

**Combinatorial maturation strategy for disease modeling and
phenotypic drug screening of Duchenne muscular dystrophy
cardiomyopathy**

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Abstract

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Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) offer great promise for regenerative medicine, preclinical drug screening, and cardiac disease modeling applications. One of the most significant hurdles towards adoption of hPSC-CM technologies, however, is cardiomyocyte developmental immaturity. Current differentiation methods produce hPSC-CMs with structural and functional characteristics most closely resembling fetal cardiomyocytes, which significantly hinders our ability to predict patient drug responses or model adult-onset cardiomyopathies. The following

dissertation addresses this challenge with the goal of engineering structurally and functionally mature cardiac tissues from hPSC-CMs for *in vitro* disease modeling and drug screening applications. Here, we present the development of a bio-inspired, combinatorial method for enhancing the maturation of hPSC-CMs that incorporates distinct physical, biochemical, and genetics cues. We began by investigating the role of surface nanotopography on hPSC-CM development and found that, similar to primary cardiomyocytes, hPSC-CMs exhibited a nanotopographic size-dependent phenotype. Utilizing the optimal nanotopographic surfaces dimensions for promoting maturation, we tested whether this maturation cue alone could improve our ability to model the cardiomyopathy associated with Duchenne Muscular Dystrophy (DMD). Although we were able to measure a blunted cytoskeletal response to the nanotopography in dystrophin-null hPSC-CMs, this difference was mild and we were unable to detect a functional disease phenotype. We therefore explored more comprehensive methods for inducing hPSC-CM maturation and developed our combinatorial maturation (ComboMat) protocol. The ComboMat protocol incorporates biomimetic nanotopography, thyroid hormone T3, and Let7i microRNA overexpression to produce hPSC-CMs with enhanced sarcomere development, improved electrophysiological and contractile function, improved mitochondrial respiratory capacity, and a transcriptome upregulated for metabolic and muscle development. When the ComboMat protocol is applied to a CRISPR-edited dystrophin knockout (KO) model of DMD cardiomyopathy, a distinctive, endogenously occurring disease phenotype emerges. Mature dystrophin KO hPSC-CMs exhibit greater propensity for arrhythmia with a higher resting cytosolic calcium content compared to Normal hPSC-CM controls. A phenotypic drug screen of

dystrophin KO hPSC-CMs using the ComboMat protocol identified compounds that mitigated arrhythmogenic behavior. The ComboMat protocol can be applied to other cardiac disease models, cardiotoxicity studies, or cardiac tissue engineering applications. *In vitro* screening assays must predict the response of the human heart with high fidelity in order to be adopted. Taken together, this research demonstrates the utility of bioengineering strategies to mature hPSC-CMs in order to develop more biomimetic, adult-like cardiac tissues for preclinical screening applications.

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Chapter 1. Introduction

Parts of this chapter have been published in the following review manuscript:

- Smith AST, **Macadangdang J**, Leung W, Laflamme MA, Kim D-H. Human iPSC-derived cardiomyocytes and tissue engineering strategies for disease modeling and drug screening. *Biotechnology Advances*. 2017; 35(1): 77–94.

1.1 Cardiovascular Disease and Inherited Cardiomyopathies

Cardiovascular disease remains the leading cause of death for both men and woman worldwide, with a rapidly growing impact on developing nations¹. The socioeconomic burden of cardiovascular disease is forecasted to exceed \$1 trillion per year in the United States alone by 2030². This devastating impact is due, in large part, to the heart's inability to regenerate, with only 1-2% cardiomyocyte turnover per year³. Inherited cardiomyopathies are a major cause of heart disease in all age groups, including children and young, otherwise healthy adults⁴. Several incidences of professional athletes collapsing in the middle of sporting events have brought inherited cardiomyopathies to the forefront of public awareness. Monogenetic inherited cardiomyopathies, which differ from family-linked diseases such as high blood pressure or coronary artery disease, are a result of genetic mutations in specific genes that cause abnormalities in the cardiac structure, conduction system, or vasculature⁵. Inherited cardiomyopathies are classified according to function and morphologic features and include hypertrophic (HCM), dilated (DCM), arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C), and channelopathies. All cardiomyopathies are genetically heterogeneous, with mutations in different genes resulting in the same

clinical presentation⁴. Due to various levels of penetrance and the timing of disease onset for individual cardiomyopathies, hope remains that therapies can be developed to prevent the emergence of a disease phenotype and improve the quality of life for these patients.

1.2 Animal Models of Cardiac Disease

Animal models, particularly small animal murine models, have yielded incredible insight into basic biology and pathogenesis of heart disease. Animal models of heart failure allow for chronic structural changes to take place with quantifiable progression into ventricular dysfunction. A wide variety of transgenic or knockout mouse models have been developed with the ability to study specific cell types and/or inducible transgene expression⁶. For instance, Arber et al. developed a DCM mouse model by selectively knocking out muscle lim protein⁷ that has been used extensively to explore molecular therapies that might rescue the disease phenotype⁶. By definition, however, no model is a perfect representation of the system in question. There are significant differences between these model organisms and humans that limit their translational utility. Major differences between animal models and humans include heart rate, sarcomeric protein expression, pharmacokinetics, genetic variability, and the age of the animals when most analyses are performed⁸. Therefore, there is a need to be able to study more physiologically relevant model systems that do not exponentially increase the costs of both basic science and translational endeavors.

1.3 Human Cardiac Tissue Engineering

Cardiac tissue engineering aims to alleviate the burdens of cardiovascular disease and address the limitations of animal models by fabricating a functional piece of human heart muscle to either a) repair or replace damaged myocardium after disease or b) to serve as an *in vitro* model of the heart to screen drugs, study disease mechanisms, or better understand heart development^{9,10}. While regenerative medicine will someday revolutionize patient treatment, from a translational perspective, *in vitro* cardiac tissue applications are a much more attainable goal in the near future. The advent of stem cell technology has been a significant leap forward for the cardiac tissue engineering field because it exponentially increased the availability and utility of human cardiomyocytes. Unlike animal models, human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs), including both embryonic (ESC) and induced pluripotent (iPSC), theoretically express the full array of ion channels, sarcomeric proteins, and metabolic machinery found in patients and should therefore more closely recapitulate the human response *in vitro*.

1.4 Applications of iPSC-CMs in Preclinical Screening

The potential applications for cardiac tissues engineered from iPSCs are vast. Although many see the development of such models as a potential means to generate autologous transplant material, such applications remain some way off. Engineered cardiac tissues have more immediate relevance to investigators seeking to better understand cardiac development¹¹, since such cells possess an innate immature phenotype. Integration of iPSC-derived cells with platforms and culture strategies that promote cardiomyocyte maturation will enable more comprehensive and detailed

studies of the signaling pathways that underpin myocardial development. In addition, such experiments will provide greater understanding of the molecular, structural, and functional changes that these cells undergo during different stages of development.

A second application for iPSC-CMs is in preclinical drug screening. The advancement of more biomimetic screening platforms will likely lead to the development of screening assays with significantly better predictive power, in terms of informing on the selection of compounds progressing to clinical trials¹². The increased availability of robust methods for generating iPSC lines also opens the door to the development of patient-specific disease modeling platforms with which to study inheritable diseases such as long QT syndrome, amyotrophic lateral sclerosis, Down's syndrome, spinal muscular atrophy, Duchenne and Becker muscular dystrophies, and Huntington's disease¹³⁻¹⁶. In the following sections, the applications of iPSCs in preclinical drug screening and modeling cardiac pathologies are discussed in more detail.

1.5 Drug Screening

Perhaps the most attainable short-term goal for iPSC-CM technology is the use of cardiac tissue analogues as *in vitro* screening assays (**Fig. 1.1A–D**). Induced PSC-CMs are ideal candidates for applications in drug screening and development. The Comprehensive *In vitro* Proarrhythmia Assay (CIPA) initiative was recently established with the intention to advance safety pharmacology from more traditional pharmacodynamic methodologies toward a combination of *in silico* and *in vitro* compound toxicity assessment¹⁷⁻²⁰. Since its inception, the program has garnered support from both government agencies and pharmaceutical companies alike, and the inclusion of *in vitro* screening in the proposed strategies serves to highlight the

importance the drug development industry is now placing on the development of accurate biomimetic cardiac tissue models.

Since primary cardiomyocytes are hard to isolate and have a short lifespan in culture before they begin to dedifferentiate, genetically transformed and/or immortalized cell lines are often utilized for drug screening applications. However, these cells lack important cardiac characteristics and ion channels, and do not fully recapitulate the cellular context of cardiomyocytes²¹. In contrast, undifferentiated iPSCs can proliferate for extended periods in culture, while iPSC-CMs express the correct electrical and physiological properties of the developing heart, mimicking primary cardiac cell functional performance. Compared with native heart tissue, cardiac cells derived from iPSCs typically exhibit slower conduction velocities and shorter action potential durations, and this is attributed to a lack of maturity in these cultured cells. Effort to improve cardiac electrophysiological function through application of suitable maturation stimuli is a heavily investigated field and discussed in more detail later. Regardless, the long-term functionality of iPSC-CMs, and their capacity to recapitulate the arrhythmogenic behavior of native cardiac muscle in response to treatment with a wide range of proarrhythmic compounds¹⁷, highlights their suitability for drug screening applications.

In pharmaceutical development, a major concern and regulatory hurdle is drug-induced cardiac toxicity. Historically, drug-induced arrhythmias have contributed heavily to reported cases of adverse drug reactions²². Consequentially, the FDA and the pharmaceutical industry in general have recently established preclinical drug screening guidelines to test for cardiac toxicity in all new chemical entities prior to market

release²³. The current *in vitro* standard for preclinical assessment is the analysis of the hERG channel response in heterologous cell types. Although this method is reliable in terms of isolating compounds with potential arrhythmogenic properties, the high safety margins associated with the hERG assay actually reduce confidence in its ability to predict arrhythmogenic potential accurately²⁴. As such, issues with false positives are a significant potential problem and may have led to a number of harmless compounds being kept in development abeyance, despite their therapeutic potential²⁵. The need for more predictive preclinical cardiac assays is strong and human iPSC-CMs offer an excellent model for screening new compounds for potentially toxic effects. For example, drugs can be tested for adverse cardiac effects by measuring the disruption of iPSC-CM electrophysiological properties²⁶. There are data reporting the electrophysiological capacity and responsiveness of iPSC-CMs in response to several cardiac and non-cardiac drugs for reference in research²⁷⁻²⁹. It has also been shown that individuals with pre-existing heart conditions are more susceptible to cardiotoxic drugs, which is reflected in disease-specific iPSC-CMs from measurements of action potential duration and drug-induced arrhythmia²³. Similarly, recent studies have shown that patients with certain genetic backgrounds exhibit increased cardiotoxic sensitivity to treatment with doxorubicin, and this susceptibility is recapitulated in iPSC-derived cardiomyocyte cultures^{30,31}. Such data highlight the potential for iPSC-CMs to be utilized in patient specific drug screens, potentially allowing doctors to personalize a patient's treatment options based on their cells' response to exposure to different compounds.

As an electromechanical organ, the heart has a number of different outputs that are important for proper function. Therefore, a plethora of screening assays exist that

can be utilized to investigate the functional performance of iPSC-CMs for drug-induced cardiotoxicity³². Cellular electrophysiology can be measured directly via patch clamp or indirectly through field potentials. Patch clamp methods suffer from low-throughput and therefore are not ideal candidates for pharmaceutical development and drug screening. Microelectrode arrays (MEAs), however, are widely applied in industry and offer the advantage of being noninvasive and can therefore be used to measure cellular responses longitudinally. The extracellular field potential of a cardiomyocyte (or cardiac tissue) correlates directly with the intracellular action potential. Thus, the field potential duration (FPD) can be used as an analogue for the cardiac action potential duration. Navarette et al. have successfully utilized MEAs to record drug-specific cardiotoxic effects in iPSC-CMs by looking at the changes in FPD³³. Importantly, MEAs also offer the added advantage of being able to investigate tissue level electrophysiological parameters, such as conduction velocity, that single cell assays inherently miss. Liang et al. recently performed a drug evaluation study using MEAs and demonstrated that the compound cisapride had no discernable effect on wild-type cardiomyocytes, but did have a pronounced effect on QT prolongation in cells from patients who suffer from long-QT syndrome²³. Cisapride is known to act on hERG channels³⁴, and so fails to pass conventional preclinical screens. Therefore, the results obtained by Liang and colleagues demonstrate the importance of screening on cardiomyocytes directly, since the effect of the drug on hERG channels may be counteracted by its effect on other ion channels. Moreover, the presented data demonstrate the value in screening across both wild type and diseased cells since compound action may vary across distinct genetic groups. Such data could prove invaluable in targeting new chemical entities to the

correct subpopulation of people for whom they are safe and hold the potential to alleviate specific symptoms.

