PDMS used in this study to other studies that utilized a 1.7 MPa blend of PDMS either as a culture material (Jung et al. n.d.; J. Kim et al. 2008; Feinberg et al. 2007) or as a support material in which a gel is cast around (Boudou et al. 2012; Ronaldson-Bouchard et al. 2018; Nunes et al. 2013; D. et al. 2013). In addition to providing a physiologically relevant stiffness, the compliant PDMS used here enabled noninvasive, live imaging mechanical outputs through the use of bright field imaging and subsequent digital image correlation analysis. As reported in our prior work (Notbohm et al. 2019), CMs seeded on 5 and 10kPa generated more contractile strain than those seeded on 50 kPa substrates. This is in good agreement with previous studies identifying an optimal stiffness for greatest contractile output (Jacot, McCulloch, and Omens 2008; Adam J Engler et al. 2008; Ribeiro et al. 2015). In this chapter we show that an increase in culture time leads to an increase in strain amplitude for the patterned hPSC-CMs. In addition to these benefits, the optical transparency of PDMS and the 2D nature of this platform make it suitable for optical imaging of cells and their internal structures. Although PDMS has the aforementioned advantages, it is a hydrophobic material and has been shown to absorb hydrophobic drugs which may complicate the interpretation of some drug studies (Toepke and Beebe 2006; Zhao et al. 2019).

To induce a more adult-like phenotype, micropatterning was added to the platform. Single cell micropatterning studies have demonstrated that myofibril organization is

dependent on the micropattern shape (Bray, Sheehy, and Parker 2008; Grosberg et al. 2011), while similar conclusions have been drawn utilizing a population of CMs and varying the lane width (McDevitt et al. 2002; Salick et al. 2014). Although these studies were effective at controlling the cytoarchitecture of CMs, they failed in their ability to create a cardiac tissue due to a lack of interconnectedness of single cells and lanes. To induce synchronous contraction over a large 2D area, micropatterned bridges were incorporated between lanes. In addition to providing myofibril alignment, the 15° chevron pattern produced anisotropic conduction velocity with speeds 2x faster in the lane direction compared to the transverse direction. Previous studies have also reported the influence of cardiac fiber orientation on the direction of conduction velocity (Roberts, Hersh, and Scher 1979; D.-H. Kim et al. 2010). The CV_L/CV_T ratio of 2 reported in this study for the patterned constructs is slower than that of ventricle ~3 and atrial ~10 tissue (Saffitz et al. 1994). Zhang et. al. also fabricated a cardiac tissue utilizing fibrin gels within a PDMS mold and demonstrated improved myofibril alignment; however, their engineered cardiac tissue displayed isotropic conduction velocity, likely due to the symmetrical orientation of the pattern used (D. et al. 2013). As a control we seeded CMs on a 10kPa PDMS monolayer and demonstrated isotropic conduction velocity which has been reported elsewhere in studies utilizing monolayers (D. et al. 2013; Herron et al. 2016). The monolayers had slightly higher conduction velocities than cells in the 15° chevron pattern. This may be a result of the limited number of cell-cell contacts afforded by the pattern and occasional gaps in the bridges/lanes. Conduction velocities of ~20 cm/sec for pattern and monolayer constructs are higher than a recent study comparing pattern and monolayer hPSC-CMs constructs (Petersen et al. 2018),

yet slower than 40 cm/sec reported by other investigators culturing hPSC-CMs in a monolayer on PDMS (Herron et al. 2016). Differences in conduction velocity could be due to the substrate stiffness, age of hPSC-CMs, or length of culture time.

SUMMARY

In this work we engineered a 2D *in vitro* cell culture platform that enables the generation of aligned human cardiac tissue in a reproducible manner. By combining patterning with physiological-relevant stiffness, a more adult-like phenotype was achieved in hPSC-CMs in terms of myofibril alignment, cell aspect ratio and anisotropic conduction velocity. This platform is amenable to already established techniques such as optical mapping and digital image correlation which will make it more easily adaptable for *in vitro* drug screening assays where electrical and mechanical function need to be measured in response to a drug. Additionally, platforms using low numbers of hPSC-CMs (~100,000/construct) are more likely to be used in high-throughput screening assays compared to 3D platforms requiring 1 million CMs per tissue.

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Chapter 3: Patterned Cardiomyocyte and Cardiac Fibroblast Co-culture

Some of the results presented in this chapter will appear in a publication that is currently in preparation and is anticipated to include the following co-authors:

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INTRODUCTION

In the previous chapter we developed a novel 2D *in vitro* cell culture platform that combines control over substrate stiffness with designed ECM patterns produced by microcontact printing and enables functional assembly of cardiac constructs. In this chapter we add an additional biomimetic cue, cardiac fibroblasts. While commercial fetal and adult cardiac fibroblasts have been available to researchers, only recent advances in the field of stem cell differentiation have led to the availability of human pluripotent stem cell-derived cardiac fibroblast (hPSC-CF) which have similar gene expression patterns as native heart CFs and distinctly alter the electrophysiological properties of the cardiomyocytes compared to co-cultures containing dermal fibroblasts (Zhang et al. 2019). Herein, we first study the behavior of hPSC-CFs when cultured alone on soft micropatterned substrates. Then, we combine hPSC-CFs with hPSC-CMs in a co-culture model.

METHODS

Methods used in this chapter are consistent with those used in chapter 2. In addition, the following methods were used:

iPSC Cardiac Fibroblast Differentiation

Human cardiac fibroblasts were differentiated from iPSCs as described previously (J. Zhang et al. 2019). Briefly, human PSCs were dissociated with 1 ml/well Versene solution (Invitrogen) at 37°C for 5 minutes, and seeded on Matrigel (GFR, BD Biosciences) coated 6-well plates at the density of 1.5x106 cells/well in mTeSR1 medium supplemented with 10 μM ROCK inhibitor (Y-27632) (Tocris). Cells were cultured for 5-6 days in mTeSR1 medium with medium change daily until reached 100% confluence when differentiation started (day 0). At day 0, the medium was changed to 2.5ml RPMI+B27 without insulin and supplemented with 12 μM CHIR99021 (Tocris) and cells were treated in this medium for 24 hrs (day 1). At day 1 medium was changed to 3ml RPMI+B27 without insulin (Invitrogen) and cells were cultured in this medium for another day (day 2). At day 2, the medium was changed to 2.5ml of the defined fibroblast culture medium supplemented with 75 ng/ml bFGF (WiCell). Cells were fed every other day and cultured until day 20 for flow cytometry analysis and subculture of cardiac fibroblasts.

hPSC-CF Seeding and DiI labeling

CF were maintained in FibroGrow media (Millipore) on tissue culture plastic. CFs were then treated with TrypLE Express (Lifetech) for 3 minutes and seeded onto both Matrigel-coated PDMS and patterned PDMS at a density of 280 CFs/mm^2 using EB20

media. Once attached to PDMS substrates, the CFs were maintained in EB2 media. In co-culture conditions, CFs were added with CMs for the Day 0 CF condition, and 4 days after CMs had been seeded for the Day 4 CF condition. The PVC tube used to control cell attachment was removed after 1 day after the addition of CFs. After removal the media was changed to EB2 media and exchanged every two days during the length of culture. In DiI labeling experiments, CFs were treated with 5 μM DiI (Invitrogen) for 30 minutes prior to being seeded on the PDMS substrates.

Decellularization of PDMS Scaffolds

To more clearly visualize ECM, PDMS scaffolds were decellularized as reported previously (Xing et al. 2014). Briefly, samples were washed with PBS prior to placing in the first decellularization solution (1 M NaCl, 10 mM Tris, and 5 mM EDTA; Sigma) and placed on an orbital shaker for 1hr at room temperature. The samples were again washed with PBS and placed in a second decellularization solution (0.5% SDS, 10 mM Tris, and 25 mM EDTA; Sigma) and shaken for 10 minutes at room temperature. After a PBS wash, the sample was rinsed in DMEM medium with 20% FBS for 48 h at room temperature and rinsed again with PBS prior to fixing and staining.

Imaging and Immunofluorescence

In addition to the primary antibodies used in the previous chapter, here we used Clone TE-7 (1:100 dilution, Millipore), Collagen 1a (1:250 dilution, Santa Cruz) and Fibronectin (1:250, Santa Cruz). Additional secondary antibodies included Alexa Fluor 488 (IgG1) goat anti-mouse and Alexa Fluor 647 (IgG2b) goat anti-mouse (Invitrogen).

RESULTS

hPSC-CF Migration and ECM Remodeling

Using time-lapse imaging and CellTracker (Piccinini, Kiss, and Horvath 2016), we followed the movements of CFs on Matrigel-coated 10kPa PDMS over the course of 13 hours. Unlike glass and tissue culture plastic in which CFs attach irrespective of protein deposition, the 10kPa PDMS did not promote CF attachment unless coated with extracellular matrix (ECM) proteins. CF attachment was restricted to the 30 μm micropatterned lanes and migration was limited to up and down movements denoted by 90 and 270 degrees from the angle of the last measurement, respectively (Fig. 1A-A'''). Due to the close spacing of the micropatterned lanes, CFs were also observed to bridge laterally between adjacent lanes, although this was less common than up and down migration. Next, CFs were seeded on a 15° chevron pattern composed of 30 μm lanes and bridges. Similar to isolated 30 μm lanes, CFs attached to micropatterned areas and migrated in the direction of the micropatterned lanes and bridges with no migration observed in areas void of ECM (Fig. 1B-B'''). In contrast, when presented with a monolayer of Matrigel CFs show no orientation preference and migrate in all directions equally (Fig. 1C-C'''). These results demonstrate CF vectors of migration can be controlled by patterning specific geometries of ECM on soft 10kPa PDMS substrates.

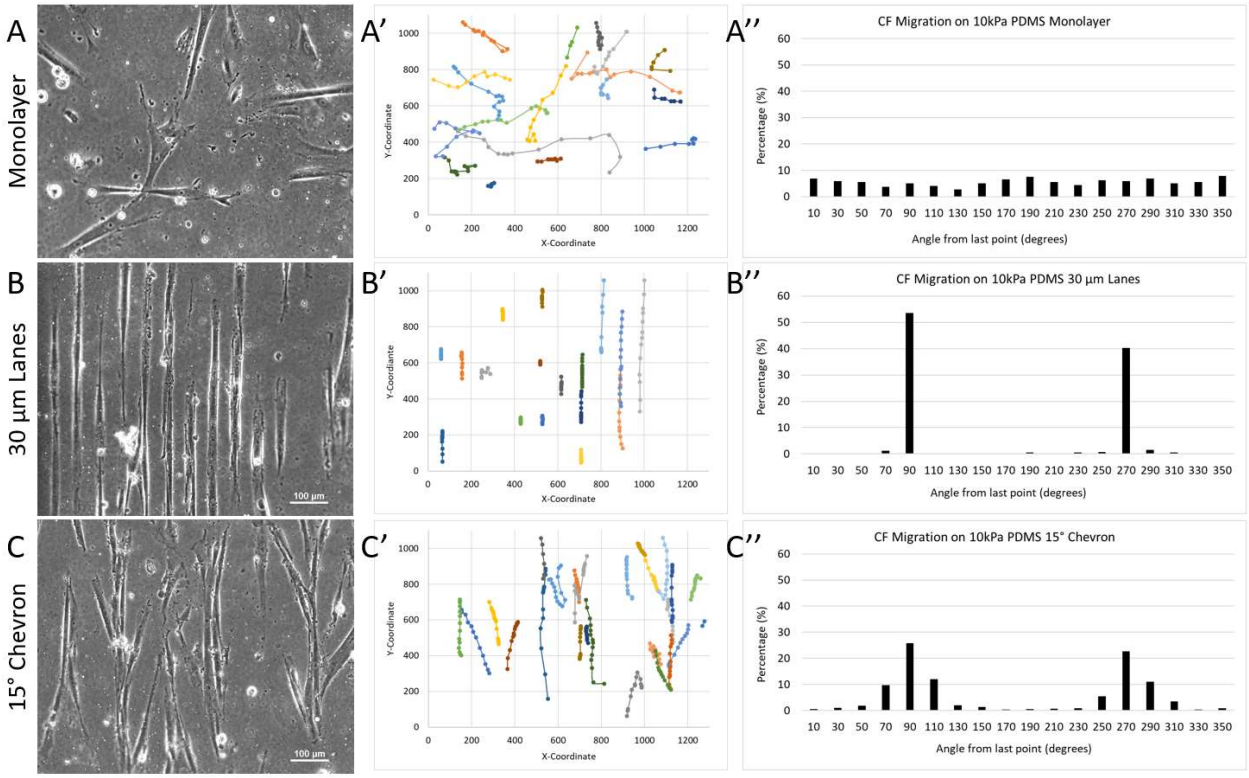

Figure 1: hPSC-CFs attached to 10kPa PDMS substrates for one day prior to 13 hour time-lapse imaging. (A-C) Representative bright field images, (A'-C') migration patterns of individual CFs and (A''-C'') angle measurements for each of the three conditions tested: 30 µm Lanes, 15°chevron pattern and Monolayer. Scale bars = 100 µm for A-C. 90 degrees indicates an upward movement and 270 degrees a downward movement from the last measurement for A'''-C'''.