As the basic function of the heart, cell contraction and beating can also be measured to screen for cardiotoxic effects. Impedance assays³⁵, muscle thin films³⁶, and video motion tracking³⁷ have all been validated as means to show changes in cardiomyocyte contractile function. Mathur and colleagues recently developed a microfluidic platform for analyzing the beat characteristics of 3D engineered cardiac tissues³⁸. Using a custom-made motion-tracking software, they were able to demonstrate physiologically relevant changes in cardiac contraction kinetics in response to increasing doses of a number of cardiotoxic compounds, including verapamil and metoprolol. The collected data showed better recapitulation of myocardial tissue responses than is achieved from analysis of 2D monolayers, indicating the importance of mature tissue structures when seeking to model drug responses effectively *in vitro*.

Cardiac excitation-contraction coupling (ECC) is another important facet of cardiac function. Calcium (Ca^{2+}) acts as a hub in ECC and can be measured *in vitro* using a number of different intracellular Ca^{2+} indicator dyes with high signal-to-noise ratios. Although Ca^{2+} measurements are typically used as a convenient surrogate for assessing changes in voltage-related properties of cells, a number of different reports have shown the ability to discover drug-induced cardiotoxicity *in vitro* through analysis of Ca^{2+} imaging data. Advances in optical mapping and image processing are allowing more intricate systems and measurements to be made in a high-throughput manner. Using 2D monolayers of stem cell derived cardiomyocytes and a custom analysis method termed kinetic image cytometry (KIC), Cerignoli et al. were able to track the

Ca^{2+} transients of all the cells in a monolayer virtually simultaneously³⁹. This allowed for detailed investigation of Ca^{2+} synchronicity between cells. When the bioactive form of triiodothyronine was added to the cells, distinct disturbances in the Ca^{2+} transients were identified. Additionally, the predicted effects of verapamil and cisapride (L-type Ca^{2+} channel and K^{+} channel blockers, respectively) could be detected using the KIC method. Furthermore, Lee et al. have demonstrated that it is possible to simultaneously record intracellular Ca^{2+} and membrane voltage using optical mapping techniques on iPSC-CMs⁴⁰.

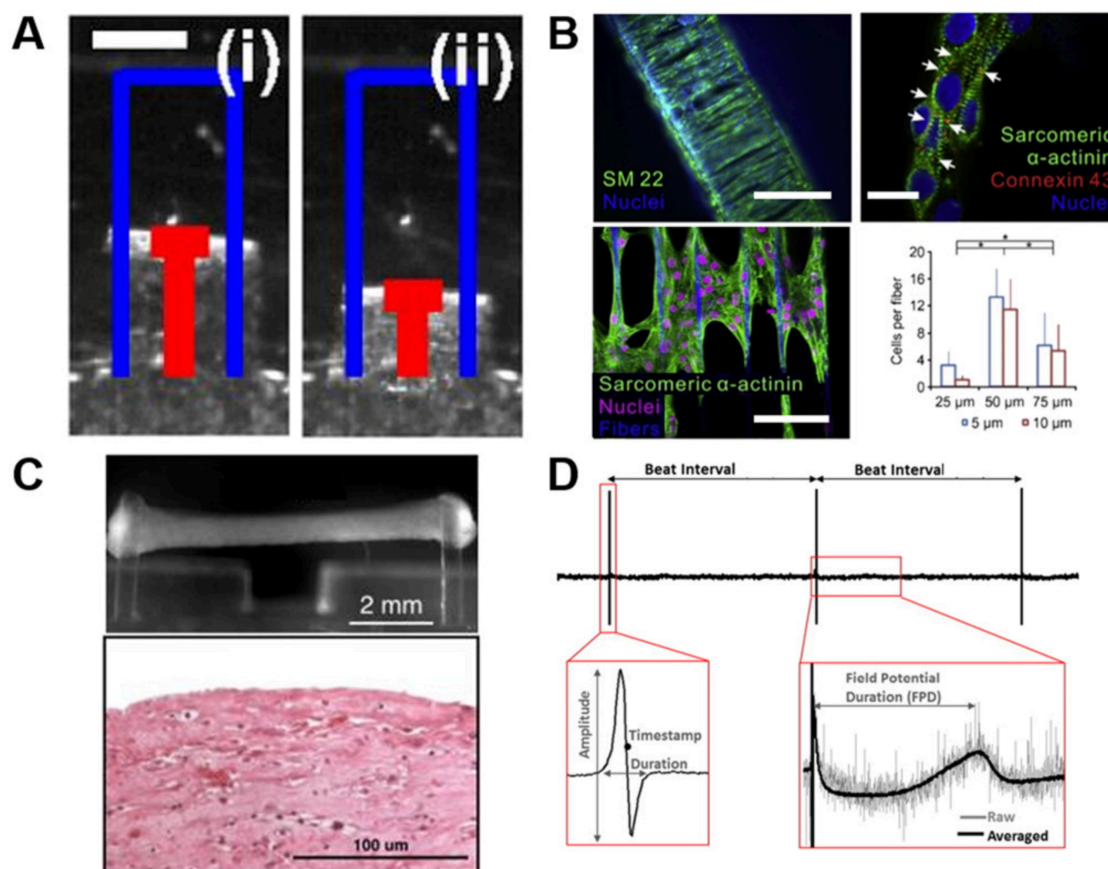


Figure 1.1. 2D and 3D technologies for high throughput cardiotoxicity screening. Drug dose response studies can be performed based on cardiac contractility platforms. (A). Images of a muscular thin film (MTF) platform incorporating flexible cantilevers supporting cultured cardiomyocytes. Measurement of changes in cantilever position between diastole (i) and during peak systole (ii) enables quantification of force generated in cultured cardiomyocytes. Scale bar = 1 mm. Reprinted with permission⁴¹. (B). A three-dimensional filamentous model of human cardiac tissue (top left) used for analysis of contractile function through measurement of filament displacement. In this image, both cardiomyocytes and myofibroblasts were stained for expression of SM22 to show cell distribution throughout the construct. Confocal images of cardiomyocytes aligned on a single fiber show advanced sarcomere structure stained by sarcomeric α -actinin and intercellular gap junctions stained by connexin 43 (white arrows, top right). Confocal images for iPSC-CMs growing on the middle layer of a filamentous matrix and aligned along the fiber direction (bottom left). In such constructs, the formation of 3D cardiac tissue was quantified by cell number on the middle layer relative to the fiber number, and matrices with 50 μ m fiber spacing were found to result in the highest value of cell per fiber (bottom right). Reprinted with permission⁴². (C). Human engineered cardiac tissues (hECTs) have been shown to mimic key aspects of the newborn human heart and thus allow functional testing for drug screening purposes. Such model systems usually incorporate a culture mold with some form of integrated endposts (top). The illustrated example shows an attached hECT on a mold from a side view. Analysis of post deflection, taking into account post length and stiffness, facilitates measurement of contractile force produced by the engineered tissue. An image is also provided showing a longitudinal section of an hECT stained with hematoxylin and eosin after 12 days in culture (bottom). Reprinted with permission⁴³. (D). Representative field potential waveforms collected from iPSC-CM monolayers using microelectrode arrays (MEAs) and details of the parameters extracted from the raw signal and the time averaged signal. Such analysis can be used to study drug-induced changes in cardiac electrophysiological function for prediction of compound efficacy and toxicity. Reprinted with permission⁴⁴.

1.6 Disease Modeling

Induced PSCs can be derived from patients with specific diseases of interest and reprogrammed into cells that retain those disease-specific traits. These cells can then be used for disease modeling and/or screening drugs *in vitro* to assess their ability to correct the observed disease phenotype⁴⁵ (**Fig. 1.2**). Below we discuss the various iPSC models of cardiac diseases that have been published to date.

1.6.1 Ion Channelopathies

Ion channelopathies are perhaps the form of cardiac disease with the most well-established iPSC-based disease models. This is due, in part, to their known impact on measurable single cell electrophysiological endpoints, such as action potential duration (APD). Long QT Syndrome (LQTS) is the most common ion channelopathy, with a prevalence of 1:2000⁴⁶. Characterized by a delayed repolarization of ventricular cardiomyocytes, and thus a prolonged QT interval on the electrocardiogram (ECG), LQTS is associated with a high risk of ventricular tachyarrhythmia and sudden cardiac death (**Fig. 1.2A**). LQTS is divided into at least 10 different subtypes according to the underlying genetic or channel mutation. LQTS type 1 (LQT1) is caused by a mutation in the KCNQ1 gene, which encodes for a subunit of the ion channel responsible for the adrenergic-sensitive, slow outward potassium current, I_{Ks} . Moretti et al. became pioneers of cardiac disease modeling using iPSCs with their 2010 publication in The New England Journal of Medicine in which they modeled the LQT1 disease phenotype using hiPSC-CMs. They demonstrated that ventricular and atrial-type hiPSC-CMs had significantly longer APDs than their healthy control counterparts, while nodal-type

hiPSC-CMs exhibited no such change in APD⁴⁷. The authors were also able to demonstrate that beta-blockade helped to protect against catecholamine-induced tachyarrhythmia, highlighting the potential utility of such disease models in helping to test therapies in an *in vitro* setting prior to their advancement to clinical trials.

Various groups have since published other LQTS subtype disease models, including another LQT1 model by Egashira et al. in which they used MEAs to measure iPSC-CM FPD in response to small molecule inhibitors, thereby demonstrating the role of I_{Ks} deficiency in the establishment of the LQT1 phenotype⁴⁸. Three groups have published models of LQT2, which is due to a missense mutation in the KCNH2 (hERG) gene⁴⁹⁻⁵¹, causing a reduction in the cardiac potassium current, I_{Kr} . Of these, Itzhaki et al. and Matsa et al. screened therapeutic agents for their ability to abolish or reduce the arrhythmogenic phenotype of the disease. Additionally, two groups have investigated a LQT3 disease model, caused by a gain-of-function mutation in the SCN5A gene responsible for the I_{NaL} current^{52,53}. Both groups utilized patch clamp methods to characterize abnormal Na^+ current inactivation in diseased hiPSC-CMs compared to control, and showed that Na^+ channel blockers helped to alleviate the disease phenotype. Furthermore, an iPSC-derived cardiomyocyte model using cells from a patient carrying an SCN5A-1795insD mutation have been published⁵⁴. This mutation gives rise to both LQT3 and Brugada Syndrome phenotypes, as well as conduction defects caused by both gain- and loss-of-function effects on the cardiac Na^+ channel, respectively. Finally, Yazawa et al. established a model of LQT8 from Timothy syndrome patients, caused by a mutation in the CACNA1C gene responsible for the Ca^{2+} influx through L-type Ca^{2+} channels⁵⁵. Similar to the other LQTS models described,

Yazawa et al. were able to demonstrate a prolongation of the APD and an alleviation of the arrhythmogenic phenotype with the addition of a therapeutic compound, in this case roscovitine.

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is another inherited channelopathy that is characterized by adrenergically mediated polymorphic ventricular tachycardia in patients without a structural cardiac pathology. Overall, CPVT is caused by improper Ca^{2+} handling, either through spontaneous Ca^{2+} release through the ryanodine receptor (RyR2), or through insufficient Ca^{2+} sequestration by calsequestrin (CASQ2)⁵⁶. To date, there have been six reports of CPVT disease models using iPSCs. Itzhaki et al., Fatima et al., Jung et al., Pasquale et al., and Paavola et al. each developed models of the more prevalent CPVT1⁵⁷⁻⁶¹, resulting from a dominant mutation in RyR2, while Novak et al. developed a model CPVT2⁶², the autosomal recessive form of the disease caused by a mutation in CASQ2. For CPVT1, all groups demonstrated aberrant Ca^{2+} handling and a propensity for delayed after-depolarizations, but each treated the hiPSC-CMs with different compounds to reduce the disease phenotype. Itzhaki et al., utilized beta-blockers to reduce Ca^{2+} overload, Fatima et al. used forskolin to increase cAMP levels, Jung et al. found that dantrolene reduced the level of Ca^{2+} spark activity, and Pasquale et al. applied a CaMKII inhibitor to reduce the disease phenotype. Meanwhile, Paavola et al. used β -agonists to slow the rate of depolarization in RyR2-mutant cells, suggesting that such treatment could constitute a marker for arrhythmogenicity. The diversity of these studies and findings highlight the fact that in order for disease modeling to be translationally relevant, a concerted effort to find the most promising therapy with consistent results across multiple cell lines is

needed before attempting to utilize the treatment in patients.