Another well-known function of CFs is ECM deposition and remodeling. Like the results above, we investigated how CFs remodel ECM when attached to Matrigel-coated micropatterned lanes, 15°chevron pattern and monolayer conditions. Matrigel is a heterogeneous mixture of ECM proteins including, but not limited to, collagen, fibronectin and laminin. In the absence of cells, collagen and fibronectin self-segregate into local, globular regions when coated on the PDMS surface. From the time-lapse observations of CF migration on micropatterned lanes we hypothesized that CFs would produce anisotropic fibers when cultured on micropatterned lanes. To test this hypothesis CFs were cultured on different micropatterned lane widths on 10kPa PDMS

substrates ranging from 20 – 200 μm (Fig. 2). After 2, 5 and 8 days of culture, the CFs were decellularized from the 10kPa PDMS micropatterned lanes in order to more clearly visualize the underlying remodeled ECM and a custom MATLAB SGFT software (Salick et al., 2019) was used to quantify ECM organization. After 2 days of culture a clear trend in ECM alignment was observed with lane width, namely smaller lane widths (those closer to 20 μm) were more aligned than larger lane widths (those closer to 200 μm) (Fig. 2). The 20 μm lane width was significantly more aligned than lane widths 60 – 200 μm at Day 2 ($P < 0.05$). With increased culture time from 2 to 8 days, all lane widths had greater than 95% fiber alignment. Although CFs in the monolayer condition were also able to convert the globular collagen and fibronectin into fibers, the amount of alignment was effectively arbitrary compared to the micropatterned lanes at 20% and 32% for Day 2 and Day 8 monolayer, respectively ($P < 0.001$) (Fig. 2).

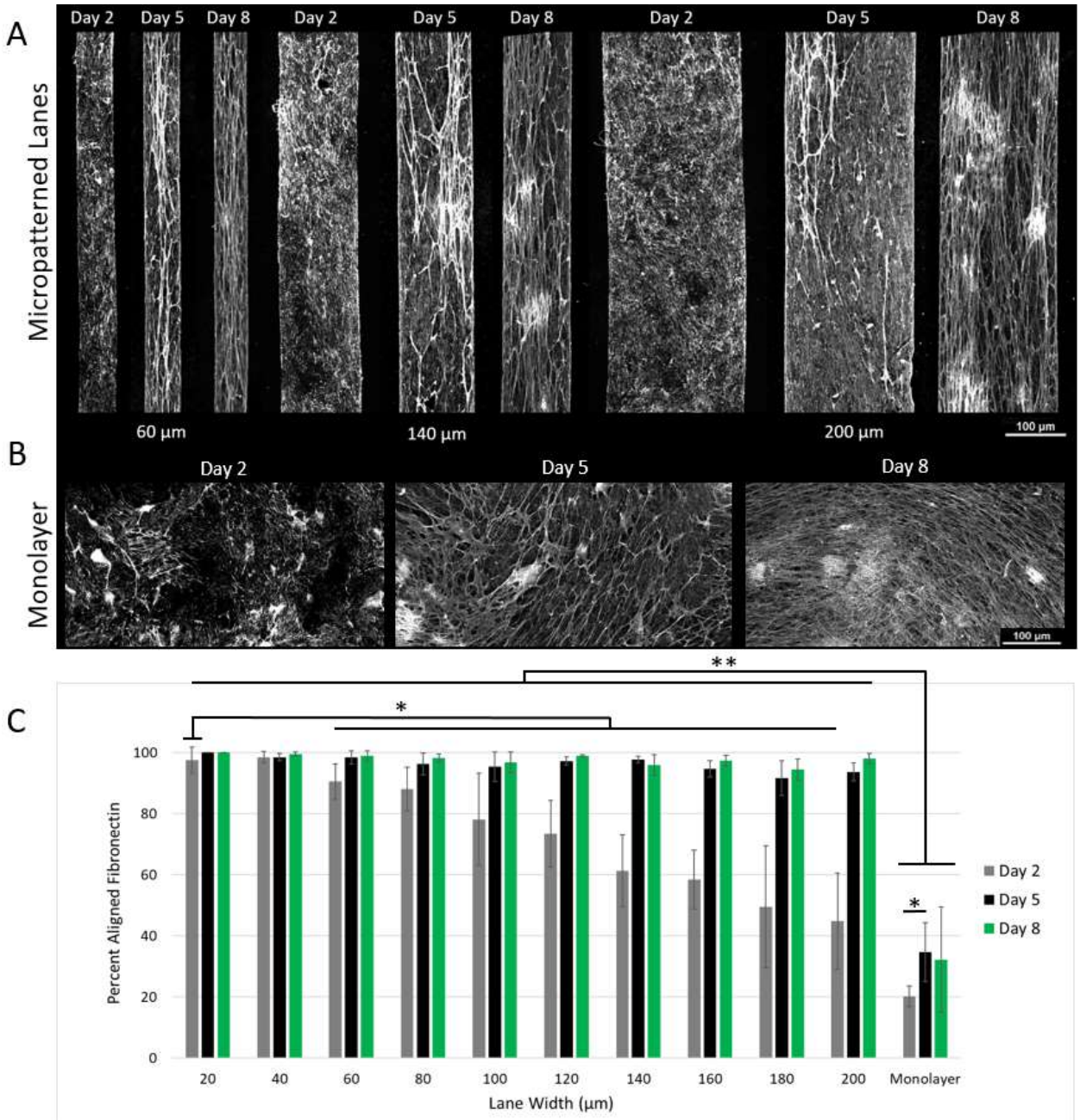

Figure 2: Decellularized ECM on (A) different micropatterned lane widths and (B) monolayers after 2, 5 and 8 days of culture with iPSC-CFs. (C) Quantification of the amount of fibronectin fibers aligned within 10 percent of the superior angle. Green = collagen and White = Fibronectin. Scale bar = 100 µm. *p < 0.05, **p < 0.001, unpaired t-test. n = 8 for each day and lane width tested.

Like the Matrigel-coated no cell condition, collagen and fibronectin appear globular when micropatterned onto 10kPa PDMS (Fig. 4A-C). When CFs were cultured on the 15°chevron, ECM fibers were produced parallel to the direction of the patterned features

(Fig. 3). After 2 days of culture the CFs had begun to remodel the ECM and produce fibers; however, as observed with the Day 2 decellularized images above, these fibers were short and did not persist the length of lanes (Fig. 4D). With longer culture of 18 days the fiber bundles were continuous throughout the length of the patterned features and the percent of area covered by ECM increased from 7 ± 1% on Day 0 to 29 ± 5% on Day 2 to 84 ± 4% on Day 18 (Fig. 4D-F).

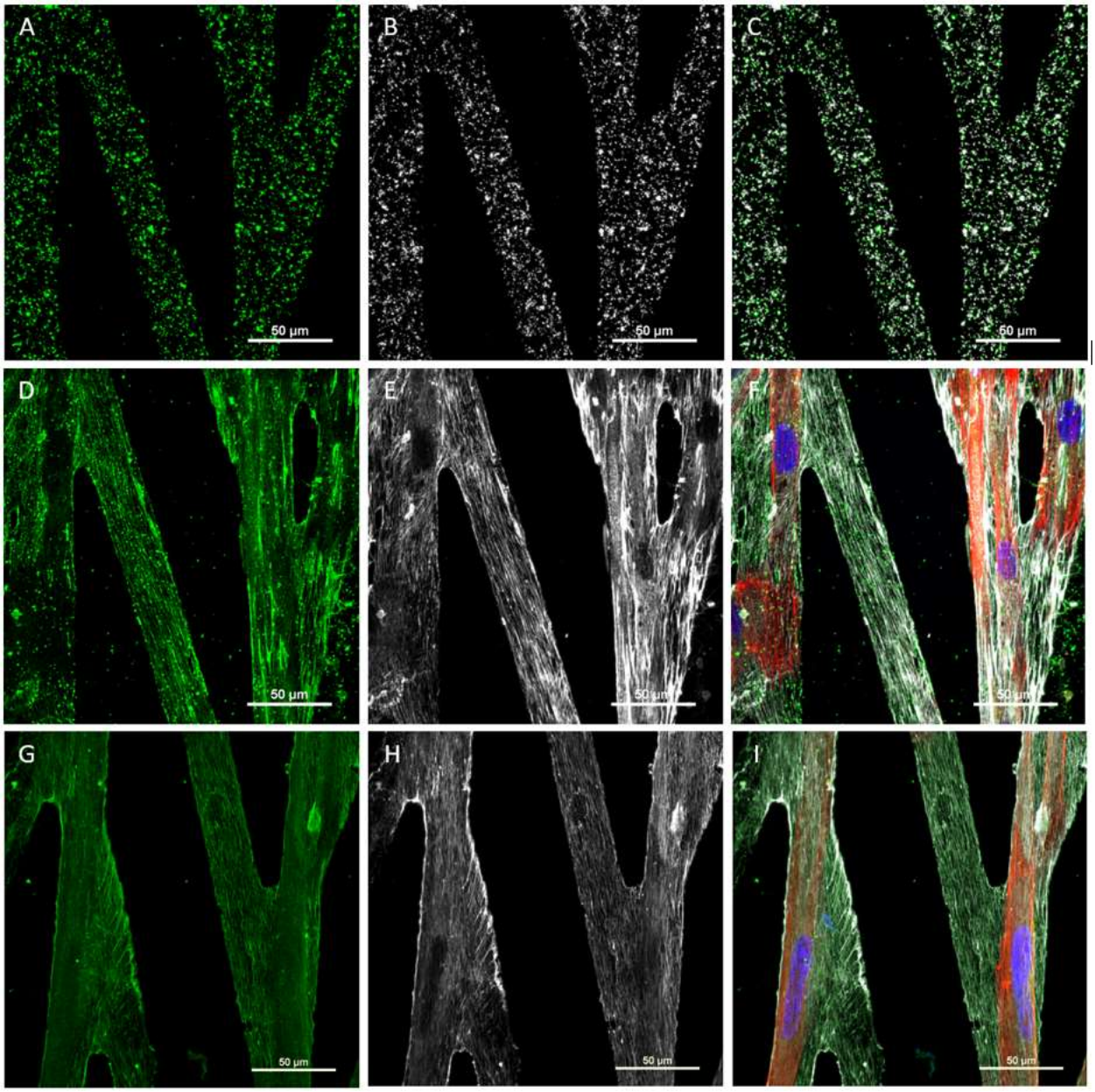

Figure 3: 10kPa PDMS substrates patterned with Matrigel and stained for collagen (green) and fibronectin (white). (A-C) Control sample with no CFs attached, (D-F) CFs attached for 2 days and (G-I) CFs attached for 18 days. Red = Actin, Blue = DAPI. Scale bars = 50 µm.

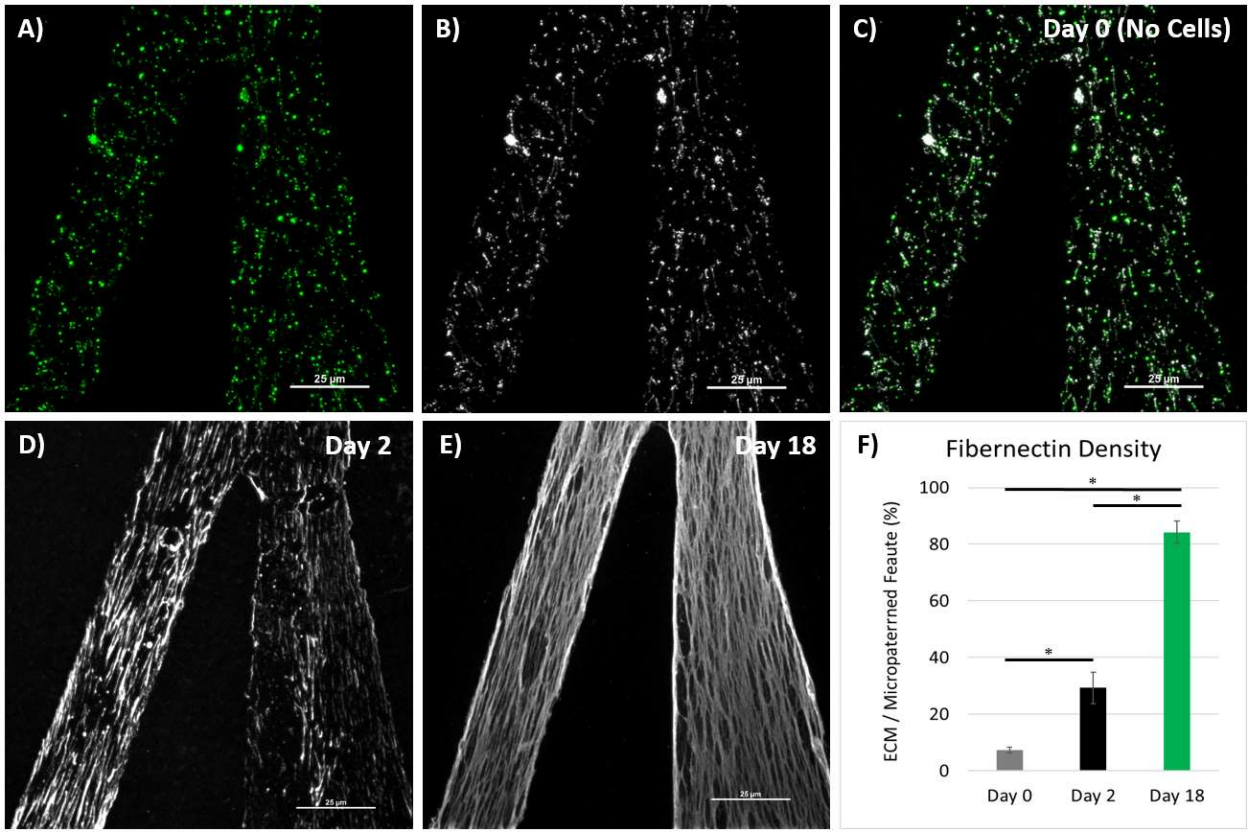

Figure 4: 10kPa PDMS substrates patterned with Matrigel using the 15°chevron pattern and stained for collagen (green) and fibronectin (white). (A-C) Day 0 control sample with no CFs attached. Scale bars = 50 μm. Decellularized scaffolds after CFs were attached for (D) 2 days and (E) 18 days. Scale bars = 25 μm. (F) Quantification of the amount of fibronectin fibers per 30 μm lane width. *p < 0.0001, one-way ANOVA with *post hoc* Tukey tests. n = 6 for all conditions.