Taken together, these reports demonstrate that iPSC technologies can be used to reliably recapitulate the pathophysiological hallmarks of arrhythmogenic disease states *in vitro*, and that these systems can be used as screening systems for new drugs or therapeutic agents. While this is an exciting prospect for the near future, to date, investigators have largely restricted their studies to demonstrating that iPSCs isolated from well-characterized monogenic diseases exhibit perturbed functional phenotypes when differentiated into cardiomyocytes *in vitro*. For the field of iPSC cardiac disease modeling to grow, such cell types need to be employed in novel mechanistic studies to help delineate the underlying causes of the observed phenotype, or as test beds with which to evaluate novel therapeutic regimens. Without progression to the use of these cells in these sorts of studies, iPSC-based cardiac disease modeling will fail to have any meaningful impact on translational health sciences and the improvement of patient care.

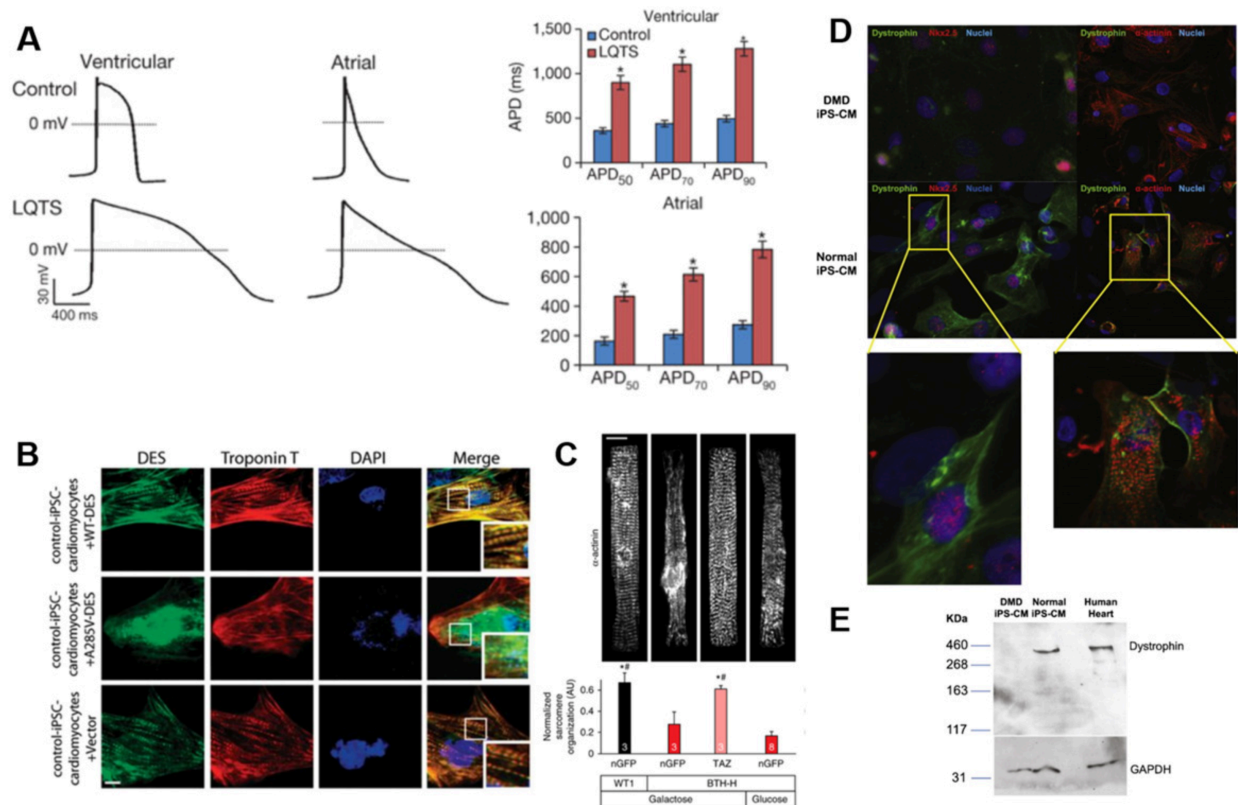


Figure 1.2. Characterization of iPSCs derived from patients with genetic cardiomyopathies for use in disease modeling applications. (A). Long Q-T syndrome (LQTS) cardiomyopathy modeling. Action potentials were recorded from control and LQTS human iPSC-CMs with ventricular-like and atrial-like morphologies. The graphs indicated prolonged action potential duration (APD) – to reach 50%, 70%, and 90% of repolarization – in both ventricular-like and atrial-like LQTS iPSC-CMs when compared to control. Reprinted with permission¹⁵. (B). Immunofluorescence staining for desmin (DES: green) and cardiac Troponin-T (red) performed in control iPSC-CMs transduced with wild-type desmin (WT-DES: top), mutant desmin (A285V-DES: middle) and vector alone (bottom) using lentivirus. Forced expression of the mutant A285V-DES in control iPSC-CMs produced a phenotype that included diffuse isolated aggregations of desmin-positive protein (green, middle right) as observed in iPSC-CMs from dilated cardiomyopathy patients, suggesting that A285V-DES exerts a dominant-negative effect. Reprinted with permission⁶³. (C). Sarcomere organization of patient-derived Barth syndrome cardiomyopathy and control iPSC-CMs. Sarcomere organization was tested in the indicated culture medium and after transfection with the indicated modified RNA. P b 0.05 vs.: *, Barth syndrome cells + nGFP modified RNA, in galactose supplemented medium; #, Barth syndrome cells + nGFP modified RNA, in glucose supplemented medium. The presented data suggest treatment with TAZ modified RNA restores sarcomere regularity. Reprinted with permission⁶⁴. (D). Cardiomyocytes derived from normal and DMD iPSCs and probed with antibodies against dystrophin (green), together with cardiac specific protein Nkx2-5 or sarcomeric α -actinin (red). Dystrophin staining was demonstrated in normal cardiomyocytes, but not in cardiomyocytes derived from DMD patients. (E). Western blot for full length dystrophin detected in the lysates from normal iPSC-CMs (lane 2) and human heart tissue (lane 3) but absent in the lysate from DMD patient iPSC-CMs (lane 1). Molecular weight markers (in kDa) are shown on the left. D and E reprinted with permission⁶⁵.

1.6.2 Structural Cardiomyopathies

Hypertrophic cardiomyopathy (HCM) is the most common inheritable heart disease, yet pharmacologic targets for treatment of the disease remain elusive. Sudden cardiac death associated with HCM is almost exclusively a result of ventricular arrhythmias degenerating into ventricular fibrillation. Like many of the channelopathies described above, a number of genetic mutations behind HCM have been identified, but the remodeling mechanisms behind the pathophysiology are complex and unknown. Therefore, HCM represents a very attractive target for *in vitro* disease modeling. When iPSC-CMs were generated from two patients with one of the most common mutations present in hypertrophic cardiomyopathy patients (LEOPARD syndrome mutation; Thr468Met in the PTPN11 gene), the resulting cells showed increased sarcomere disorganization and were larger than those of wild-type controls⁶⁶. Lan et al. have produced an iPSC model of HCM and again demonstrated aberrant electromechanical function⁶⁷. Using patch clamp methods, Lan et al. showed an increased propensity for delayed afterdepolarizations in HCM cardiomyocytes compared to controls. Using confocal line scanning techniques with fluo-4 to look at Ca²⁺ transients, the group also showed aberrant Ca²⁺ handling that was alleviated with the addition of the L-type Ca²⁺ channel blocker, verapamil.

Familial dilated cardiomyopathy (DCM) is characterized by an increase in chamber size and a thinning of the chamber walls, resulting in volume overload and systolic dysfunction. Sun et al. were the first group to develop an iPSC-CM model of DCM, resulting from a mutation in the TNNT2 gene⁶⁸. Since then, two other models of DCM have been published. Siu et al. created hiPSC-CMs from patients with a mutation

in LMNA (lamin A/C)⁶⁹ while Tse et al. used whole exome sequencing to find a mutation in the desmin gene in patients with DCM⁶³ (**Fig. 1.2B**). In these reports, DCM iPSC-CMs exhibit a predictable increase in cell size and abnormal sarcomere structure and organization. Similar to the iPSC models of LQTS, successful derivation of iPSC-CMs from patients carrying structural gene mutations serves to highlight the power of such technologies to recreate the pathologic phenotype of the disease. Such work has now opened the door for further studies looking at possible mechanisms and, subsequently, the identification of more effective therapeutic targets.

1.6.3 Other Cardiomyopathies

A number of reports have presented the development of iPSC-based models for inherited diseases that do not traditionally fit as channelopathies or structural cardiomyopathies. Pompe disease is a result of a mutation in the GAA gene encoding for the lysosomal glycogen-degrading enzyme, acid alpha-glucosidase. As a result, myocytes have an accumulation of membrane-bound glycogen, increased cytoplasmic glycogen particles, mitochondrial aberrance, and progressive autophagic buildup⁷⁰. Huang et al. were able to establish an iPSC model of Pompe disease and produced “cardiomyocyte-like cells” (CMLCs). These Pompe-CMLCs exhibited increased glycogen content, and could be rescued by treatment with recombinant GAA or L-carnitine⁷⁰.

Freidreich's ataxia (FRDA) is a recessive neurodegenerative disease that is also associated with HCM, resulting from a mutation in the gene encoding frataxin (involved in mitochondrial function). Hick et al. used an iPSC model of FRDA to investigate both the neural and cardiac pathologies associated with Freidreich's ataxia⁷¹. Both the iPSC-

derived neurons and cardiomyocytes demonstrated signs of mitochondrial dysfunction, as measured via mitochondrial membrane potential and structural abnormalities. Barth syndrome is another inherited mitochondrial disorder, resulting from a mutation in the TAZ gene encoding for the protein tafazzin. Wang et al. were able to show that hiPSC-CMs with the TAZ mutation exhibit abnormal sarcomere structure and mitochondrial function, and were able to apply various cardiac tissue engineering approaches to characterize the cardiac dysfunction⁶⁴ (**Fig. 1.2C**).

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) has also been modeled using iPSC-CMs. ARVD/C is characterized by fatty infiltration (especially within the right ventricle) and is usually caused by mutations in desmosomal proteins⁷². As with many cardiomyopathies, ARVD/C is also associated with an elevated risk of arrhythmia. Induced PSC models of ARVD/C exhibit abnormal nuclear translocation of junction plakoglobin proteins (Pkg), as well as very low β -catenin activity compared with wild-type controls⁷³⁻⁷⁵. Mutant Pkp2 proteins in these cells fail to anchor Pkg to the sarcolemmal membrane, resulting in Pkg nuclear translocation and downregulation of β -catenin activity. Despite this clear *in vitro* phenotype, no exaggerated lipogenesis or apoptosis in H9 hESC- or mutant iPSC-CMs was found following 3 months in culture⁷⁴. The authors suggest that this observation is consistent with the delayed, adult-onset clinical course of ARVD/C. If accurate, this result serves to highlight the inadequacy of immature iPSC-derived cells for modeling late-onset disease states, and the need for suitable maturation strategies *in vitro*.

Finally, combination studies using iPSC-CMs for simultaneous disease modeling and drug screening have been performed. Sharma and colleagues infected

cardiomyocytes with Coxsackievirus B3 in order to induce a myocarditis phenotype for use as an antiviral drug screening platform⁷⁶. Through a combination of bioluminescence imaging, calcium imaging, cell metabolism and vitality assays, and gene expression studies, the authors were able to assess the effect of a number of compounds, including interferon- β 1, ribavirin, pyrrolidine dithiocarbamate, and fluoxetine, for their capacity to abrogate CVB3-Luc proliferation and influence cell function and survival in cultured hiPSC-CMs. Studies such as these demonstrate how drug screening and disease modeling applications for iPSC-CMs can be combined to further our understanding of cardiac pathologies and help identify potential drug candidates for improving patient well-being.