Pattern CM-CF Co-culture

Using the 15° chevron pattern, we next sought to make a co-culture model composed of hPSC-CMs and hPSC-CFs that maintained the anisotropic features observed when culturing hPSC-CMs alone in the micropattern (i.e. the CM Only condition). Multiple ratios of CM:CF were tried ranging from 100:3 – 100:25. The lower 100:3 ratio resulted in CMCF co-cultures with similar pattern fidelity as CM Only patterns after 18 days in culture, while CFs in the higher 100:25 ratio quickly overgrew the pattern and merged adjacent lanes, ultimately leading to pattern deterioration and detachment after 18 days in culture (Fig. 5). Therefore, a CM:CF ratio of 100:10 was chosen which allowed for

pattern remodeling while also maintaining CM and CF cellular attachment. This CM:CF ratio used here also matches ratios used in 3D collagen gel compaction studies (Zhao et al. 2019) and is the same density of CFs used in the CF work described above.

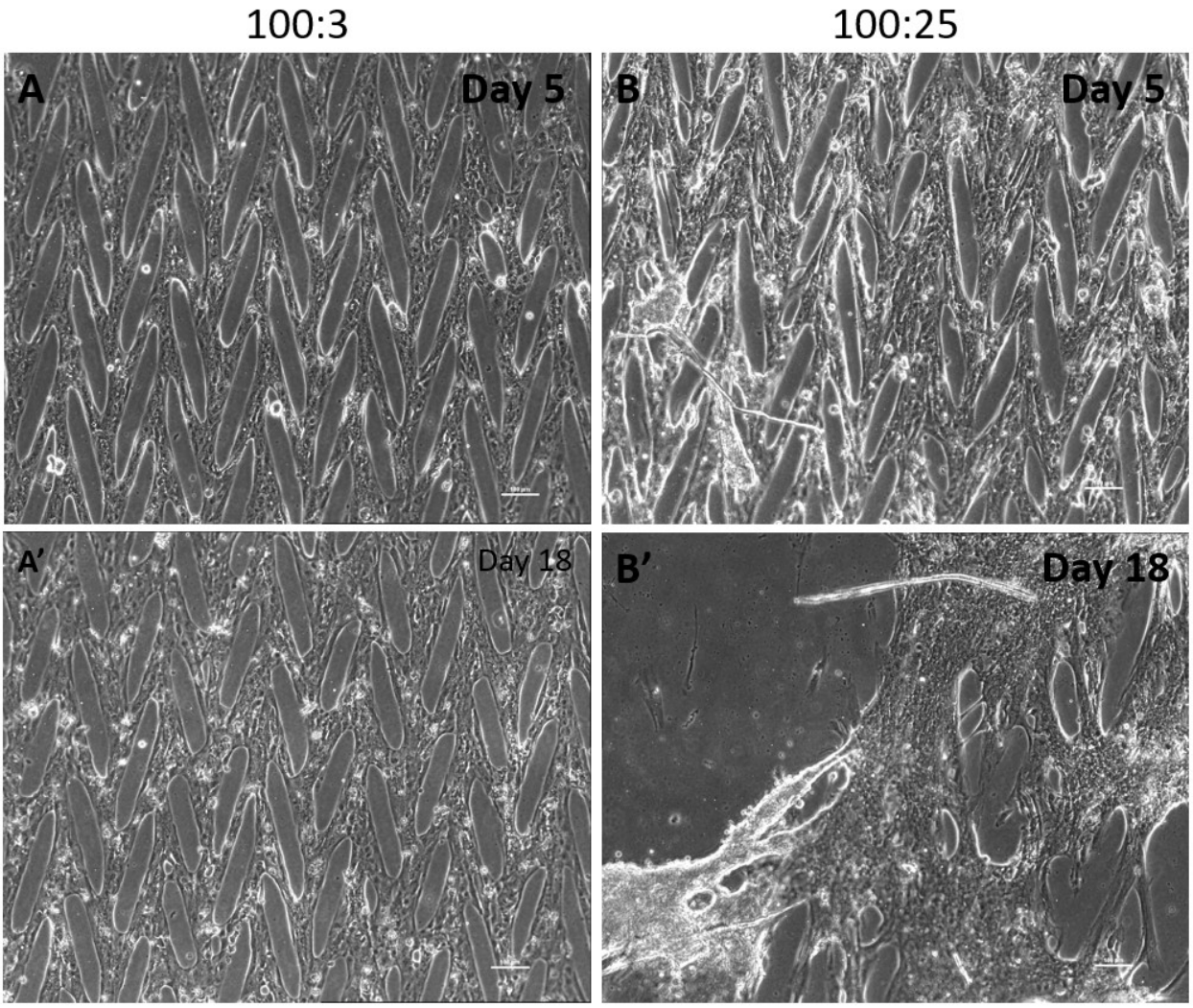

Figure 5: Bright field images of patterned CMs and CFs on Day 5 (A-B) and Day 18 (A'-B') of co-culture. (A-A') CM:CF ratio of 100:3 had little pattern divergence while (B-B') a ratio of 100:25 resulted in pattern deterioration. Bright field locations are the same in A-A' and B-B' so that the same cells can be tracked. Scale bar = 100 μm.

To explore the impact of co-culture with CMs, hPSC-CFs were either (1) combined with hPSC-CMs on Day 0, or (2) on Day 4 after CMs had already been patterned, referred to as Day 0 CF and Day 4 CF, respectively. In the Day 0 CF condition, the CMs and CFs

initially confined themselves to the micropatterned lanes after 1 day of co-culture (Fig. 6); however, with extended time in culture the cells migrated and merged adjacent lanes of the pattern, forming almost a complete monolayer after 18 days in culture leaving little of the underlying micropattern still visible (Fig. 6). In contrast, the CM only condition displayed little merging of adjacent lanes during the 18 days of culture. Like the CM Only and Day 0 CF conditions, cells in Day 4 CF were also constrained to the micropatterned lanes after 1 day of culture (Fig. 6). The Day 4 CF condition showed some pattern remodeling with the formation of merged lanes but also had areas where the pattern remained visible after 18 days of co-culture (Fig. 6). Quantification of cell area coverage at the start and end of co-culture confirmed bright field observations (Fig. 6B-C). Namely, the CM Only group showed the smallest increase after 18 days with 15.35% more of the pattern occupied by cells ($P < 0.0001$), followed by 21.69 ($P < 0.0001$) and 30.36 % ($P < 0.0001$) for the Day 4 CF and Day 0 CF conditions, respectively.

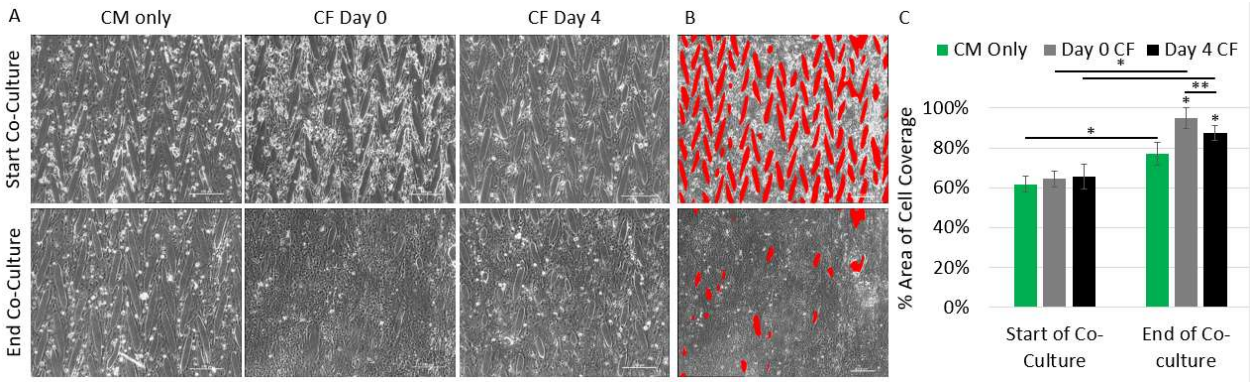

Figure 6: Bright field images of patterned hPSC-CMs at the (A-C) start and (A'-C') end of co-culture. (A-A') The CM Only control group. (B) CFs were seeded concurrently with CMs on Day 0 and cultured until (B') Day 18. (C) CFs were seeded on top of patterned CMs on (C) Day 4 and cultured until (C') Day 18. Bright field locations are the same in A-A', B-B' and C-C' so the same cells can be tracked during culture. Scale bars = 250 μm. *$p < 0.0001$, **$p < 0.001$, two-way ANOVA with *post hoc* Tukey tests. n = 9 for CM Only, n = 11 for Day 0 CF and n = 11 for Day 4 CF.

CF Attachment, Migration and ECM production in Pattern Co-culture

To visualize attachment and integration, CFs were stained with DiI prior to co-culture with CMs. After 1 day of co-culture, more CFs were observed in the Day 0 CF compared to the Day 4 CF conditions, although this number was not significantly different (P-Value = 0.155 , Fig. 7). In order to determine how migratory the CFs were in the co-culture conditions, samples were tracked for 13 hours using time-lapse imaging. Similar to when seeded alone on the 15°chevron pattern, CFs in both co-culture conditions were influenced by the micropatterned features and migrated on top and along the edges of lanes (Fig. 8A). To determine how many fibroblasts had integrated and remained at the end of co-culture, samples were fixed at Day 18 and fibroblast were identified with the TE-7 antibody (Kaaya et al. 1995). As expected, the CM Only group had no CFs present (Fig. 9A-A'). After 18 days of culture CFs were more abundantly expressed in the Day 0 CF condition (Fig. 9B-B') compared to the Day 4 CF condition (Fig. 9C-C'), even though both were seeded at the same 100:10 ratio. As observed during live time-lapse imaging, Day 18 IHC showed fibroblasts on top of and alongside CMs both in the Day 0 and Day 4 CF co-culture conditions (Fig. 10).

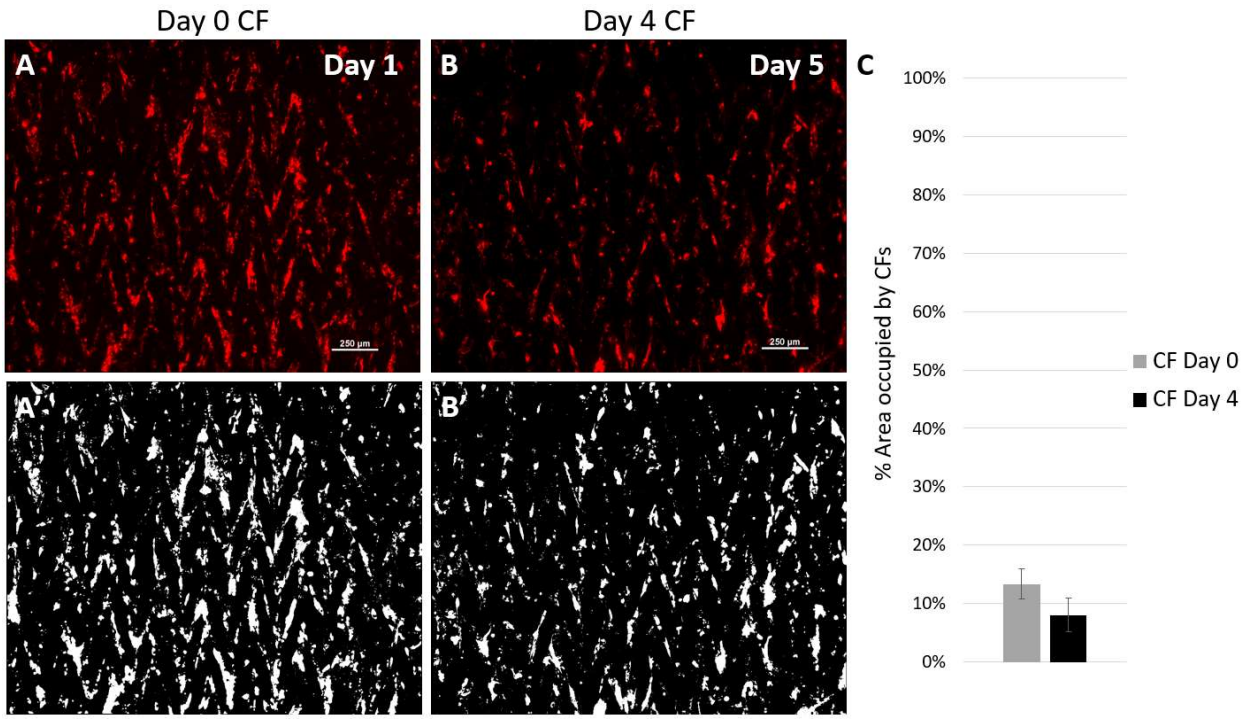

Figure 7: (A) Day 0 CF and (B) Day 4 CF DiI labeled CFs co-cultured with CMs for 1 day. (A'-B') Thresholding and binning of the TexasRed channel using CellProfiler allowed for (C) quantification of CF attachment and integration after 1 day of co-culture (n = 8, P-value = 0.155). n = 8, scale bars = 250 µm.