1.6.4 Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disease affecting ~1:3500 live born males. Frameshift mutations in the dystrophin gene result in the expression of a truncated, nonfunctional dystrophin protein. Without dystrophin, muscle cells are particularly prone to mechanical stress and rupture⁷⁷. Over time this results in muscle scarring and degeneration, eventually leading to premature death. With improved palliative treatment and prolonged lifespans, cardiomyopathy is becoming the leading cause of death in patients with DMD. While it is known that patients with DMD exhibit diastolic dysfunction and arrhythmias, the pathogenesis of this disease phenotype is not fully understood. The *mdx* mouse model of DMD has provided valuable insights into the disease but, paradoxically, *mdx* mice exhibit minimal cardiac dysfunction, limiting translational cardiac therapies⁷⁸. In fact, a recent clinical trial with sildenafil, which showed promise in alleviating cardiac dysfunction in the *mdx* mouse⁷⁹, exacerbated

cardiac symptoms in DMD patients and had to be terminated early⁸⁰. Therefore, the need for an accurate human cardiac model of dystrophin cardiomyopathies has never been greater. Guan et al. were able to develop a model of DMD from urine-derived iPSCs, and found that cardiomyocytes derived from these cells exhibited abnormal Ca^{2+} handling compared to controls, an increased sensitivity to hypotonic stress, and altered contractile mechanics⁶⁵ (**Fig. 1.2D,E**). Similarly, Lin and colleagues demonstrated that cardiomyocytes derived from DMD patient iPSCs exhibited characteristic dystrophin deficiency, as well as elevated levels of resting Ca^{2+} , mitochondrial damage, and cell apoptosis. Additionally, they demonstrated that treatment with the membrane sealant Poloxamer 188 significantly decreased resting cytosolic Ca^{2+} levels, repressed caspase-3 (CASP3) activation and consequently suppressed apoptosis in these cells⁸¹.

1.7 Current Limitations of iPSC-CMs for Modeling Cardiomyopathy

While cells differentiated from pluripotent sources using the methods described so far possess a definite cardiac or cardiomyopathic phenotype, they typically exhibit immature structural and functional properties. This creates problems with regard to the use of these cells in clinically relevant settings (testing novel therapies or evaluating the efficacy and/or toxicity of new drugs), since cell therapy, cardiotoxicity screening, and human heart disease models depend on the cells' ability to recapitulate the properties of their adult *in vivo* counterparts accurately⁸². As such, various methods have been investigated as means to improve cardiomyocyte maturation *in vitro*. For example, a recent study showed that treating human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) with the growth hormone tri-iodo-L-thyronine (T3) increased maturation in terms of contractile output and structural development⁸³.

However, it has been suggested that this approach is somewhat limited in practice, since the common cardiac medium supplement, B27, contains T3 anyway⁸⁴. Promoting complete cardiomyocyte maturation from stem cell sources has yet to be achieved, but is likely to require combinatorial approaches mimicking various aspects of the native myocardial niche.

Despite recent advances in the derivation of iPSC-CMs, the lack of cell and tissue maturity, as well as low induction efficiencies, remain significant hurdles to the more widespread application of these cells in preclinical and potential clinical applications. To that end, methods to engineer more accurate and biomimetic cardiac niches *in vitro* to improve iPSC-CM development and maturation have become a focal point of many bioengineering research programs worldwide. Currently, the most advanced systems rely on the application of complex bioengineering strategies⁸⁵, which are discussed in more detail in the next section. Examples of improved drug screening outcomes or more accurate modeling of disease phenotypes in response to specific bioengineering solutions are provided.

1.8 Biomimetic Strategies for Human Cardiac Tissue Engineering

The central tenet of tissue engineering is that by generating an environment that more closely recapitulates the *in vivo* niche, it is possible to develop *in vitro* culture systems that promote the physiological and functional maturation of cells in a more biomimetic manner. Previous work has shown that long-term culture of cardiomyocytes leads to the development of phenotypically mature cells with more adult-like structural properties, gene expression profiles, and functional characteristics⁸⁶. This is not surprising given the time scale of cardiac development *in vivo*. However, these timescales are

impractical for high throughput screening technologies or evaluation of patient responses to specific drugs/therapies, where the shortest time frame possible is desired. In terms of promoting cardiac maturation *in vitro*, it is likely that no perfect surrogate for a six month to 1-year culture period will be found. Nevertheless, bioengineering techniques can be applied to manipulate cultured cells and enhance development as much as possible. Integration of such advanced culture systems with methods to evaluate functional performance enables the development of platforms capable of providing predictive data on human tissue responses to chemical, mechanical, or pathological challenges. Such technology holds huge potential for advancing preclinical drug screening and disease modeling applications⁸⁷. The validation of biomimetic human tissue models for predictive mechanistic studies will enable movement away from flat tissue culture plastic-based models (which may offer inadequate representations of the native biological environment) and animal models (which can produce data that often does not translate adequately to human studies). Below, various methods for improving the maturation or physiological accuracy of engineered cardiac tissues are presented and examples of the use of these systems for enhancing preclinical screens are provided.

1.8.1 Substrate Topography and Stiffness

Engineering cardiac tissues for *in vitro* modeling purposes requires overcoming a plethora of hurdles, such as cell selection, cell localization, phenotypic maturation, regulation of endogenous regeneration, development of electromechanical function, and optimization of mechanical properties⁸⁸⁻⁹². Native cardiac tissue consists of arrays of cells aligned in parallel to facilitate directionally controlled macroscopic contraction⁹³⁻⁹⁵,

whereas conventional *in vitro* culture platforms lack any form of anisotropic signal, leading to random alignment of the developing cells⁹⁶. Therefore, in order to promote correct physiological and functional development, cardiac engineering strategies must promote the uniaxial alignment of the cultured cells to mimic native tissue structure⁹³⁻⁹⁵, and enable the subsequent maturation of cardiomyocytes toward an adult phenotype⁹⁶.

To improve the performance of cultured iPSC-CMs, investigators make use of stem cells' ability to sense and respond to exogenous microenvironmental stimuli. These signals can influence cell fate, proliferation, and differentiation. It has been suggested that cell adhesion proteins (including integrins, cadherins, and non-muscle myosin II) play a pivotal role in sensing other cells as well as properties of the extracellular matrix (ECM) such as elasticity, porosity, and topography⁹⁷. In response, cells conform to the microenvironment by modulating intracellular mechanics⁹⁸, reorganizing cytoskeletal architecture, and inducing changes in transcriptional regulation^{99,100}. Knowing that stem cells sense and respond to their environment, incorporation of biomimetic substrates and exposure to correct physiological stimuli has been shown by many to be able to improve the differentiation efficiency of iPSCs down the cardiac lineage, and the subsequent maturation of the developed cardiomyocytes toward an adult phenotype.

One example of an attempt to mimic the native myocardial niche *in vitro* is the utilization of biomimetic nano-structured surfaces to improve cell adhesion, proliferation, and migration, as well as cardiomyogenic differentiation and structural development^{101,102}. Parallel nano-grooves and nano-ridges help maintain the cell polarity and alignment important for organ development by providing contact guidance

which influences the organization of microtubules, focal contacts, and actin filaments in parallel with the underlying topography¹⁰³. Investigations with cardiomyocytes on dynamically shifting topographies demonstrate the capacity for such cells to reorient themselves in real-time, according to changes in substrate cues, mirroring cells' capacity to remodel *in vivo* in response to damage or alterations in mechanical strain¹⁰⁴. Similarly, cells cultured on ECM fibers and microgrooves aligned with electrical stimulation patterns have also shown increased elongation, alignment, and electrical functionality^{103,105} (**Fig. 1.3A–C**). In addition to improving cellular alignment, nanotopographic cues that match the dimensions of native ECM fibers have been shown to promote substantial improvements in cardiomyocyte maturation, leading to more adult-like cellular morphologies and functional parameters^{103,105,106}. This observation lends credence to the theory that accurate recapitulation of the native cellular niche, in this case through recreation of ECM alignment and fiber dimensions, promotes the development of seeded cells toward a more mature phenotype¹⁰³.

While a culture substrate's anisotropic properties can have a profound impact on cellular alignment and maturation, its rigidity can also influence cell shape, which is crucial for modulating cell survival and differentiation¹⁰⁷. It has been shown that substrate rigidities matching the estimated physiological range of the native heart tissue ECM can control the proliferation, differentiation, and morphogenesis of endothelial cells through endogenous regulation of the activating protein p190RhoGAP¹⁰⁸. Similarly, studies have demonstrated that isolated embryonic cardiomyocytes cultured on matrices that mimic the elasticity of the developing myocardial microenvironment are optimal for transmitting contractile work to the matrix and for promoting striation

development and contractile function¹⁰⁹. The cited study also highlighted that matrices with mechanical properties representative of a post-infarct fibrotic scar tissue causes cells to overstrain themselves, leading to a lack of striated myofibrils and the cessation of contraction over time. Data such as these demonstrate how modulation of substrate stiffness can be used to recreate pathological phenotypes in cardiac cells for downstream applications. Furthermore, dynamic hydrogels with time-dependent stiffening properties support greater cardiac progenitor cell differentiation and maturation down the cardiac lineage compared to cells maintained in static hydrogels lacking specific biomechanical cues¹¹⁰.

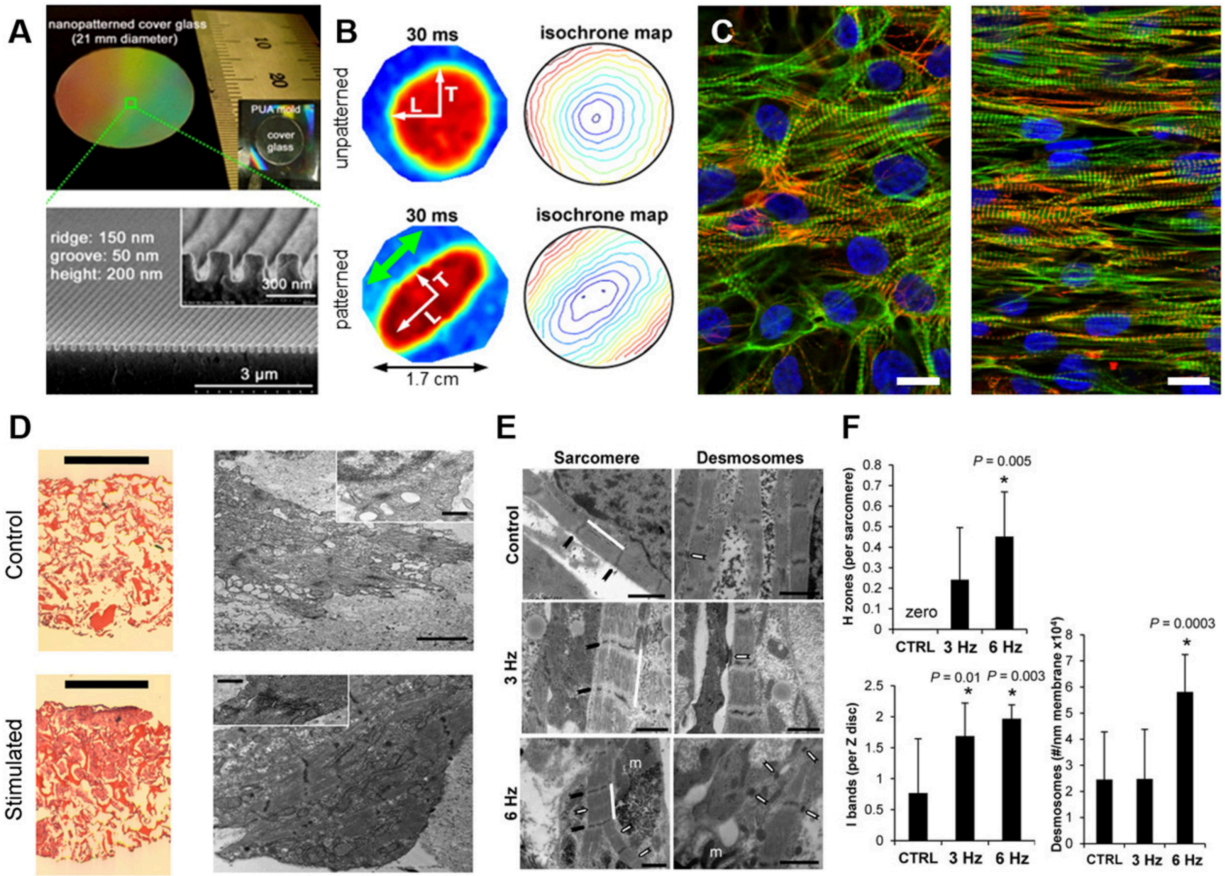


Figure 1.3. Bioengineering of the myocardial niche. (A). Low magnification image of a glass coverslip patterned with nanogrooves (top). Cross-sectional scanning electron microscopy (SEM) of the same surface illustrating the nanopatterned substratum (bottom). Reprinted with permission¹⁰³. (B). Optical mapping data show anisotropic propagation of action potentials in monolayers cultured on nanofabricated substrata, and isotropic propagation in cells cultured on flat surfaces. Point stimulation at 3 Hz was applied to the center of the cardiac monolayers as indicated by white arrows at 0 ms. The green arrow indicates the alignment of cardiomyocytes on the patterned substrates. Reprinted with permission¹⁰³. (C). Human stem cell-derived cardiomyocytes on flat (left), and nanopatterned (right) substrates highlighting the improvement in structural alignment in cells maintained on suitable topographies. Cells were stained for actin (green), actinin (red), and nuclei (blue). Scale bars: 50 μ m. Reprinted with permission¹¹¹. (D). Cardiac constructs maintained without (control), and with (stimulated) electrical stimulation. (Left) Hematoxylin and Eosin (H&E) staining of unstimulated engineered tissue constructs and constructs stimulated with monophasic square wave pulses of 3 V amplitude, 3 Hz frequency and 2 ms duration. Scale bar = 1 mm. (Right) Transmission electron microscopy images of the tissues presented in the H&E images, with insets of sarcomeres. Scale bar = 2 μ m in main image, 500 nm in inset. Reprinted with permission¹¹². (E). Ultrastructural analysis of engineered cardiac biowires highlights that electrical stimulation at 6 Hz induces cardiomyocyte self-organization. Representative images of non-stimulated (control), electrically stimulated biowires illustrate sarcomere structure (Sarcomere panel: white bar; Z disks, black arrow; H zones, white arrows; m, mitochondria), and presence of desmosomes (Desmosomes panel, white arrows). Scale bar = 1 μ m. Reprinted with permission¹¹³. (F). Morphometric analysis (average $\pm$ s.d.) showing ratio of H zones to sarcomeres (CTRL vs. 6 Hz, $P = 0.005$), ratio of I bands to Z disks (CTRL vs. 3 Hz, $P = 0.01$; CTRL vs. 6 Hz, $P = 0.003$), and number of desmosomes per membrane length (CTRL vs. 6 Hz, $P = 0.0003$). In normal adult cells, the ratio of H zones to sarcomeres is 1 and the ratio of I bands to Z disks is 2. Reprinted with permission¹¹³.

1.8.2 3D Tissue Culture

An important factor to consider when attempting to faithfully recreate the myocardial niche, is the inclusion of a 3D culture environment to more closely mimic cell-cell and cell matrix mechanics and structural organization. The majority of novel platforms designed to mimic the three-dimensional structure of the mammalian myocardium rely on manipulation of mechanical signals to facilitate the organization and anisotropic alignment of cultured cardiomyocytes within an exogenous matrix. Typically, cells are suspended in a scaffold matrix cultured within a platform that infers directional and structural parameters upon the developing tissue¹¹⁴⁻¹¹⁷. Cardiomyocytes have repeatedly demonstrated the capacity to respond to these physical stimuli, and orient themselves along the lines of principal strain within such cultures.

Scaffolds can be engineered from polymeric materials of synthetic origin, which include polylactide (PLA), polyglycolide (PGA), lactide and glycolide copolymer (PLGA), poly(ϵ -caprolactone) (PCL), and poly(N-isopropylacrylamide) (PNIPAAm), as well as combinations of these polymers¹¹⁸. Alternatively, they can be engineered from natural polymers and polymer combinations, such as collagen, gelatin, Matrigel, cellulose, chitosan, hyaluronic acid, or silk fibroin. Examples of synthetic, three-dimensional cardiac tissues include disk-shaped PLGA constructs, honeycomb scaffolds, and electrospun matrices, while examples of natural constructs include hydrogel scaffolds (circular hydrogel molds mechanically and electrically stimulated), and decellularized native tissue^{90,119} (**Table 1.1**). The use of both biological and synthetic scaffolds have been extensively employed in various cardiac engineering strategies, and each possesses inherent advantages and disadvantages¹²⁰⁻¹²⁴. Synthetic scaffolds are more

controllable and reproducible, and often exhibit stronger mechanical properties, which can be advantageous *in vivo* for repairing damaged tissue. Biological scaffolds offer a more accurate representation of the native cardiac ECM. They therefore potentially enable activation of cell signaling pathways to aid cellular development through presentation of antigens recognized by specific cell surface receptors. More in depth discussions of the benefits and weaknesses of different scaffold techniques are discussed elsewhere in detail^{121,124}. Due to the wide array of scaffolding approaches investigated so far (each with inherent advantages and disadvantages), a single “optimal” scaffold has yet to be established, highlighting that there currently remains substantial room for further development. Nevertheless, 3D cardiac drug screens with moderate throughput^{125,126} are currently in development and represent the means to reliably quantify contractile force outputs in a manner not possible in 2D culture. Such platforms constitute a viable option for further refinement toward effective, next generation preclinical analysis platforms for evaluating new chemical entities.

Although strategies for generating aligned cardiac tissues *in vitro* typically rely on the application of an exogenous scaffold material, the development of detachable cell monolayers through manipulation of tunable stimuli-responsive polymers has recently led to the possibility of generating three-dimensional scaffold-free tissue constructs with physiologically relevant cell densities¹²⁷⁻¹²⁹. Detachment of these cell populations can be achieved through a controlled shift in an exogenous stimulus, such as temperature, and the subsequent stacking of cell monolayers represents a reliable method for generating cell dense 3D constructs for various *in vivo* and *in vitro* applications. Such tissue constructs do not require integration with scaffold materials, leading to greater cell-cell

contact and improved cellular communication. Furthermore, the integration of nanotopographic substrate cues and stimuli-responsive substrates has been shown to facilitate the production of cell dense, scaffold-free constructs with controllable cellular orientation and anisotropic contraction patterns¹²⁸. Oxygen diffusion gradients within such cell dense constructs limit the thicknesses that can be achieved using such methods. However, studies have shown that co-culture with endothelial cells can create vascular beds within 3D, cell sheet- based constructs, thereby enabling the synthesis of thicker cardiac tissues for downstream applications¹³⁰.

Cardiac spheroids represent an alternative method for generating scaffold-free 3D tissues, albeit without the structural alignment present in cell sheet engineering techniques. Recent work from Beauchamp and colleagues details methods for generating 3D spheroid cultures of cardiomyocytes derived from pluripotent stem cell sources¹³¹. These constructs are free of necrotic cores and exhibit spontaneous contractile activity, calcium transients, and sarcomeric development. Similar 3D spheroid microtissues have been used for studying cardiac disease development in Chagas patients¹³². Specifically, investigators used spheroid tissues of cardiac cells to investigate the matrix altering effects of cardiac exposure to 5% serum from patients with Chagas disease. Although not using iPSCs, this study demonstrated that exposure to trypanosome-infected blood caused an increase in cardiac ECM component expression in 3D cardiac cultures. Moreover, the data suggest that MMP-2 and MMP-9 are correlated with the cardiac spheroid remodeling induced by serum of patients with Chagas disease. Given the importance of ECM production for studying matrix remodeling in disease states, these results highlight the value of 3D culture systems for

investigating pathological mechanisms associated with ECM production and regulation.

Table 1.1. Examples of different scaffold types for cardiac regeneration

Categories	Example of scaffolds	References
2-Dimensional scaffolds	PDMS coated surfaces are imprinted with ECM proteins (laminin/fibronectin/gelatin) using micro-contact printing techniques to generate anisotropic cardiac tissues	133,134
3-Dimensional scaffolds	- Cells are cultured on temperature-sensitive polymer surfaces (which allow for detachment of intact cell sheets without using enzymes) to create scaffold-free “stackable” cell sheets - Nano-fibrous meshes synthesized using electrospinning methods are employed to create highly porous substrates - PLGA scaffolds coated with ECM proteins are seeded with cardiomyocytes and used to generate multilayered cardiomyocyte tissue	90,119,135-138
Hydrogel-based scaffolds	- Accordion-like honeycomb microstructures yield porous scaffolds with controllable stiffness and anisotropy - Cardiomyocytes encapsulated in ring-shaped hydrogels (mixture of collagen and Matrigel) are subjected to phasic mechanical stretch – driving cells toward highly differentiated and more mature states capable of increased force generation - Hydrogel-encapsulated cells are cultivated on porous collagen scaffolds and subjected to electrical stimulation to achieve synchronously contracting cardiac patches	112,139,140
Decellularized native tissue	- Tissues are decellularized and used as scaffolds, leaving extracellular matrix with preserved composition, architecture, and mechanical properties for repopulation of cardiac stem cells - Decellularized ECM can also be disintegrated and formed into ECM-based hydrogels that are then used as scaffolds for cardiac engineering applications	141,142

1.8.3 Electromechanical Conditioning

Given the electrically and mechanically active nature of cardiac tissue, perhaps the most important factors to consider when seeking to develop biomimetic functional profiles in engineered myocardial tissue are physiological representations of mechanical strain and electrical activation¹⁴³. Studies have demonstrated that prolonged exposure of engineered cardiac tissue to continuous pacing promotes improvements in contractile force production, sarcomere structural development (**Fig. 1.3D**), and Ca^{2+} responsiveness, as well as a reduction in spontaneous beating activity^{112,144-148}. Furthermore, treatment of constructs with a combination of electrical pacing and insulin-like growth factor 1 (IGF-1) supplementation has been shown to further improve the contractile properties and connexin-43 expression in engineered heart constructs over and above levels observed in response to either factor applied independently¹⁴⁹. The integration of three-dimensional cardiac biowires (derived from pluripotent stem cell sources) with long-term stimulation protocols for up to 4 weeks *in vitro* promotes improvements in sarcomeric banding (**Fig. 1.3E,F**), excitation thresholds, and conduction velocities^{113,150}. Cells from these preparations also exhibit larger hERG currents, more negative resting membrane potentials, and larger capacitances (suggesting larger cells) than unstimulated controls, further indicating the improved functionality of cardiomyocytes exposed to long-term electrical stimulation¹¹³.

Similarly, studies of engineered cardiac construct responses to mechanical conditioning have indicated that application of uniaxial cyclic strain regimes promote physiological hypertrophy and cardiomyocyte proliferation above levels achievable in unstrained constructs¹⁵¹. Additionally, work with primary cells has also indicated that

cyclic stretch promotes cardiomyocyte proliferation through activation of p38-mitogen-activated protein kinase¹⁵².

Given the apparent importance of mechanical strain in promoting cardiomyocyte development, it is not surprising that modulation of this stimuli can have a negative impact on cardiac development *in vitro*. McCain et al. designed and built a system to mimic mechanical overload in cultured cardiomyocytes by applying cyclic stretch to engineered laminar ventricular tissue on a stretchable chip¹⁵³. The authors showed that the gene expression profiles activated in response to cyclic stretch were characteristic of pathological remodeling, and included a decrease in α - to β -myosin heavy chain ratios, as well as maladaptive changes to myocyte shape and sarcomere alignment. Examined calcium transients were found to resemble those reported in failing myocytes and peak systolic stress was significantly reduced. As such, the presented data demonstrate how application of pathologically relevant stretch regimens can create tissues with disease relevant phenotypes.

While electrical pacing and mechanical stretch have been shown to have profound impacts on cardiac development in isolation, exposure of engineered cardiac tissues to combined electrical and mechanical stimuli can be used to further improve structural and functional maturation^{154,155}. In such protocols, application of the electrical stimulus was timed to occur after the beginning of mechanical stimulation in order to mimic the biophysical environment present during isovolumetric contraction. Specifically, application of combined electromechanical stimuli was found to improve construct function by promoting the expression of proteins responsible for cell-cell communication and contractility, namely SERCA2a, troponin T, and Akt¹⁵⁵. These data

serve to highlight the significant effect electrical and mechanical stimulation can have on developing engineered cardiac constructs. Furthermore, they demonstrate that combinatorial stimulation programs can have further additive effects on cardiomyocyte maturation, highlighting the need for a multi-stimulus approach when seeking to model adult myocardial tissue effectively.

1.8.4 Biochemical Modulation of Cardiac Phenotype

In addition to recreation of the physical myocardial niche, biomolecular and biophysical studies have looked at small molecule treatment^{83,156} to further improve the phenotype of cultured cardiomyocytes. Using such methods, researchers have shown that treatment of engineered cardiac tissues with either insulin-like growth factor 1 or the β 2 agonist clenbuterol led to significant increases in contractile force production in a time dependent manner¹⁵⁶. In addition, multiple adrenergic receptor agonists have long been known to enhance hypertrophy of cultured cardiac cells^{157,158}. Although more typically associated with models of pathological hypertrophy, the possibility of optimizing treatment with such factors to bring about the formation of a non-pathological hypertrophied phenotype is an exciting potential avenue of research.

Treatment of stem cell-derived cardiomyocytes with the thyroid hormone T3 has been shown to upregulate markers of the cardiac contractile apparatus, correlating with improved force production and calcium handling in these cells⁸³. Such treatment mimics the spike in thyroid hormone release after birth, which has been linked to correct cardiac development in humans. As such, this treatment offers an excellent example of mimicking cardiac developmental cues *in vitro* to bring about the establishment of a more mature cardiac state.