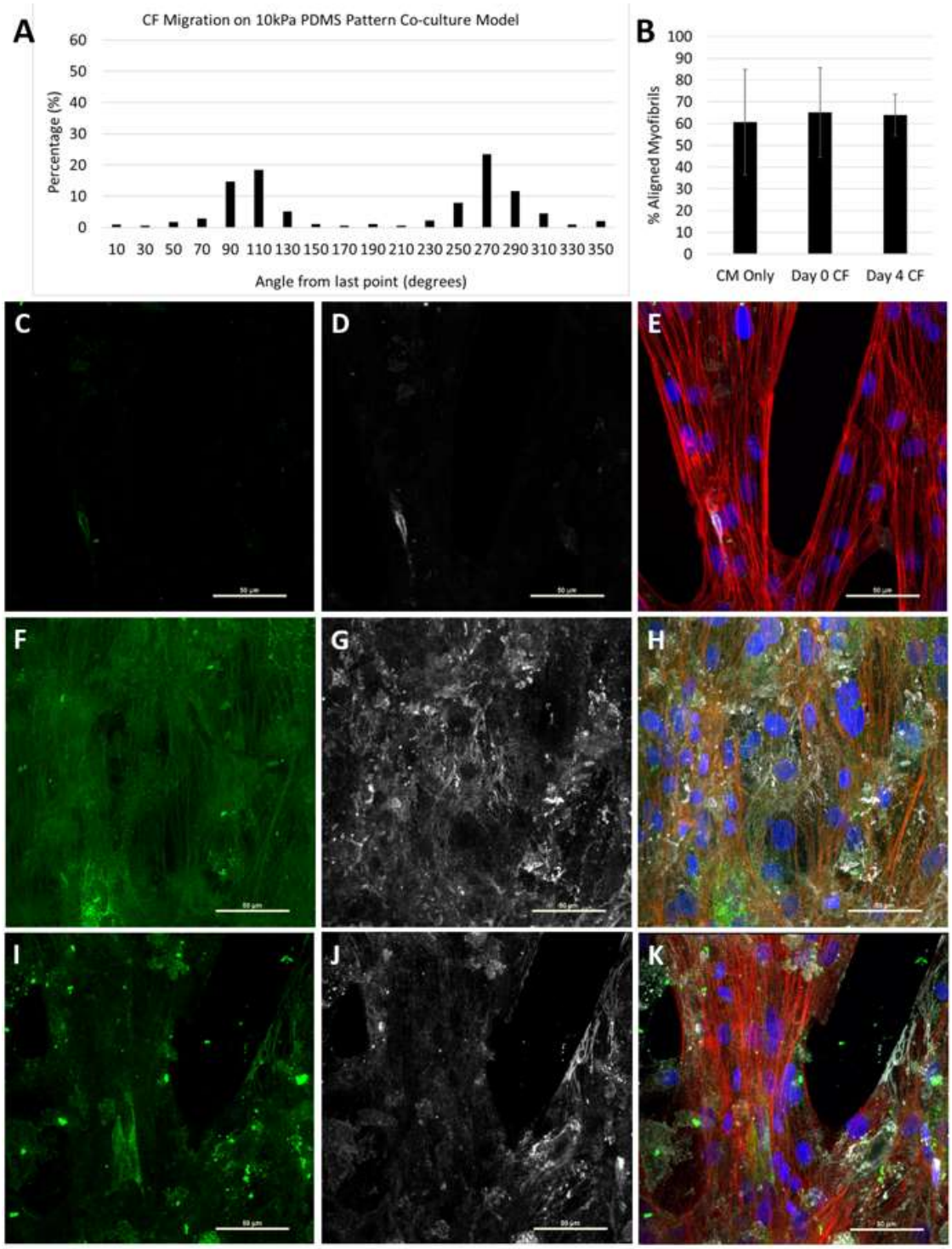

Figure 8: (A) Migration patterns of individual CFs when co-cultured with CMs on the 15°chevron pattern. 90 degrees indicates an upward movement and 270 degrees a downward movement from the last measurement. (B) Quantification of the percent of myofibrils aligned within 10 degrees of the superior angle for the three conditions tested (n = 4). Collagen and fibronectin expression after 18 days of culture for the CM only (A-C), Day 0 CF (D-F) and Day 4 CF conditions (G-I). Collagen = green, Fibronectin = white, Actin = red, DAPI = blue. Scale bars = 50 μm.

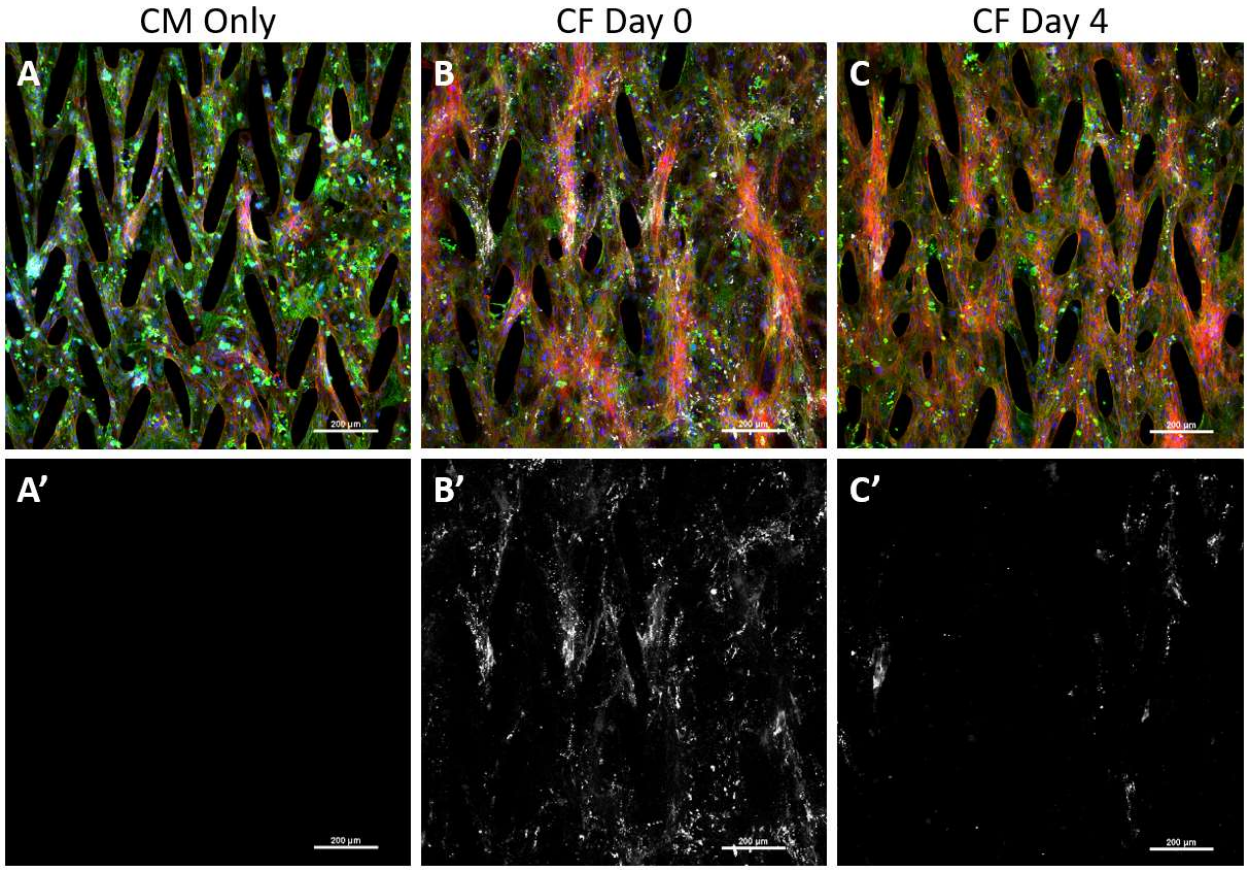

Figure 9: Aligned CMCF co-cultured for 18 days on 10kPa PDMS substrates patterned with 15 degree chevron. CFs (white) are not present in (A-A') the CM only group, (B-B') abundantly expressed in the Day 0 CF condition and (C-C') sparse in the Day 4 CF condition. Alpha-actinin = green, TE-7 = white, Actin = red, DAPI = blue. Scale bars = 200 µm.

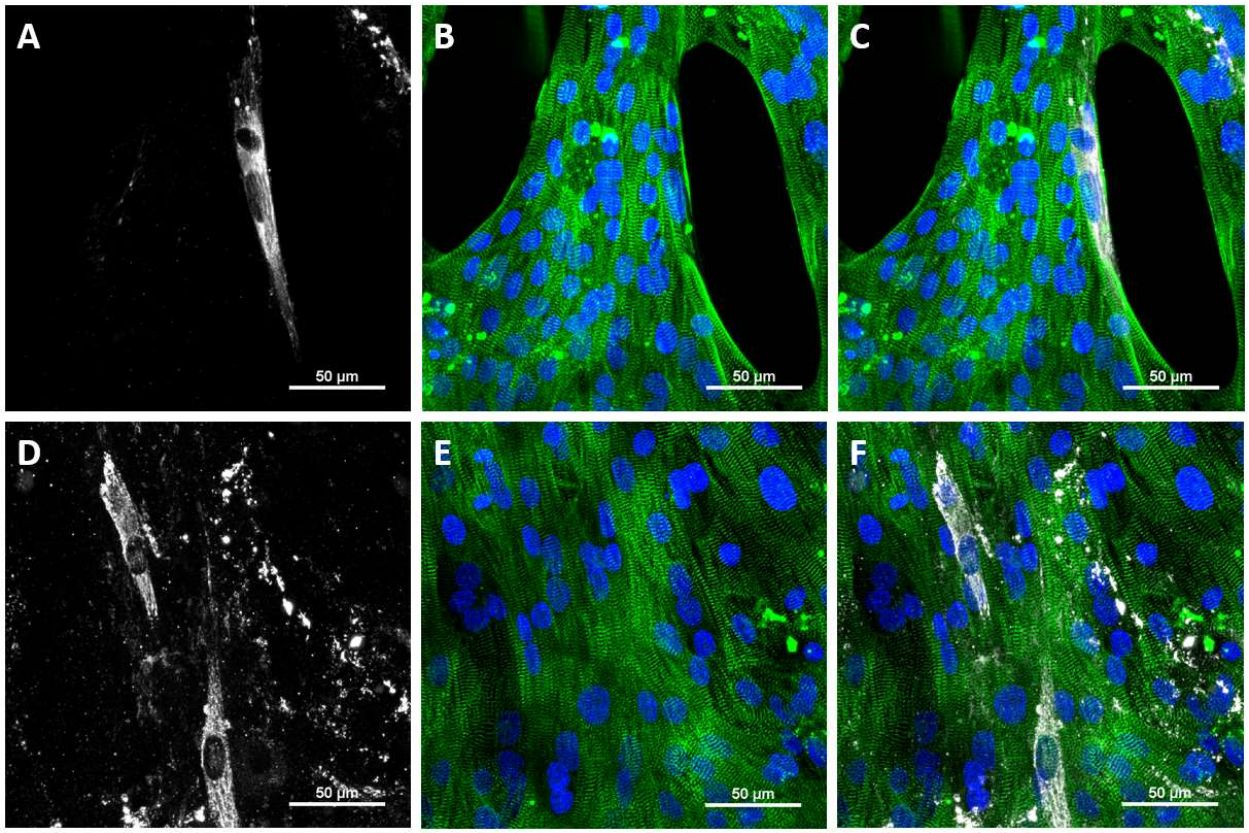

Figure 10: CFs attach and integrate into the co-culture system and can be seen alongside (A-C) and on top (D-F) patterned CMs after 18 days of co-culture. Alpha-actinin = green, TE-7 = white, Actin = red, DAPI = blue. Scale bars = 50 µm.

Myofibril alignment was quantified in the co-culture conditions using SGFT. While myofibril alignment was expected in areas of high pattern fidelity, we also found that myofibrils were aligned in areas of the co-culture conditions that were visibly merged (Fig. 11). For the Day 0 CF and Day 4 CF conditions, the amount of aligned myofibrils was 65.16 ± 20.61% and 63.95 ± 9.48%, respectively, which is in close agreement with 60.63 ± 24.25% reported for the CM Only condition (Fig. 8B). 3D volume rendering of confocal z-stack images revealed a larger thickness in the co-culture conditions (z-height = 11 µm), representing roughly the height of two cells, compared to the CM only condition (z-height = 6 µm; Fig. 12).