Lastly, microRNA modulation has been shown to modulate cardiomyocyte maturation. Specifically, miRNA-499 overexpression in embryonic stem cell-derived cardiovascular progenitors promotes a significant increase in the yield of ventricular cardiomyocytes, and an increase in contractile protein expression¹⁵⁹. By contrast, miRNA-1 transduction does not alter cardiomyocyte yield but shortens action potential duration and promotes the development of a hyperpolarized resting membrane potential as signs of functional maturation¹⁵⁹. Also, miRNA-1 but not -499 has been shown to augment immature Ca^{2+} transient amplitudes and kinetics¹⁵⁹. Similarly, miR208 has been highlighted as a potential candidate that also may promote cardiac maturation, due to its involvement in thyroid hormone responsiveness and the myosin isoform switch^{160,161}.

More recently, the Let-7 family of miRNAs was found to be the most highly upregulated miRNA during cardiac maturation *in vitro*. Based on this observation, overexpression of Let-7 family members in human ESC-derived cardiomyocytes leads to enhanced cell size, sarcomere length, force of contraction, and respiratory capacity¹⁶². The authors of this study used large-scale expression data, target analysis, and metabolic flux assays to suggest that Let-7-driven cardiomyocyte maturation could be a result of down-regulation of the phosphoinositide 3 kinase (PI3K)/AKT protein kinase/insulin pathway and an up-regulation of fatty acid metabolism.

Although miRNAs can be used to promote cardiac development, they also hold the potential to recreate cardiomyopathic phenotypes. While forced overexpression of stress-inducible miRNAs is sufficient to induce hypertrophy in cultured cardiomyocytes, overexpression of miR-195, which is up-regulated during cardiac hypertrophy, results in

pathological cardiac growth and heart failure in transgenic mice¹⁶³. Although yet to be applied in iPSC cultures, such methods constitute a viable option for future studies seeking to use iPSC-CMs to model pathological cardiac development.

1.8.5 Multiple Cell Type Culture

Although the majority of engineered cardiac tissues focus on the form and function of cardiomyocytes, accurate recapitulation of the myocardial niche requires integration of multiple cell types, including cardiac fibroblasts and endothelial cells. Additionally, the integration of supporting vascular cell types in 3D engineered cardiac tissues would enable to researchers to model cardiac vascularization in order to study coronary disease and infarction *in vitro*¹⁶⁴. Given the prevalence of heart disease in the world today, such models would likely yield data that would be of tremendous value to modern healthcare professionals and would offer a useful *in vitro* model for the high throughput evaluation of novel heart disease therapeutics.

Co-culture of engineered cardiac tissues with endothelial cells has been shown to improve production of vessel forming cytokines and can lead to the generation of capillary networks capable of linking engineered tissues to the host vasculature during *in vivo* studies^{130,165}. In addition, data has been published suggesting that maintaining co-cultures of endothelial cells and cardiomyocytes can induce improvements in cardiomyocyte viability and function¹⁶⁶. Electrophysiological studies of cardiomyocyte and cardiac fibroblast co-cultures suggest that the development of mechanical interactions between these cell types during cardiac injury may act to impair cardiac conduction through increased activation of mechanosensitive channels in response to tension applied to the myocyte by the myofibroblast¹⁶⁷. Such studies demonstrate how

interplay between different myocardial cell types can lead to significant changes in overall function in engineered tissues. Moreover, the presented data illustrate the importance of such interactions when seeking to understand changes in performance in disease states and ischemic tissues.

In addition to co-culture with supporting myocardial cell types, co-culture of iPSCs with primary cardiac cells has also been shown to increase yields of iPSC-CMs¹⁶⁸. Such data suggests cardiac cells may help modify their *in vitro* niche to promote the development of an environment better suited to promoting cardiac differentiation.

1.9 Instrumented Cardiac Functional Assays

Once recapitulation of adult tissue structure and function is achieved, measurement of functional performance is a further important consideration if the system is to be effectively utilized in a broad spectrum of studies. Functional performance should be quantifiable in order to enable comparison to the output of the native tissue, and to validate the improvement of the engineered construct over conventional cell culture methods. In the case of cardiac muscle, this means the developed system must possess the means to measure contractile and/or electrophysiological performance. Desire to accurately investigate functional outputs in engineered cardiac constructs has led to the development of novel cardiac biological microelectromechanical systems (BioMEMS) capable of monitoring these parameters in real-time.

The potentially fatal nature of channelopathies in human cardiac disease¹⁶⁹, coupled with the importance of assessing changes in ion channel performance as a means of predicting drug-induced arrhythmogenesis¹⁷⁰, has led to extensive

characterization of iPSC-CM electrophysiology *in vitro*. Patch clamp recordings offer the means to gain high-resolution data pertaining to the functional maturity of single cells, as well as a means to characterize cardiomyocyte subtype, response to stimuli, and pathology. However, such methods are invasive, extremely low throughput, and incapable of providing measurement of whole tissue function. In their place, cardiac screening technologies are increasingly focusing on the use of either microelectrode arrays (MEAs) or optical mapping techniques to assess the electrophysiological output of cultured cardiomyocytes. MEAs permit recording of electrical field potentials from cardiac monolayers similar to whole heart analysis by electrocardiography^{33,171,172}, whereas the use of either genetically encoded or exogenously applied voltage-sensitive dyes enable real-time monitoring of action potential propagation through cultured cells³². Ca^{2+} sensitive dyes have also been employed for analysis of cardiac function^{173,174}. However, it should be noted that Ca^{2+} dyes do not directly report electrophysiological endpoints, and are actually used as a surrogate for direct voltage measurements in most cases.

Using MEAs and optical mapping, estimations of cardiac conduction velocity, action potential duration, and beat frequency/regularity can be made, providing in depth analysis of the health and functional stability of engineered cardiac tissues. Through assessment of parameters such as these, investigators have demonstrated the capacity for *in vitro* models of human cardiac tissue to recapitulate physiological responses to cardiotoxic compounds^{33,172}. Specifically, the use of MEAs has been shown to be advantageous in correctly selecting out false positive and negative compounds as identified using more conventional ion channel studies. The ability for such screening

systems to provide more accurate appraisals of compound action *in vitro* holds promise for the use of these technologies for improving the efficacy of current preclinical screening protocols.

In addition to measurement of electrophysiological performance, assessment of contraction within *in vitro* platforms represents a vital secondary metric for accurately predicting whole heart function. The majority of engineered myocardial models measure contractile function either through direct measurement of force using a transducer¹⁷⁵, or through analysis of fixed post deflection^{126,176,177} or substrate deformation in response to cardiomyocyte contraction^{32,42}. Use of microscale cantilevers is becoming increasingly common as a means to monitor cardiomyocyte contractile properties reliably and reproducibly in a multiplexed, and therefore higher throughput, manner^{36,41,123,178,179} (**Fig. 1.1A**). Deflection recordings can be used to extrapolate measurements of contractile performance in such systems and so provide feedback on the functional development of the engineered tissue.

More recently, Pioner et al. presented the means to isolate individual myofibrils from hiPSC-CMs in order to assess their mechanical and kinetic properties¹⁸⁰. Moreover, the authors demonstrated the feasibility of using this system in conjunction with hiPSC-CMs to study the development of contractile abnormalities for disease-associated mutations in sarcomere proteins. Among other metrics, analysis revealed a marked impairment of maximal isometric tension generated by myofibrils from cells derived from patients carrying a MYH7 mutation. The collected data highlight the capacity for this novel functional assay to stratify disease phenotypes and potentially screen for novel therapeutics to alleviate symptoms associated with myofibrillar

mutations.

Although many of these *in vitro* cardiac assays focus on a single cell type, integration of heart-on-a-chip models with microfluidics^{181,182} has opened the possibility of linking multiple *in vitro* tissue models to create body-on-a-chip devices capable of modeling tissue-tissue crosstalk in response to drug treatment or specific pathologies¹⁸³⁻¹⁸⁵. The importance of cardiac tissue for accurate predictions of system toxicity makes integration of heart-on-a-chip models into such complex systems a vital component for effective modeling of whole-body responses to compound exposure¹⁸⁵. However, the ability for such complex systems to accurately model system responses to drug treatment has yet to be demonstrated effectively.

1.10 Unmet Needs and Future Perspectives

Although iPSCs are good candidates for drug screening and disease modeling applications, they are not without limitations. As detailed in this review, iPSC-CMs are developmentally immature, often making it difficult to relate data collected from such sources to that of their fully mature counterparts in the adult human heart. The late onset nature of many of the diseases discussed means that phenotypically immature iPSC-CMs likely fail to recapitulate disease development accurately, since they are incapable of reaching the stage of development where the disease typically manifests *in vivo*. Moreover, complex cellular interactions in human metabolic processes often cannot be recapitulated in these culture systems, not to mention the confounding factor of human genetic variation between cells of different donors. A large panel of patient-derived iPSCs needs to be assessed in order to take into account the range of responses that a given drug may elicit when applied to a heterogeneous human

population. Such a library could also be used to refine clinical trial designs by indicating whether a stratified subpopulation will respond uniformly to a specific drug¹⁸⁶.

For these reasons, and despite their substantial promise, patient-specific iPSC models have not yet replaced animal models and traditional cell line assays in the development of new drug compounds. The persistent issue with maturity and accurate recapitulation of adult-onset disease phenotypes has led to an explosion of bioengineering strategies to improve engineered cardiac tissue development *in vitro*. Such techniques seek to mimic multiple aspects of the cardiac microenvironmental niche, using manipulation of ECM alignment and topography, small molecule doping, and electromechanical stimulation to improve the differentiation, development, and maturation of cultured cardiomyocytes.

Looking to the future, integration of cardiomyocyte culture systems with other cell types to model cellular interactions within the heart as well as between organs will enable more precise comparisons with the *in vivo* condition to be made. Such research may lead to the establishment of new *in vitro* disease models for conditions that do not directly target cardiomyocytes. For example, co-culture of cardiomyocytes and patient fibroblasts within a suitable tissue engineered platform may allow researchers to model cardiac defects brought about by connective tissue disorders such as Marfan syndrome¹⁸⁷ and scleroderma¹⁸⁸. Although not a cardiac-specific condition, Marfan syndrome can cause defective aortic media, defective valve cusps, interatrial communication, and sunken chest (pectus excavatum)¹⁸⁹. The establishment of a suitable Marfan syndrome iPSC-based model of the myocardium would enable in-depth analysis of how connective tissue physiology contributes to the formation of

physiologically-correct cardiac structures during development. Regardless of the disease, improvements in our ability to model the intricate cell-cell and cell-matrix interactions that occur within the mature and developing myocardium will likely lead to more accurate and complex models of the human heart for numerous downstream applications.

Despite the wide array of iPSC-based cardiac disease models produced to date, recent improvements in gene editing technologies also hold promise for furthering our capacity to model cardiac disease effectively. The clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9 system uses short guide RNAs to drive CRISPR-associated endonucleases (Cas9) to a target locus for precision editing of genetic sequences¹⁹⁰. This revolutionary technology is more efficient, easier to use, and simpler to generate than earlier gene editing methods that require the synthesis of custom proteins for each DNA target. As such, CRISPR/Cas9 technology is now widely used to edit the genome of a wide variety of cell types, including iPSCs¹⁹¹. Recent work has demonstrated the feasibility of creating inducible gene knock out lines through a combination of CRISPR/Cas9 gene editing and Cre/LoxP or Flp/FRT recombination¹⁹². In doing so, the authors were able to create iPSC lines in which precise temporal control of SOX2, PAX6, OTX2, and AGO2 could be exerted. The potential for such technology to advance cardiac disease modeling is clear. Pluripotent cells could be driven down the cardiac lineage and matured before knocking out the gene of interest and observing the changing phenotype. Alternatively, knock out of the gene at an early stage would enable study of how expression of this gene influences cardiac development. Work with iPSC-CMs and skeletal muscle cells from DMD patients has shown that CRISPR also offers a

powerful tool for correcting deleterious mutations. Cardiomyocytes derived from DMD iPSCs were edited with CRISPR/Cas9 to restore the reading frame of dystrophin, leading to improved membrane integrity and restoration of the dystrophin glycoprotein complex¹⁹³. Combination of iPSC, gene editing, and combinatorial tissue engineering technologies to create more accurate models of cardiac disease is a daunting prospect. However, the successful combination of such strategies will have a dramatic effect on our capacity to generate meaning preclinical assessment of novel therapies. Furthermore, such model systems will simultaneously expand our capacity to study the mechanisms underlying disease onset and development accurately.

1.11 Dissertation Overview and Significance

The research presented in this dissertation sought to address the challenges of hPSC-CM maturation, cardiac disease modeling, and phenotypic drug screening as described throughout Chapter 1. Chapters 2 through 5 describe the rationale, scientific methods, and results of the following three specific aims:

Aim 1: Characterize a dystrophin-null human pluripotent stem cell-derived cardiomyocyte phenotype on biomimetic nanotopographic surfaces (Chapters 2 & 3).

Aim 2: Develop a combinatorial maturation protocol to accelerate the structural and functional development of hPSC-CMs (Chapter 4).

Aim 3: Establish a mature human cardiac tissue model of DMD cardiomyopathy for phenotypic drug screening (Chapter 5).

The widespread adoption of human induced pluripotent stem cell-derived cardiomyocytes in preclinical screening has the potential to make a significant contribution to the understanding of cardiac disease onset and progression and to accelerate the development of new drugs for the betterment of human health. The work presented in the following four chapters addresses major hurdles for adoption of such strategies. The results of Aim 1 provide insight into the role of extracellular matrix contact guidance cues on hPSC-CM structural development and serve to highlight the need for more holistic approaches to cardiac maturation and functional disease state stratification. In Aim 2 we develop a combinatorial strategy to advance the maturation of hPSC-CMs employing distinct biophysical, biochemical, and genetic cues. These three factors work in a synergistic fashion to improve the transcriptional, structural, electromechanical, and metabolic properties hPSC-CMs and limit the cell line variable response of single-factor maturation strategies. In Aim 3, applying our combinatorial maturation strategy to a dystrophin-null model of Duchenne Muscular Dystrophy, we found a distinct maturation-dependent electrophysiological disease phenotype that corresponds well with early disease presentation in patients. Utilizing our custom microelectrode array platform, we screened the ability of various classes of drugs to prevent the electrophysiological dysfunction and found that Ca^{2+} antagonists reduced diastolic Ca^{2+} overload and restored function to healthy baseline levels. This work represents seminal progress for the advancement of hiPSC-CMs towards preclinical disease modeling and drug screening applications.

Chapter 2. Anisotropic Nanopatterned Substrates Enhance the Structural Development of Human Pluripotent Stem Cell-Derived Cardiomyocytes

Parts of this chapter have been published in the following manuscripts:

- **Macadangdang J**, Lee HJ, Carson D, Jiao A, Fugate J, Pabon L, Regnier M, Murry C, Kim D-H. Capillary Force Lithography for Cardiac Tissue Engineering. *Journal of Visualized Experiments*. 2014; (88): 1–8.
- Pioner JM, Racca AW, Klaiman JM, Yang K-C, Guan X, Pabon L, Muskheli V, Zaunbrecher R, **Macadangdang J**, et al. Isolation and Mechanical Measurements of Myofibrils from Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *Stem Cell Reports*. 2016; 6(6): 885–896.

2.1 Abstract

Cardiovascular disease remains the leading cause of death worldwide¹⁹⁴. Cardiac tissue engineering holds much promise to deliver groundbreaking medical discoveries with the aims of developing functional tissues for cardiac regeneration as well as *in vitro* screening assays. However, the ability to create high-fidelity models of heart tissue has proven difficult. The heart's extracellular matrix (ECM) is a complex structure consisting of both biochemical and biomechanical signals ranging from the micro- to the nanometer scale¹⁰³. Local mechanical loading conditions and cell-ECM interactions have recently been recognized as vital components in cardiac tissue engineering^{133,151,195}.

A large portion of the cardiac ECM is composed of aligned collagen fibers with nano-scale diameters that significantly influences tissue architecture and electromechanical coupling¹⁰³. Unfortunately, few methods have been able to mimic the organization of ECM fibers down to the nanometer scale. Recent advancements in nanofabrication techniques, however, have enabled the design and fabrication of scalable scaffolds that mimic the *in vivo* structural and substrate stiffness cues of the ECM in the heart¹⁹⁶⁻¹⁹⁹.

Here we present the development of a reproducible, cost-effective, and scalable nanopatterning process for the functional alignment of cardiac cells using a polyurethane (PU) based polymer. These anisotropically nanofabricated substrata (ANFS) mimic the underlying ECM of well-organized, aligned tissues and can be used to investigate the role of nanotopography on cell morphology and function^{107,200-203}. Both primary and human pluripotent stem cell-derived cardiomyocytes align and elongate in the direction of the underlying nanotopography, resulting in more physiologic cell morphology and tissue structure. We found that ANFS with 800 nm dimensions were optimal for promoting the structural development of hPSC-CMs and resulted in faster Ca^{2+} influx during contractions. Finally, when cultured long-term on ANFS, hPSC-CMs displayed advanced structural development and maturation with the appearance of well-organized sarcomeres and transverse (T) tubule-like structures. Taken together, these data highlight the value of recapitulating *in vivo*-like microenvironments in the dish in order to advance the structure and function of hPSC-CMs.

2.2 Rationale

Cardiovascular disease is the leading cause of morbidity and mortality in the world and present a weighty socio-economic burden on an already strained global health system^{2,194}. Cardiac tissue engineering has two distinct goals: (1) to regenerate damaged myocardium after ischemic disease or cardiomyopathy or (2) to create a high fidelity model of the heart for *in vitro* drug screening or disease modeling.

The heart is a complex organ that must work constantly to supply blood to the body. Densely packed laminar structures of cardiomyocytes and supportive tissues are arranged in helical patterns throughout the heart wall^{93,204}. The heart is electromechanically coupled in a highly coordinated fashion via excitation contraction coupling (ECC)²⁰⁵ to efficiently eject blood to the body²⁰⁶. Several major hurdles remain to be addressed, however, before nature's intricate design can reliably be recapitulated *in vitro*. First, although robust cardiomyocyte differentiation methods continue to be developed²⁰⁷, hPSC-CMs still exhibit rather immature phenotypes. Their electromechanical properties and morphology most closely match fetal levels²⁰⁸. Second, when kept in traditional culture conditions, both stem cell-derived and primary cardiomyocytes fail to assemble into native, tissue-like structures. Rather, cells become randomly oriented and do not exhibit the banded rod-shaped appearance of adult myocardium²⁰⁹.

The extracellular matrix (ECM) microenvironment with which cells interact plays a significant role in numerous cellular processes^{107,202,210}. The ECM consists of complex, well-defined molecular and topographical cues that significantly influence the structure and function of cells^{105,196}. Within the heart, cellular alignment closely follows the

underlying nanometer scale ECM fibers¹⁰³. The impact of these nanotopographical cues on cell and tissue function, however, is far from completely understood. Preliminary studies of nanometer scale cell-biomaterial interaction indicate the potential importance and impact of sub-micron topographic cues for cell signaling²¹¹, adhesion²¹²⁻²¹⁴, growth²¹⁵, and differentiation^{216,217}. However, due to the difficulty in developing reproducible and scalable nanofabricated substrates, such studies could not reproduce the multi-scale cellular effects of the complex *in vivo* ECM microenvironment. Understanding how cardiomyocytes interact with the nanoscale ECM microenvironment could allow for the ability to control cellular behavior to more closely mimic native tissue function.

In this chapter, we design and optimize an anisotropic nanofabricated substratum (ANFS) for culture of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) via a straightforward and cost-effective capillary force lithography technique. We explore the role of nanotopography in hPSC-CM maturation and demonstrate that similar to primary cardiomyocytes, hPSC-CMs display a nanotopography size-dependent phenotype, with optimal structure and function on 800 nm ANFS. Finally, when cultured on ANFS long-term, hPSC-CMs develop advanced structural features such as densely packed, in-register sarcomeres and transverse (T) tubule-like structures. Such substrates allow for more detailed studies of myofibril contraction/relaxation dynamics and ECC¹⁸⁰, complex tissue-level behavior in a two-dimensional platform^{103,218-220}, and could be used to improve cellular graft function when implanted into the heart for regenerative purposes⁹⁶.

2.3 Scientific Methods

* Note: All procedures are conducted at room temperature (~23 °C) unless otherwise noted.

2.3.1 Fabrication of Silicon Master

- 1.1.) Clean silicon wafer with 100% ethanol or xylene and dry under O₂/N₂ gas.
- 1.2.) Place silicon wafer in spin-coater at rotation speeds of 2000-4000 rpm to produce a 0.3-0.5 µm thick film.
- 1.3.) Pattern the photoresist film with the correct dimensions by using a photolithography system
- 1.4.) Fully immerse the patterned photoresist-coated silicon wafers in an appropriate volume of photoresist developing solution.
- 1.5.) Rinse the developed photoresist-coated silicon wafers with deionized water.
- 1.6.) To form arrays of sub-micron scale ridges with near vertical side walls, deep reactive ion etch the exposed silicon using an etching system.
- 1.7.) Remove the remaining photoresist by placing the silicon wafer in a plasma asher system.
- 1.8.) Cut the silicon wafers with a diamond-tipped cutter into the appropriately sized silicon masters for subsequent replica molding.

2.3.2 Fabrication of PUA Mold from Silicon Master

* Note: Volume to be added to silicon master for nanofabrication will vary depending on the area of the nanopatterned master to be replicated as well as the viscosity of the polyurethane acrylate (PUA) solution.

- 2.1.) Clean silicon master surface with 100% ethanol or xylene and dry under O₂/N₂ gas.
- 2.2.) Place silicon master pattern side up in a petri dish.
- 2.3.) For a silicon master with a 2 cm x 2 cm surface pattern, pipette 40 μL of PUA to the pattern surface.
- 2.4.) Place a sheet of 4 cm x 4 cm transparent polyester (PET) film over the dispensed PUA.
- 2.5.) Press down on the PET sheet and spread the PUA underneath the sheet across the pattern face using a roller or flat edged surface (such as a card) so that the entire pattern is covered by the PUA prepolymer.
- 2.6.) Place silicon master, prepolymer, and PET approximately 10cm below a 20 Watt (115V) UV light ($\lambda = 365$ nm) for 50 seconds. To be effective, the UV light wavelength can be anywhere between 310-400 nm. The intensity of the light is 10-15mW/cm² at the surface of the substrate.
- 2.7.) After curing, remove PET film slowly with forceps. PUA should attach to the PET film with a negative of the silicon master nanopattern.
- 2.8.) Cure PUA/PET nanopatterns under UV for at least 12 hours prior to use. Overexposure is not an issue.
- 2.9.) To clean silicon masters, place another film of PET on top of the master without the addition of PUA and expose to UV light ($\lambda = 365$ nm) for 50 seconds and remove PET film. This will remove any unreacted monomers.
- 2.10.) Rinse silicon master with 100% ethanol or xylene and dry under O₂/N₂ gas.

2.3.3 Nanopatterning Polyurethane Polymer

3.1.) Prepare 25 mm diameter circular glass slides by placing in an ozone treatment chamber for 10 minutes.

3.2.) Place ozone-treated glass slides onto small PDMS block for easy handling.

3.3.) Apply thin layer of surface adhesion promoter with paintbrush to glass slides. Air dry glass slides for 30 minutes.

3.4.) Place the glass slide on a piece of printer paper.

3.5.) Drop dispense 10 μ l of polyurethane (PU) pre-polymer (NOA 76) to center of glass slide. Make sure no bubbles are present after addition.

3.6.) Place PUA mold, pattern face down, onto the glass slide. Disperse the PU uniformly across the surface of the glass slide by rolling a rubber cylinder roller along the PUA mold. Printer paper will absorb polymer overflow.

3.7.) UV-cure for 60 seconds under 20 Watt UV lamp. Polymerization time of the PU is dependent on power of UV source.

3.8.) Remove the sample from UV light source and carefully peel the PUA mold from PU coated glass slide. Polymerization of the PU is considered complete when the PUA mold peels cleanly away from the sample and the PU glass slide has an iridescent appearance.

3.9.) Place finished samples in desiccator for storage for as long as a month.

2.3.4 Cell Seeding and Culture

* This protocol describes the culture of neonatal rat ventricular myocytes (NRVMs) and H7 human embryonic stem cell-derived cardiomyocytes (hESC-CMs) but other cell sources may be used.

4.1.) Attach the ANFS coverslips (PU or PLGA) to a 35 mm tissue culture polystyrene dish. Pipette 20 μ L of Norland Optical Adhesive (NOA83H) to the bottom of the dish and gently place the ANFS coverslip on top of the NOA. Allow glue to spread out and cover entire coverslip bottom. Cure NOA by exposing dish to UV for 10 min.