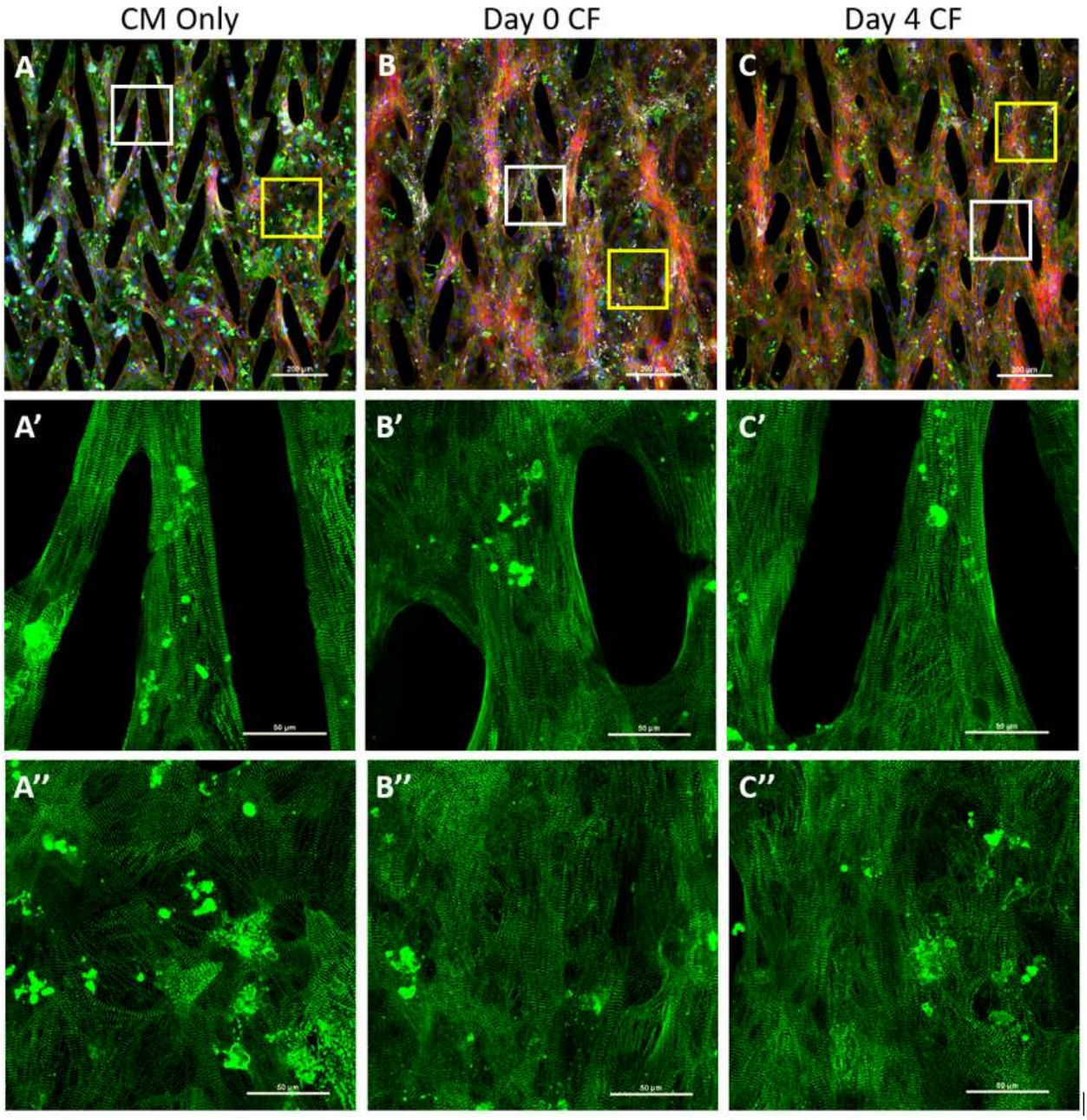

Figure 11: CMs cultured on 15° chevron pattern 10kPa PDMS substrates for 18 days in the presence of (A) 0%CF, (B) 10% CF added on Day 0 and (C) 10% CF added on Day 4. (A'-C') Immunostaining for alpha-actinin in areas where the pattern is still visible (white boxes in A-C). (A''-C'') Immunostaining for alpha-actinin in areas where the pattern had merged (yellow boxes in A-C). Alpha-actinin = green, TE-7 = white, Actin = red, DAPI = blue. Scale bar = 200 μm for A-C and A'-C', scale bar = 50 μm for A'-C' and A''-C''.

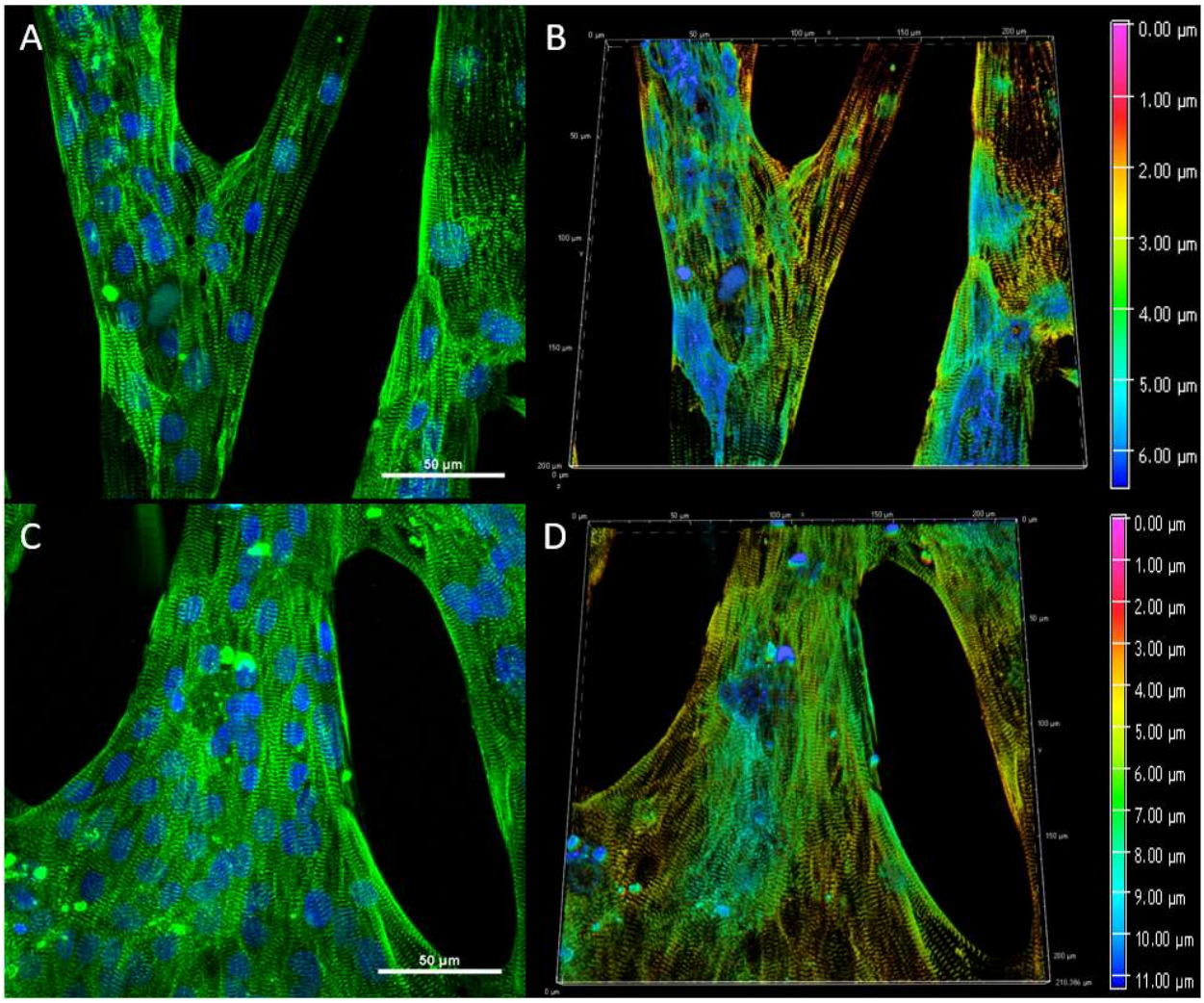

Figure 12: Construct thickness for the CM only (A-B) and Day 4 CF conditions (C-D). (B) is a 3D z-depth coded volume rendering of (A) while (D) is a rendering of (C). Alpha-actinin = green and DAPI = blue for A and C. Scale bar = µm. Color scale bar for B goes from 0 – 6 µm and for D goes from 0 – 11 µm.

Next we looked at the ECM present after 18 days of culture. As expected, the CM Only condition displayed little collagen and fibronectin, while both the Day 0 CF and Day 4 CF conditions showed an abundance of these ECM proteins (Fig. 8C-K). Decellularization of these constructs after 18 days of culture also confirmed a large amount of ECM remodeling and deposition in the co-culture conditions with little remodeling and no discernable deposition of ECM in the CM Only condition (Fig. 13).

These results highlight the ability of the underlying ECM pattern to influence myofibril alignment in unison with further ECM remodeling and deposition within a CF co-culture.

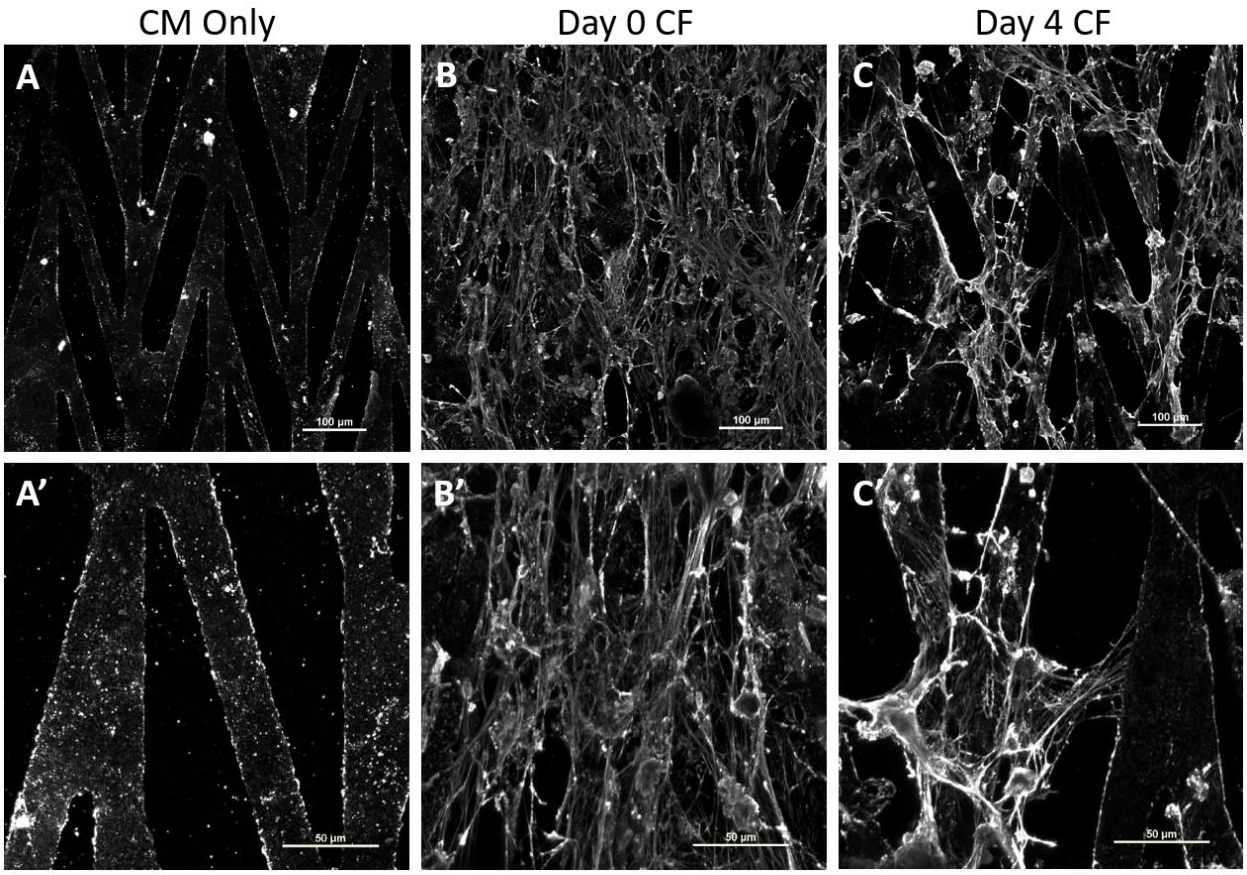

Figure 13: Fibronectin (white) expression after decellularization of Day 18 (A-A') CM Only, (B-B') Day 0 CF and (C-C') Day 4 CF constructs. A-C scale bars = 100 µm, A'-C' scale bars = 50 µm.

Electrophysiological Properties of Pattern Co-culture

Optical mapping was used to assess the electrical properties of the patterned co-culture conditions. Though not significant, the calcium duration time was faster in the Day 0 CF condition (651 ± 80 ms) and Day 4 CF condition (601 ± 62 ms) compared to the CM Only (687 ± 29 ms) (Fig. 14A). There was a significant difference in Ca^{2+} release when evaluating the rise time of CaT for both co-culture conditions compared to the CM Only with speeds of 37, 30 and 86 ms for the Day 0 CF, Day 4 CF and CM Only conditions,

respectively (P < 0.0001; Fig 14B). Lastly, there was no significant difference in conduction velocity for CM Only (10.5 ± 1.7 cm/sec) compared to the Day 0 CF (14.9 ± 3 cm/sec) and Day 4 CF (12.5 ± 4.6 cm/sec) (Fig. 14C).

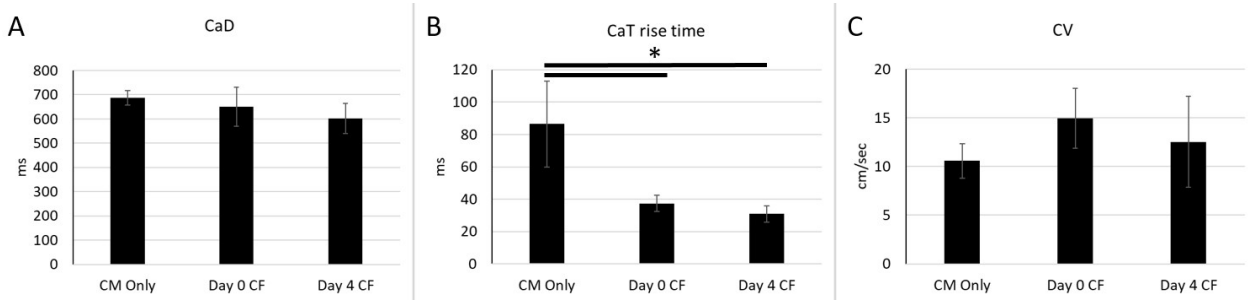

Figure 14: Optical mapping results for calcium duration (CaD), calcium release (CaT) and conduction velocity (CV) for the different conditions tested. *p < 0.0001, one-way ANOVA with *post hoc* Tukey tests. n = 7 for all conditions. Optical mapping courtesy of Di Lang.

Contractile Strain in Pattern Co-culture

As in the previous chapter we assessed contractile strain to measure mechanical output, which is an indicator of hPSC-CM maturation. A repeated measures, two way analysis of variance (ANOVA) was performed on all samples to determine significant differences (p < 0.05) for the three conditions, day of strain analysis and strain output. For each time point tested, there was a significant difference between the amount of strain generated in the co-culture conditions compared to the CM Only condition (P < 0.0001; Fig. 15). Over the three time points tested the strain produced by the CM Only condition remained around ~2%. Likewise, there was no significant difference in strain values (3-3.5%) for the CF Day 0 condition during 18 days of culture. Strain in the CF Day 4 condition increased from 2.73 ± 0.39% at Day 6 to 3.32 ± 0.66% at Day 12 (P < 0.05) and remained at 2.94 ± 0.61% for Day 18.

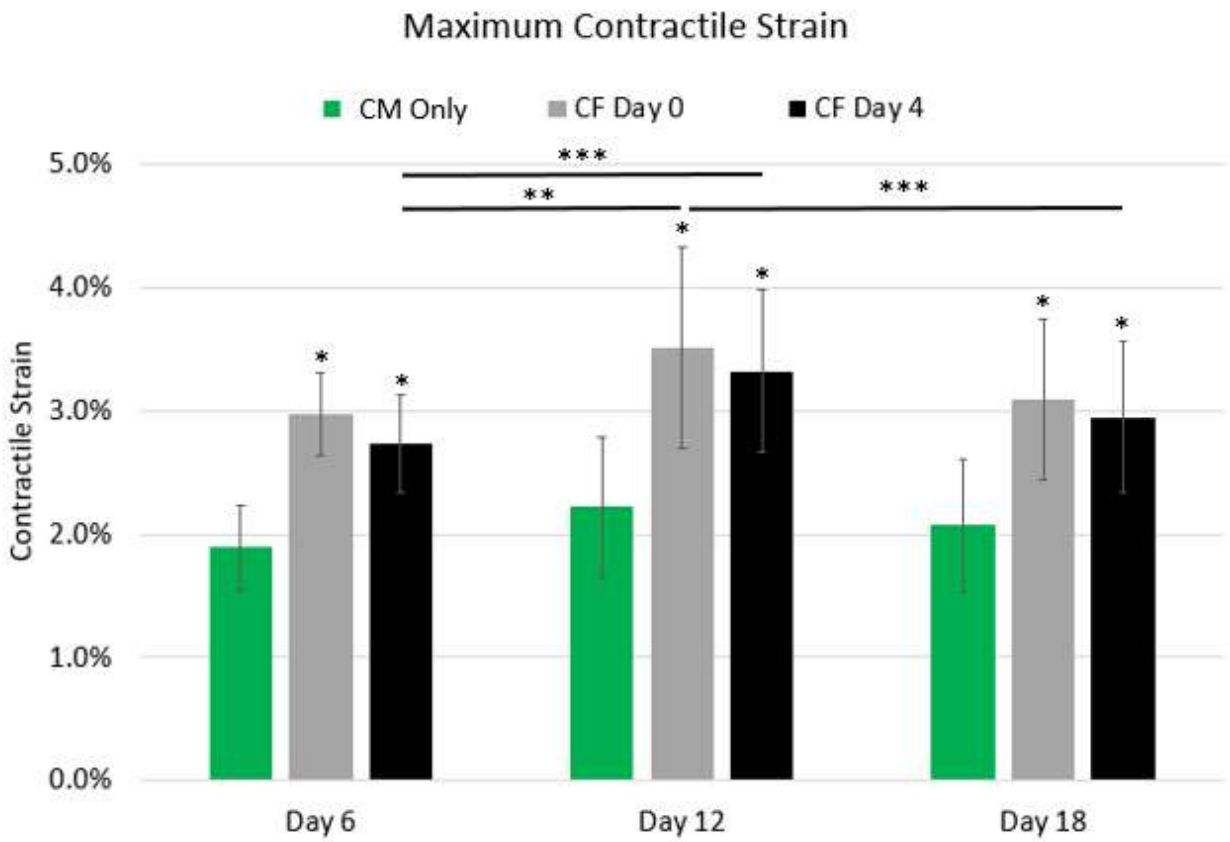

Figure 15: Development of contractile strain for the three patterned conditions tested on Day 6, 12 and 18 of culture.*p < 0.0001, **p < 0.0005, ***p < 0.05, two-way ANOVA with *post hoc* Tukey tests. n = 19 for CM Only, n = 19 for CF Day 0 and n = 22 for CF Day 4. Strain analysis courtesy of Alana Stempien.

DISCUSSION

Most efforts to improve hPSC-CM maturation have utilized cues stemming from the *in vivo* cardiac milieu which include physiologically-relevant substrate stiffness (Hazeltine et al. 2012; Ribeiro et al. 2015), electrical and mechanical conditioning (Eng et al. 2016; Ronaldson-Bouchard et al. 2018; Shadrin et al. 2017; Zhao et al. 2019), micropatterning (Salick et al. 2014; Notbohm et al. 2019) and co-culture (C. Kim et al. 2009; Tiburcy et al. 2017). While these studies have resulted in modest hPSC-CM maturation improvements, individually they fail to fully recapitulate the adult cardiomyocyte phenotype. Thus, the field has gravitated towards the creation of more biomimetic

microenvironments that have the ability to combine multiple signaling factors into one *in vitro* culture platform. Good combinatorial approaches include electrical stimulation inside a soft collagen gel (Nunes et al. 2013) and electrical stimulation combined with mechanical stress (Ruan et al. 2016). This chapter describes the investigation of the influence of micropattern geometry on the behavior of human pluripotent stem cell-derived cardiac fibroblast (hPSC-CF) cultured independently and in combination with human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) on soft micropatterned substrates.

Previous studies have demonstrated that nano and micro grooved channels influence fibroblast migration (D.-H. Kim et al. 2009; Londono et al. n.d.). More recent work has demonstrated an anisotropic ECM scaffold can be generated by culturing fibroblasts on grooved PDMS surfaces followed by decellularization (Xing et al. 2014). Unlike the previous studies, we show that microscale confinement alone, with no z-dimension, is sufficient to control CF migration and produce anisotropic fibrillar ECM spanning 100s of micrometers. At lower seeding densities CFs are able to migrate in different directions in the 200 μm lane width; however, as the CFs continue to proliferate their density increases within the micropattern feature limiting their migration parallel to the lane direction. In contrast, CFs seeded on unpatterned monolayers were able to align the ECM locally but showed no global organization. While the current work documents the effects of CF migration and ECM production on micropatterned lanes on a 10kPa PDMS substrate, future work will investigate other factors that influence ECM production and organization.

Having characterized both CMs and CFs separately on the 10kPa PDMS 15° chevron pattern, we next combined these cell types to produce a co-culture model. Surprisingly, few studies have combined CMs and CFs systematically and with defined ratios (C. Kim et al. 2009; Kensah et al. 2011; Tiburcy et al. 2017; Miragoli, Gaudesius, and Rohr 2006a). In most co-culture studies, CFs are considered part of the supporting cast necessary for matrix compaction and remodeling of three-dimensional matrices (Nunes et al. 2013; Boudaoud et al. 2014; Tiburcy et al. 2017; Ronaldson-Bouchard et al. 2018). Although such three-dimensional culture techniques are capable of better replicating the three-dimensional cell-cell interactions found *in vivo*, deconvolving differences in heterogeneous cell populations is more challenging and can lead to difficulties with reproducibility. Additionally, the small cell input in this study (i.e.,~0.1 million/pattern) compared to the large number of cells (i.e., 0.5 – 2 million cells/construct) required for three-dimensional studies makes this platform more scalable for high throughput screening applications.

It is known that during embryonic development cardiac fibroblast are derived mainly from the endocardium (Wessels et al. 2012) and epicardium (Moore-Morris et al. 2014), two distinct compartments generated after the formation of a looped heart (Gittenberger-de Groot et al. 2012; Furtado et al. 2016). Therefore, to mimic development we patterned CMs on the 15° chevron pattern first and then added CFs four days later (Day 4 CF condition). To the best our knowledge, this is the first co-culture study to test the sequential addition of fibroblasts and to use CMs and CFs derived from pluripotent stem cells on soft micropatterned substrates. We also consistently seeded young CMs 30 days after they were differentiated as recent reports

suggest the responsiveness of CMs to physical stimuli declines as differentiation progresses (Ronaldson-Bouchard et al. 2018).

In comparison to the Day 0 CF condition, more of the PDMS not initially stamped with ECM was visible in the Day 4 CF condition after 18 days of culture. After decellularizing all conditions, it was found that the lane ECM in the Day 4 CF condition resembled the lane ECM in the CM Only condition. This led us to conclude that once CMs adhere to the Matrigel-coated PDMS, fibroblasts are unable to penetrate beneath the CMs and instead attached to the top of the CMs, migrate, and produce additional ECM. In comparison, the Day 0 CF condition had lane ECM in a semi-fibrillar form indicating the CFs were able to remodel the ECM prior to CM attachment, although not as extensively as when CFs were cultured alone on micropatterned lanes for 8 days. Future work will investigate whether the increase in cell area coverage for the Day 0 CF condition was due to movement of the cells from the lanes or hypertrophy of CMs.

In addition to ECM production, fibroblasts are also known to influence cardiac electrophysiology. Here we report improved calcium dynamics with the addition of CFs. The calcium rise time was significantly faster in the co-culture conditions (30-37 ms) compared to the CM Only (86 ms) condition and more physiologically relevant to values of the human left ventricle (~25 ms) (Lang et al. 2015). Though not significantly different, the co-culture conditions also had a shorter calcium duration trending in the direction towards values reported for the human left ventricle (~400 ms) (Lang et al. 2015). This data supports the conclusion that the co-culture conditions had improved calcium kinetics compared to the CM Only . In addition, we also assessed electrical

impulse propagation and reported faster conduction velocities in the co-culture conditions compared to the CM Only condition, though statistically not significantly different. In contrast, most prior literature shows a negative correlation with slower conduction velocity and increased percentage of cardiac fibroblast in co-culture (D. et al. 2013; Xie et al. 2009; Thompson et al. 2011). One possible explanation for the reported differences could be the small percentage of fibroblast used in this study. It has been reported that percentages of fibroblast less than 10% can increase conduction velocity by modulating the resting membrane potential in the well-known phenomenon of “supernormal conduction” (Kagiyama, Y., Hill, J.L. and Gettes 1982; Shaw and Rudy 1997; Miragoli, Gaudesius, and Rohr 2006b). Another possible explanation could be the use of hPSC-CFs in our co-culture model as it has been reported fetal CFs increase CV more so than co-cultures containing adult CFs (Liau et al. 2017).

CFs also have been shown to increase the amount of force generated by cardiomyocytes in comparison to when cardiomyocytes are cultured alone (Naito et al. 2006; Radisic et al. 2008; D. et al. 2013). In this study we report similar findings with both co-culture conditions producing more contractile strain compared to the CM Only condition. While the CF Day 0 strain remained the same for the three days tested, there was an increase in strain for the Day 4 CF condition when comparing Day 6 and Day 12. This may suggest that the CMs need to be cultured with the CFs for more than two days before reaching a maximum contractile strain. Future work will investigate earlier time points in the CM Only and Day 0 CF condition to determine how earlier the maximum strain value reaches a plateau for these conditions.

SUMMARY

While cardiomyocytes make the tissue of the heart contract, they are not the only cell type present in the native myocardium. Here we demonstrate the importance of cardiac fibroblasts and their role in ECM production and maintenance. When cultured alone on soft micropatterned substrates, hPSC-CFs are confined to the micropatterned features and remodel the ECM into anisotropic fibers. Similar remodeling and ECM production occurs when cultured with hPSC-CMs in a co-culture model. In addition to ECM, our results indicate hPSC-CFs influence hPSC-CM function with improved calcium kinetics and greater strains in the co-culture conditions compared to when hPSC-CMs are cultured alone. These combined observations highlight the important role cardiac fibroblasts play *in vivo* and co-culture models like the one presented here will allow for more accurate *in vitro* cardiac constructs to be generated.

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Chapter 4: Electrical Conditioning of Patterned Constructs

Some of the results presented in this chapter will appear in a publication that is currently in preparation and is anticipated to include the following co-authors:

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INTRODUCTION

In order to improve the electrical properties of hPSC-CMs, we subjected hPSC-CMs seeded on the 15°chevron micropatterned platform to electrical conditioning via field stimulation. A fundamental property of cardiomyocytes *in vivo* is their electromechanical excitability, with electrical signals pervasive throughout life. Immature hPSC-CMs cultured *in vitro* have a spontaneous contractions and beat arrythmically. In comparison, fetal cardiomyocytes *in vivo* maintain a rhythmic heart rate of ~3Hz and adult cardiomyocytes have a heart rate ~1Hz [1]. It has been demonstrated that electrical stimulation improves electrical function of cardiomyocytes by enhancing connexin expression and sarcomeric structure [2,3]. To this end, we chose the following two stimulation frequencies to mature hPSC-CMs: 1 and 2 Hz, with an unstimulated control. hPSC-CMs on the 15°chevron micropatterned platform were also compared to unpatterned monolayers, both of which received electrical stimulation at 1 or 2 Hz for 2 weeks.

METHODS

Methods used in this chapter are consistent with those used in chapter 2. In addition, the following methods were used:

Electrical Field Stimulation

Electrical pacing was achieved by field stimulation (8 V, 37°C) at frequencies of 1 and 2 Hz using a C-Pace system (IonOptix). PDMS constructs under pacing conditions were placed in 6-well plates with a C-dish 6 well top (IonOptix) and stored at 37°C. Nonstimulated controls were placed in a normal 6-well plate and also stored at 37°C. Ravi Vaidyanathan and Sara Abozeid from the Eckhardt,Lab assisted with pacing.

RESULTS

Electrical stimulation of Patterned and Monolayer hPSC-CMs

As in the proceeding chapters, Day 30 hPSC-CMs were seeded on the 15° chevron pattern. As a control, 15° chevron patterns were compared to unpatterned monolayer hPSC-CMs. Initial experiments initiated pacing one day after the cells were attached to the 10 kPa PDMS substrates; however, the hPSC-CMs were nonresponsive and did not capture with the external stimulation. Therefore, hPSC-CMs in both the patterned construct and monolayer control were allowed to attach and spread on the 10 kPa PDMS substrates for four days. On the fourth day, electrical stimulation was initiated and continued for two weeks, making it a total of 18 days the hPSC-CMs were on the substrate (Fig. 1).

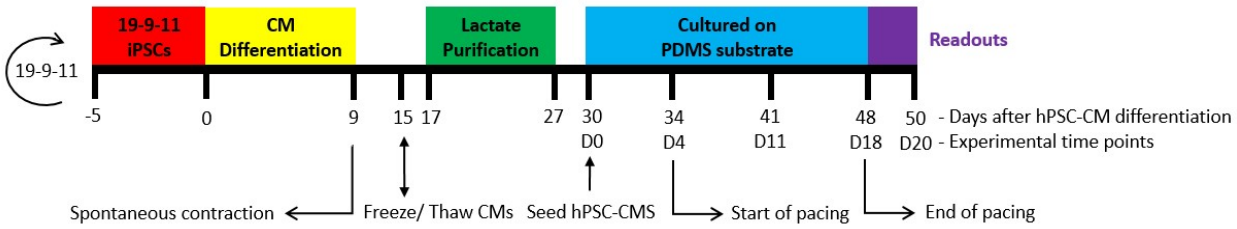

Figure 1: Schematic of experimental time line.

After two weeks of electrical stimulation, Day 18 hPSC-CMs in the patterned construct and monolayer control were fixed and stained for alpha-actinin and n-cadherin to mark the sarcomere apparatus and cell boundaries, respectively (Fig. 2A-B). hPSC-CMs in the patterned constructs were significantly longer and had an average length-to-width ratio of 4:1 while hPSC-CMs in the monolayer controls were 2:1 (Fig. 2C). Pacing frequency did not change aspect ratio. Using custom MatLAB code developed in our lab [4] we analyzed the degree of myofibril alignment in both Patterned and Monolayer hPSC-CMs using the cardiac specific marker alpha-actinin. As expected given our prior research, hPSC-CMs in the patterned construct had a more mature aligned phenotype compared to those in the monolayer control (Fig. 2D). For hPSCs in the patterned constructs, electrical stimulation increased the amount of myofibrils aligned within 10 degrees of the superior angle from 61% in the unpaced condition to 64% and 85% in the 1Hz and 2Hz paced conditions, respectively. Alignment in the patterned constructs was oriented along the lane and bridge direction. Although close to random for the monolayer condition with an alignment score of 18% for the unpaced monolayer controls, this was modestly improved to 28% and 34% when paced at 1Hz and 2Hz, respectively. hPSC-CMs in the patterned constructs increased from 61% in the unpaced condition to 64% and 85% in the 1Hz and 2Hz paced conditions.

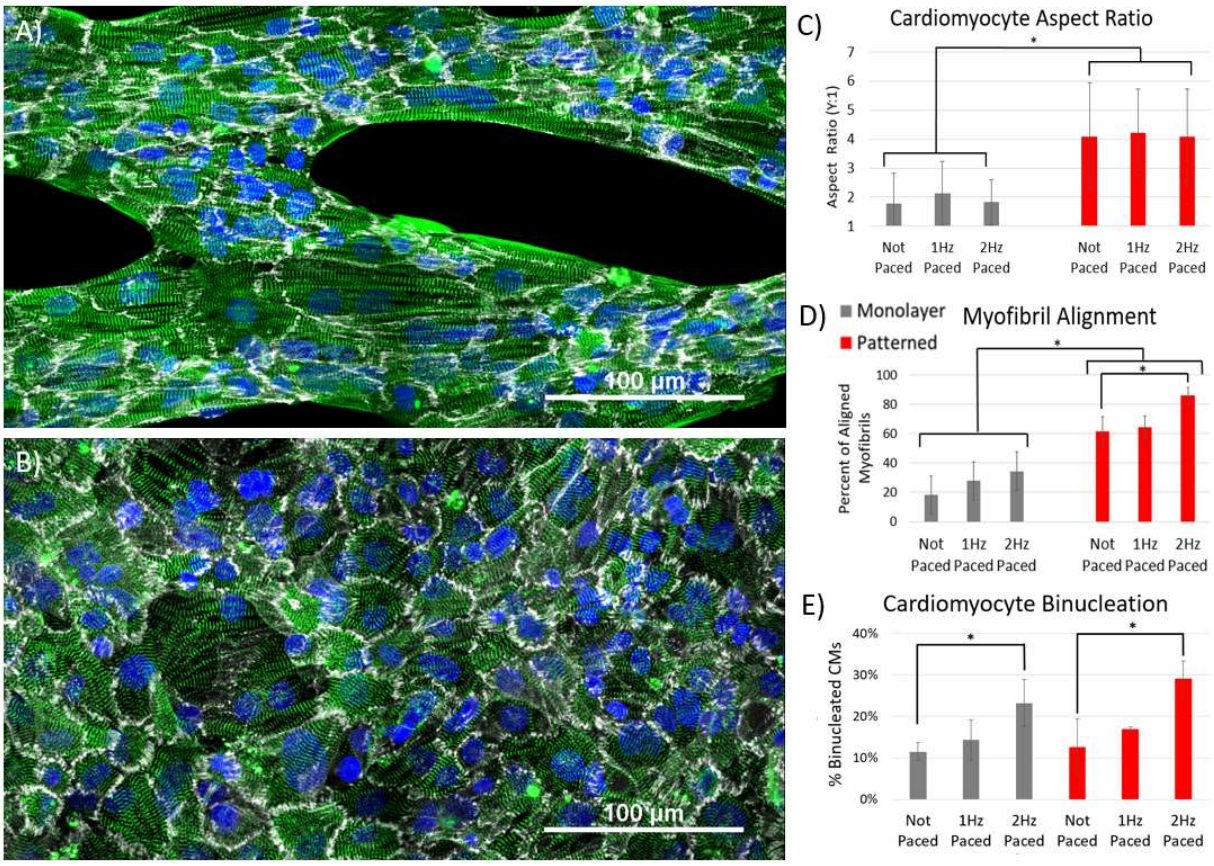

Figure 2: (A) Patterned construct and (B) monolayer control with hPSC-CMs at Day 18 Green = Alpha-actinin, White = N-Cadherin, Blue = DAPI. Scale bars = 100 µm. (C) Aspect ratio, (D) myofibril alignment, and (E) binucleation are compared for patterned construct and monolayer control under 1 and 2 Hz pacing regimes. (D) Percent of myofibrils aligned within 10 degrees of the primary axis. *p < 0.05, two-way ANOVA with *post hoc* Tukey tests. For cardiomyocyte aspect ratio and binucleation n = 4 for all conditions. For myofibril alignment, n = 6 for all conditions.

High magnification imaging allowed for the assessment of hPSC-CM sarcomere organization. The confinement of hPSC-CMs to micropatterned lanes, irrespective of pacing stimuli, produced sarcomere alignment in the direction of the pattern features; conversely, sarcomeres of hPSC-CMs in the monolayer controls were disarrayed (Fig. 3). With the addition of pacing more sarcomere alignment was observed locally within an individual CM for the Monolayer hPSC-CMs; however, globally the sarcomeres were not aligned. Although not quantified, visually there appeared to be more z-line registry in the 2Hz Paced Patterned and Monolayer hPSC-CMs conditions compared to the 1Hz

Paced and unstimulated controls. The amount of binucleated hPSC-CMs also increased in the 2Hz Patterned and Monolayer hPSC-CMs conditions, though no statistical difference was seen between 2Hz Patterned to 2Hz Monolayer hPSC-CMs (Fig. 2E).

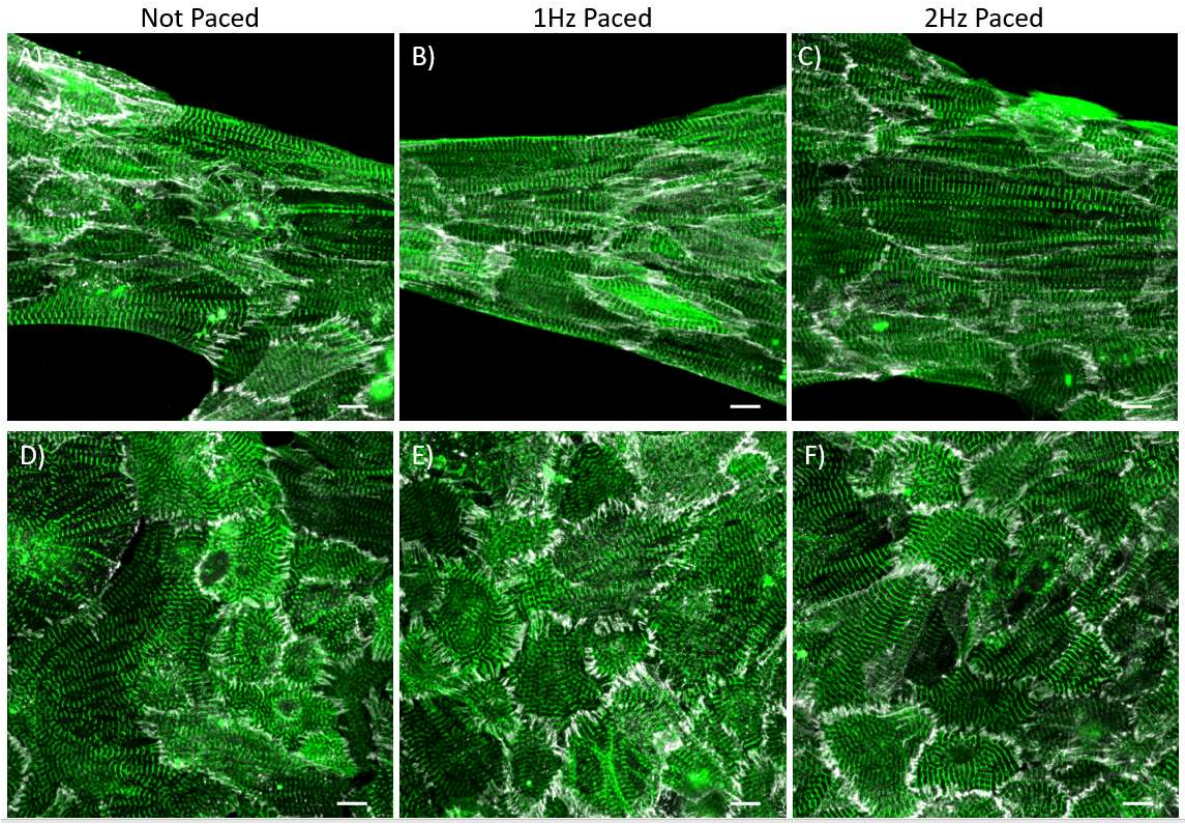

Figure 3: Sarcomere organization in (A-C) Patterned and (D-F) Monolayer hPSC-CMs under different pacing frequencies. Green = Alpha-Actinin, White = N-Cadherin. Scale bars = 10 μm.

Electrical Impulse Propagation in Patterned and Monolayer hPSC-CMs

Next we analyzed electrical impulse propagation using optical mapping methods. The human heart displays anisotropic electrical impulse propagation (Fig. 4A). In comparison, monolayer controls exhibit isotropic electrical impulse propagation (Fig. 4B). The patterned samples recapitulated anisotropic electrical impulse propagation, with speeds 2x faster in the direction of the lanes compared to the transverse direction (Fig. 4B). The same anisotropic behavior was observed in all patterned conditions

irrespective of pacing frequencies. The CV_L/CV_T ratio of ~2 reported for the pattern hPSC-CMs also was not impacted by pacing frequencies. In addition to directionality, we also tested the conduction velocity under the different pacing regimes (Fig. 4C). When optical mapping was performed at 1Hz, the 1Hz Monolayer (24.4 ± 10 cm/sec), 1Hz Pattern (26.4 ± 11 cm/sec) and 2Hz Pattern (27.9 ± 9.6 cm/sec) were all significantly faster than the Not Paced Monolayer (13.7 ± 4.5 cm/sec)($P < 0.05$; Fig. 4C). When optical mapping was performed at 2Hz, there was no significant difference among the groups tested (Fig. 4D). For both the monolayer and patterned constructs, preliminary results indicate that external electric field stimulation increased conduction velocity. Monolayers tended to have a higher conduction velocity when compared to the patterned constructs under the same pacing frequency.

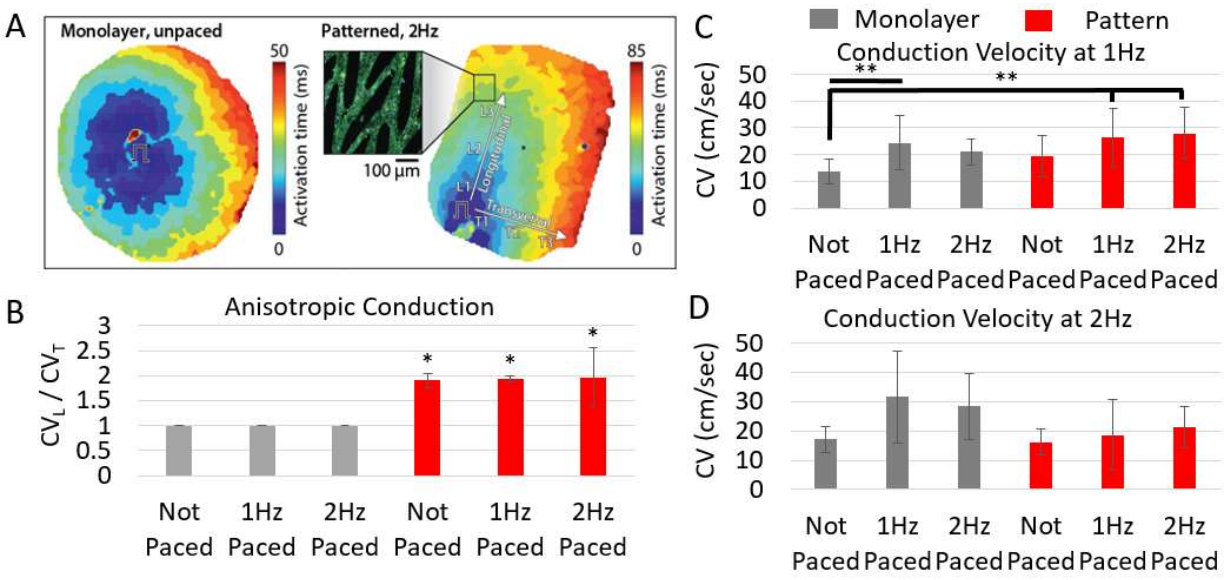

Figure 4: Optical mapping of the (A) monolayer and patterned constructs. (B) Anisotropic conduction determined by comparison of the longitudinal (CV_L) and transverse (CV_T) conduction velocity speeds. Conduction velocities for monolayer and patterned constructs when externally paced at (C) 1Hz and (D) 2Hz. Optical mapping data courtesy of Alexey Glukhov and Di Lang. *$p < 0.0001$, **$p < 0.05$, two-way ANOVA with *post hoc* Tukey tests. n = 14 for Not Paced Monolayer, n = 11 for 1Hz Monolayer, n = 7 for 2Hz Monolayer, n = 10 for Not Paced Pattern, n = 6 for 1Hz Pattern, n = 6 for 2Hz Pattern.

DISCUSSION

A fundamental property of cardiomyocytes is their electrical and mechanical excitability. Without exogenous stimulation, hPSC-CMs contract spontaneously, albeit the rate is often irregular. In comparison, with electrical conditioning at 1 and 2 Hz the hPSC-CMs beat in a regular, rhythmic manner.

Using alpha-actinin to mark the sarcomere structure of hPSC-CMs, we show that the 15°chevron pattern induces myofibril alignment in the feature direction. In comparison, hPSC-CMs grown in monolayer controls display myofibril disarray. This is in good agreement with our previous results that show a correlation between the micropattern lane width and the alignment of hPSC-CM myofibrils (Salick et al. 2014). When electrical conditioning was added to the platform, we saw a significant increase in myofibril alignment for both the monolayer and patterned constructs. Specifically, the 2 Hz condition produced more aligned myofibrils than the 1 Hz or the unstimulated condition. We hypothesize the 2 Hz had better alignment than the other groups tested because the spontaneous rate of the unstimulated control was of similar frequency to the 1Hz condition. Improvements in myofibril alignment have also been observed in other studies that used electrical conditioning (Nunes et al. 2013; Eng et al. 2016).

Next we used n-cadherin to label cell boundaries. hPSC-CMs in the patterned construct had a more adult, rod-shaped phenotype with an aspect ratio of 4:1. While this was better than the 2:1 aspect ratio reported in monolayers, it is still well below the 7:1 aspect ratio reported for adult CMs (Lieu et al. 2009). The 1 Hz and 2 Hz pacing frequencies did not alter the aspect ratio of hPSC-CMs compared to unstimulated

controls. This is in contrast to other reports that indicate electrical conditioning improves aspect ratio (Radisic et al. 2004; Ronaldson-Bouchard et al. 2018). One plausible explanation for this difference could be the seeding density. It is possible that the seeding density used in this study was too high to provide adequate room for cell hypertrophy. Another explanation could be time in culture. A recent report showed no difference in cell area until 3 and 4 weeks after stimulation (Ronaldson-Bouchard et al. 2018). Future studies that investigate how different seeding densities and time in culture effect electrical output would benefit the field.

One notable observation from this pacing study was the amount of multinucleated cells present in the 2 Hz condition compared to the unstimulated control and 1 Hz condition. This observation was present in both the monolayer and patterned constructs. To the best of our knowledge, a correlation between binucleation and pacing frequency has not been reported previously. In humans, it is known that cardiomyocytes proliferate for the first few months after birth and the estimate of multinucleated cells afterwards is ~25% (Laflamme and Murry 2011). We hypothesize the 2Hz rate is similar to the 3Hz neonatal heart rate (Arduini 1995) and causes the hPSC-CMs to mature and exit the cell cycle and become multinucleated. Since this platform is amenable to live-imaging, future studies that tag the nucleus and watch how binucleation occurs *in vitro* may shed light on how this process happens *in vivo*.

In addition to providing myofibril alignment, the 15° chevron pattern produced anisotropic conduction velocity with speeds 2x faster in the lane direction compared to the transverse direction. Previous studies have also reported the influence of cardiac

fiber orientation on the direction of conduction velocity (Roberts, Hersh, and Scher 1979; Kim et al. 2010). Zhang et. al. also fabricated a cardiac tissue utilizing fibrin gels within a PDMS mold and demonstrated improved myofibril alignment; however, their engineered cardiac tissue displayed isotropic conduction velocity, likely due to the symmetrical orientation of the pattern used (D. et al. 2013). As a control we seeded CMs on a 10kPa PDMS monolayer and demonstrated isotropic conduction velocity which has been reported elsewhere in studies utilizing monolayers (D. et al. 2013; Herron et al. 2016). Electrical stimulation increased conduction velocities for both the monolayer and patterned constructs, which is in good agreement with other reports (Badie and Bursac 2009; Ronaldson-Bouchard et al. 2018). A recent report achieved one of the best examples of hPSC-CM by pacing for two weeks at a frequency increasing from 2 Hz to 6 Hz by 0.33 Hz per day, followed by one week at 2 Hz (Ronaldson-Bouchard et al. 2018). Such pacing intervals can easily be adapted with the work presented here.

SUMMARY

In this chapter we demonstrate the feasibility of adding electrical stimulation as an additional pro-maturation cue to the 2D patterned *in vitro* cell culture platform. Both electrical and mechanical outputs need to be amenable in *in vitro* platforms to assess the response of hPSC-CMs when stimulated with a drug. In this study we report improved sarcomere organization and increased conduction velocities for both monolayer and patterned constructs when exogenously electrically stimulated. These results demonstrate that additional cues present in the *in vitro* environment can improve the functional properties of hPSC-CMs compared to isolated, individual cues.

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Chapter 5: Future Work

The 2D micropattern platform in this study was engineered for *in vitro* cardiotoxicity and drug screening testing. Although the preceding chapters were focused on substrate stiffness, micropattering, co-culture and electrical stimulation, this platform is amenable to other known pro-maturation cues that may further improve the functional properties of hPSC-CMs. The ability to test these factors individually and concomitantly in one biomimetic platform will aid in the directed maturation of hPSC-CMs for both basic biology studies and applications in drug and toxicity testing. The following sections describe potential promising avenues of research for further investigation.

Modulating PDMS substrate stiffness

We strategically picked PDMS as our substrate material used in this platform due to its biocompatibility and tunability of Young's modulus from 5 kPa – 1.7 MPa (Palchesko et al. 2012). In most of the work in the proceeding chapters we focused on 10kPa PDMS, with some additional work on 5 and 50 kPa PDMS. In our recent paper we showed that hPSC-CMs seeded on 5 and 10 kPa PDMS generated more strain than those on 50 kPa PDMS (Notbohm et al. 2019). Although we tested mechanical strain, we did not assess electrophysiology with different PDMS elastic moduli. With many forms of heart disease the myocardium stiffens and conduction velocity is impaired (Miragoli, Gaudesius, and Rohr 2006b). Replicating this phenomena in vitro by changing stiffness may help identify molecular changes in hPSC-CMs that can then be targeted in drug discovery.

Additional studies that would benefit from the modulation of substrate stiffness involve CFs. It has been shown that matrix stiffness regulates the conversion of CFs into α-smooth muscle actin (α-SMA) expressing myofibroblasts (Hinz 2009; Huang et al. 2012). These myofibroblasts secrete different ECM proteins than CFs and modify the balance of metalloproteinases and their inhibitors to promote fibrosis (Berk, Fujiwara, and Lehoux 2007). It would be of interest to compare the function of CMs seeded on a deceulluzarized scaffold of ECM proteins that were produced by CFs on (a) a healthy stiffness ~10 kPa and (b) a diseased stiffness ~50 kPa. In terms of a co-culture study, TGF-β1 could be used to induce myofibroblast conversion (K. Chen et al. 2004) and cardiac function could be assessed pre and post addition of TGF-β1. Such 2D *in vitro* cardiac fibrosis platforms would provide novel, non-animal platforms to elucidate pathways governing ECM remodeling and cardiac function during disease.

Decellularized ECM Scaffolds

One of the more striking findings in this work revolved around the ability of hPSC-CFs to remodel micropatterned ECM. We found that collagen and fibronectin had a globular phenotype when coated or patterned onto the PDMS substrates in the absence of cells. However, CFs converted the ECM into fibers reminiscent of the native myocardium ECM, which were well aligned when the CFs were confined to a pattern. While we have shown here and in our prior work the importance of microtopology on hPSC-CMs (Salick et al. 2014), other labs have demonstrated the importance of nanoscale topogology on cardiomyocyte function (D.-H. Kim et al. 2010; Carson et al. 2016). The ability to combine micropatterned lanes embedded with nanoscale ECM fibers may provide an optimal environment for hPSC-CM maturation. To do this, CFs would be

seeded onto micropatterned lanes and allowed to remodel the ECM. Afterwards, the PDMS scaffold would be decellularized leaving behind fibrillar, nanoscale ECM (Xing et al. 2014). Then CMs would be seeded and cultured on the decellularized ECM fibers.

Another potential deceullarization strategy involves the creation of anisotropic ECM sheets. Schmuck and colleagues demonstrated an ECM patch can be generated by CFs seeded at a high density and then decellularized (Schmuck et al. 2014). The Feng lab took this a step further and generated an anisotropic ECM sheet by culturing a high density of CFs on nanograted 1.7 MPa PDMS substrates, followed by decellularization (Xing et al. 2014). In the preceding chapters when CFs were cultured on 30 μm lanes spaced apart by 30 μm, there was time-lapse video evidence of CFs bridging and migrating from one lane to another. I hypothesize that if the lane spacing is smaller than 30 μm, then more bridging will occur. If you then over seed CFs on such a pattern I hypothesize the CFs would produce an anisotropic fibrillar ECM on top of the compliant PDMS substrate that could then be decellularized and seeded with another cell type.

Going from 2D to 3D

The micropatterned platform discussed thus far creates a 2D anisotropic cardiac tissue. While this method mimics the cytoarchitecture within one cross-section of the heart, it does not recapitulate the 3D structure of the whole heart. Only a decellularized heart will give the complete 3D structure found *in vivo* and some groups have pursued this direction (Rieder et al. 2005; Ott et al. 2008). While 2D has its advantages, it does not fully represent the 3D cell-cell interactions which occur in the native myocardium. Fortunately, thanks to cardiac fibroblasts, the platform described in our work can easily

be scaled up from one cell layer to multiple cell layers. In our co-culture work we found evidence of areas containing cells stacked on top of each other, compared to only 1 cell layer in the CM Only condition. We concluded that CFs secreted ECM on top of CMs which allowed other CMs to attach and form multiple layers. These multiple layers formed spontaneously with only one seeding of CMs and CFs. To create multiple cell layers, a second seeding of CMs can be added on top of the first layer of cells after CFs have secreted additional ECM. Presumably, since aligned ECM has been produced by the CFs on top of the first layer of CMs, the second seeding of cells will be able to attach on top of the first layer. If successful, this process may be repeated multiple times depending on the desired level of tissue layers/thickness. It will be of interest to see if the organization provided by the bottom, aligned layer of CMs is maintained with the addition of layers on top.

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Chapter 6: Concluding Remarks

Cardiomyocytes and cardiac fibroblasts, along with the rest of the cells that make up the human body, are living organisms that respond to the complex signals provided by their surroundings. In this work, our goal was to provide a 2D substrate platform that mimicked the myocardial microenvironment and could be used in *in vitro* cardiotoxicity and drug screening assays. Our hypothesis was that the combination of multiple signaling factors would improve the functional properties of hPSC-CM more so than individual, isolated cues. Our results indicate functional improvements in hPSC-CMs as evident by improved calcium kinetics and increased contractile strain when hPSC-CMs are cultured on a physiological-relevant substrate stiffness, micropatterned topology and co-cultured with hPSC-CFs. Although a viable *in vitro* culture system for cardiac-related investigations was the end goal, it was important to know what factors contributed to the improved maturation. The platform described here allows for the systematic addition and subtraction of signaling cues which may lend new insights and aid in the directed maturation of immature hPSC-CMs. As the field progresses and hPSC-CM maturation improves, their inclusion in drug discovery and toxicity testing platforms will increase, ultimately leading to the development of new drugs which will improve health outcomes and save lives.