4.2.) Sterilize ANFS by rinsing with 2 mL of 70% aqueous ethanol solution for 5 min, twice. Remove ethanol by aspiration. Allow ANFS to completely air dry for ~1 hr under the UV sterilization lamp (λ = 200-290 nm) in the biological safety cabinet.

4.3.) Cellular adhesion is enhanced by coating the ANFS in fibronectin overnight. Dilute fibronectin in DI water to 5 μ g/mL. Pipette 2 mL of fibronectin solution into dish. Place in incubator at 37 °C and 5% CO₂ overnight (at least 6 hrs).

4.4.) Obtain NRVMs, hESC-CMs, or other cardiac cells of interest according to previous protocols²⁰⁷.

4.5.) Centrifuge cell sample at 1,000 rpm for 3 min to pellet the cells.

4.6.) Carefully remove the supernatant by aspiration. Make sure not to disturb the pellet.

4.7.) Resuspend cells in appropriate culture media to a concentration of 4.6×10^6 cells/mL.

4.8.) Carefully pipette 200 μ L of cell suspension onto sterilized ANFS. Make sure cell suspension remains on the coverslip.

4.9.) Place cells in incubator at 37 °C and 5% CO₂ for 4 hrs to allow cells to attach to ANFS.

4.10.) Add 2 mL of warm culture media to dish and replace cells in incubator under the same conditions.

4.11.) After 24 hrs, remove media and wash with 2 mL of DPBS twice to remove excess cells.

4.12.) Add 2 mL of warm culture media to dish and replace cells in incubator under the same conditions. Culture the cells to confluence. Replace media every other day.

2.3.5 *Ca²⁺ Imaging*

Calcium transients were recorded as previous published²²¹. Briefly, cells were loaded with 5 μM Fluo-4 AM (ThermoFisher) in Tyrode's buffer for 20 minutes at 37°C. Fluo-4 containing Tyrode's was then removed and the cells were washed with Tyrode's for 10 minutes. Cells were then brought to the confocal microscope at 23°C. Cells were field stimulated at 1 Hz using a MyoPacer stimulator (IonOptix) and platinum wire electrodes. Using the 40x air objective, confocal line scans (1.54 ms per line) were recorded on rhythmically beating cells for 10 seconds. MATLAB was then used to analyze Ca²⁺ transient amplitude and kinetics (upstroke velocity, decay constant, and relaxation times).

2.3.6 *Quantitative real-time PCR*

High quality total RNA was isolated using the RNeasy Miniprep Kit (Qiagen). cDNA was reverse transcribed using the Superscript III first-strand cDNA synthesis kit (Invitrogen). Primers for KCNJ2, cTnT, and HCN4 were purchased from BioRad and qPCR was

performed using SYBR green chemistry and an ABI 7900HT instrument. Samples were normalized to GAPDH as a housekeeping gene.

2.3.7 Immunohistochemistry

Cells were fixed in 4% paraformaldehyde for 15 minutes and blocked with 5% goat serum in PBS for 1 hour at room temperature. Cells were then incubated with mouse anti-a-sarcomeric actinin monoclonal antibody (1:500, Sigma-Aldrich) diluted in 1% goat serum in PBS overnight at 4°C. The next day, cells were washed with PBS followed by staining with Alexa-594 conjugated goat-anti-mouse secondary antibody (1:200, Invitrogen) and Alexafluor 488 phalloidin (1:200, Invitrogen). Counterstaining was performed with Vectashield containing DAPI (Vector Labs). Images were captured with a Nikon A1R confocal microscope using a Nikon CFI Plan Apo VC 60X water immersion objective.

For caveolin-3 (CAV3), cells were incubated with 1.5% normal goat serum before labeling with anti-CAV3 primary antibody 1:500 (AV09021, Sigma) and the day after with Alexa Fluor 488 goat anti-rabbit 1:200 (A-11008, Life Technologies). Each staining with secondary antibody was incubated for 1 hour and later PBS washed prior to nuclei staining with DAPI (Vectashield). Nanopatterned coverslips were then flipped over a normal microscope slide for confocal images (Nikon A1R confocal microscope).

2.3.8 Morphological Analysis

Images captured by confocal microscope were analyzed for average cell area, cell perimeter and sarcomere length (SL) in Fiji (ImageJ) Software using standard analysis plugin. Myofibril alignment resulted from Z-bands density and bi-dimensional

organization was analyzed using an algorithm based on the Fast Fourier Transform (FFT) similarly to previously described method²²². Single cells were discernible from each other for the quantification of myofibril alignment. The periodicity of Z-bands along the entire cell surface were reflected in the peak components of the power spectrum profile. The area of the first order of spatial frequency components centered at 0.55 ± 0.05 corresponding to the measured resting SL was normalized for the entire area under the power spectrum curve. The resulted value is as much greater as the myofibril alignment index of each single cell.

2.4 Results

2.4.1 Capillary Force Lithography Yields High Fidelity Anisotropic Nanopatterned Surfaces for Cell Culture

Figure 2.1 is a schematic overview of both UV-assisted and solvent-mediated capillary force lithography fabrication methods.

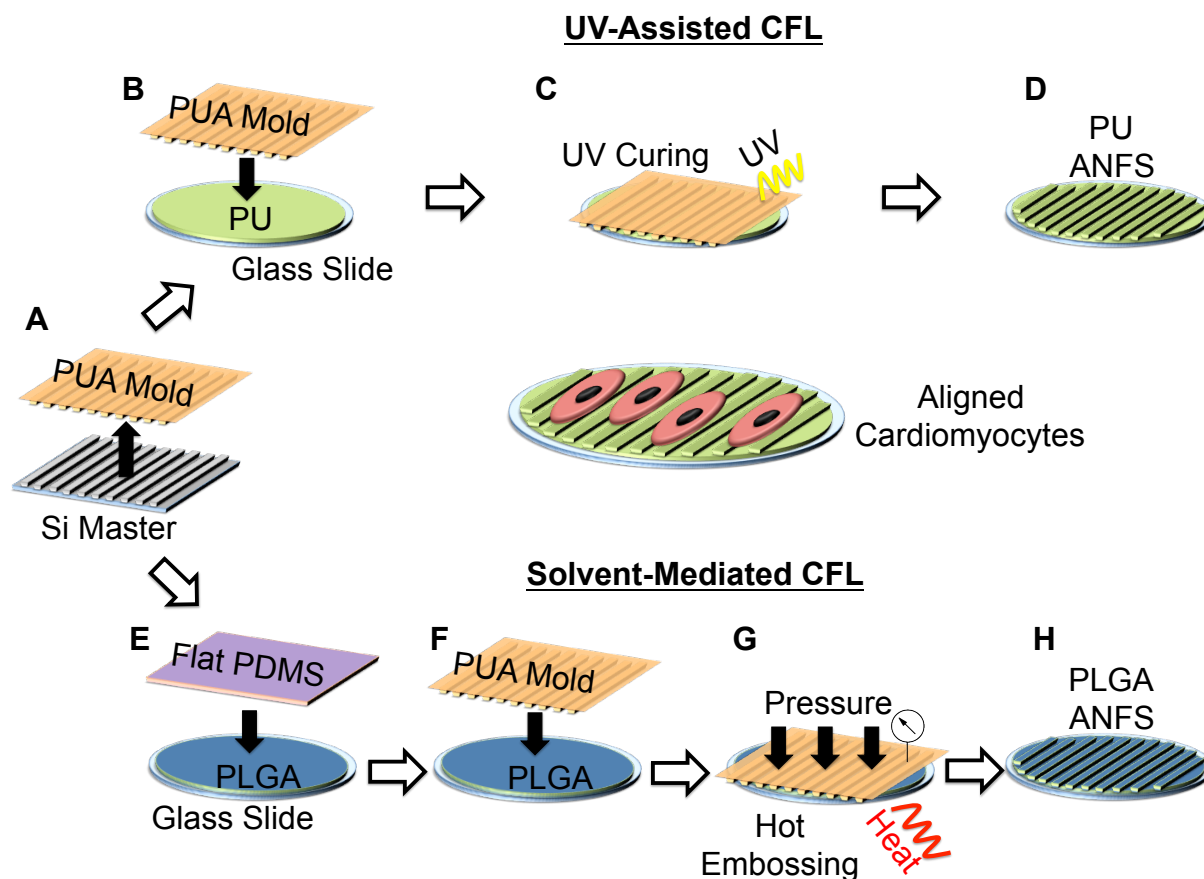


Figure 2.1. Nanofabrication schematic. Diagram of the two nanofabrication processes. (B-D) Depict UV-assisted capillary force lithography (CFL), while (E-H) depict solvent-mediated CFL. (A) PUA negative is made from a silicon master. (B) PU pre-polymer is drop-dispensed onto a glass slide and the PUA mold is placed on top. (C) A PUA mold and PU coverslip are then exposed to UV light to cure the PU. (D) PLGA polymer solution is drop-dispensed onto a glass slide and a flat PDMS mold is used to create a flat PLGA surface. (E) A PUA mold is placed on top of the flat PLGA and (F) constant heat and pressure are applied to hot-emboss the NP into the PLGA. (G) After peeling away the PUA mold, a NP-PU or PLGA substrate is left behind. Cardiac cells can then be seeded onto this NP surface to create aligned arrays of cells.

Due to the diffraction of light caused by the nanoscale topography, anisotropic nanopatterned surfaces, or simply nanopatterns (NPs), appear iridescent under white light. **Figure 2.2** depicts this iridescent surface on a well-patterned 25 mm NP-polyurethane coverslip (**Fig. 2.2A**) with 800 nm ridge and groove widths (**Fig. 2.2B**). Scanning electron micrographs reveal the NPs are highly ordered and the CFL methods faithfully reproduce the desired nanotopography with precision and fidelity over large surface areas.

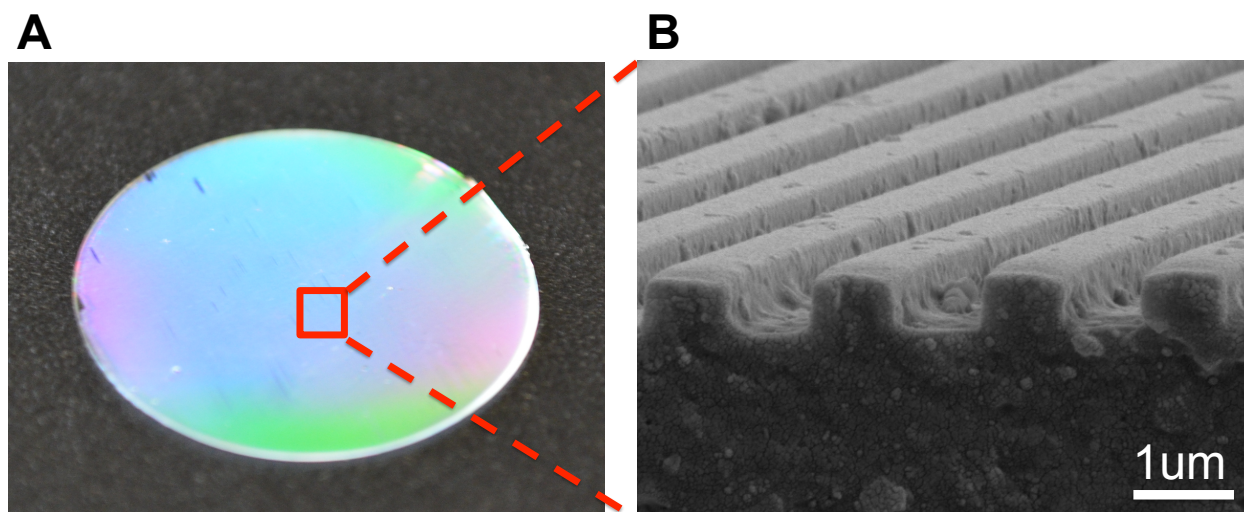


Figure 2.2. Nanopatterned PU Substrate. (A) Photograph of large area NP surface. The iridescent appearance of the coverslip is caused by the nanotopography. (B) SEM image of the cross-section of the underlying nanopography.

2.4.2 Anisotropic Nanopatterned Surfaces Promote Structural Anisotropy in Primary and Human Pluripotent Stem Cell-Derived Cardiomyocytes

After seeding onto the NPs, primary and human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) begin to align in the direction parallel with the ridges of the surface. **Figure 2.3** depicts representative bright field images of neonatal rat ventricular myocytes (NRVMs) after seven days of culture and H7 human embryonic stem cell-derived cardiomyocytes (hESC-CMs) after 48 hours of culture. NRVMs and hESC-CMs show obvious structural anisotropy and alignment (**Fig. 2.3B,D**) whereas cardiomyocytes on traditional unpatterned flat surfaces are randomly oriented (**Fig. 2.3A,C**).

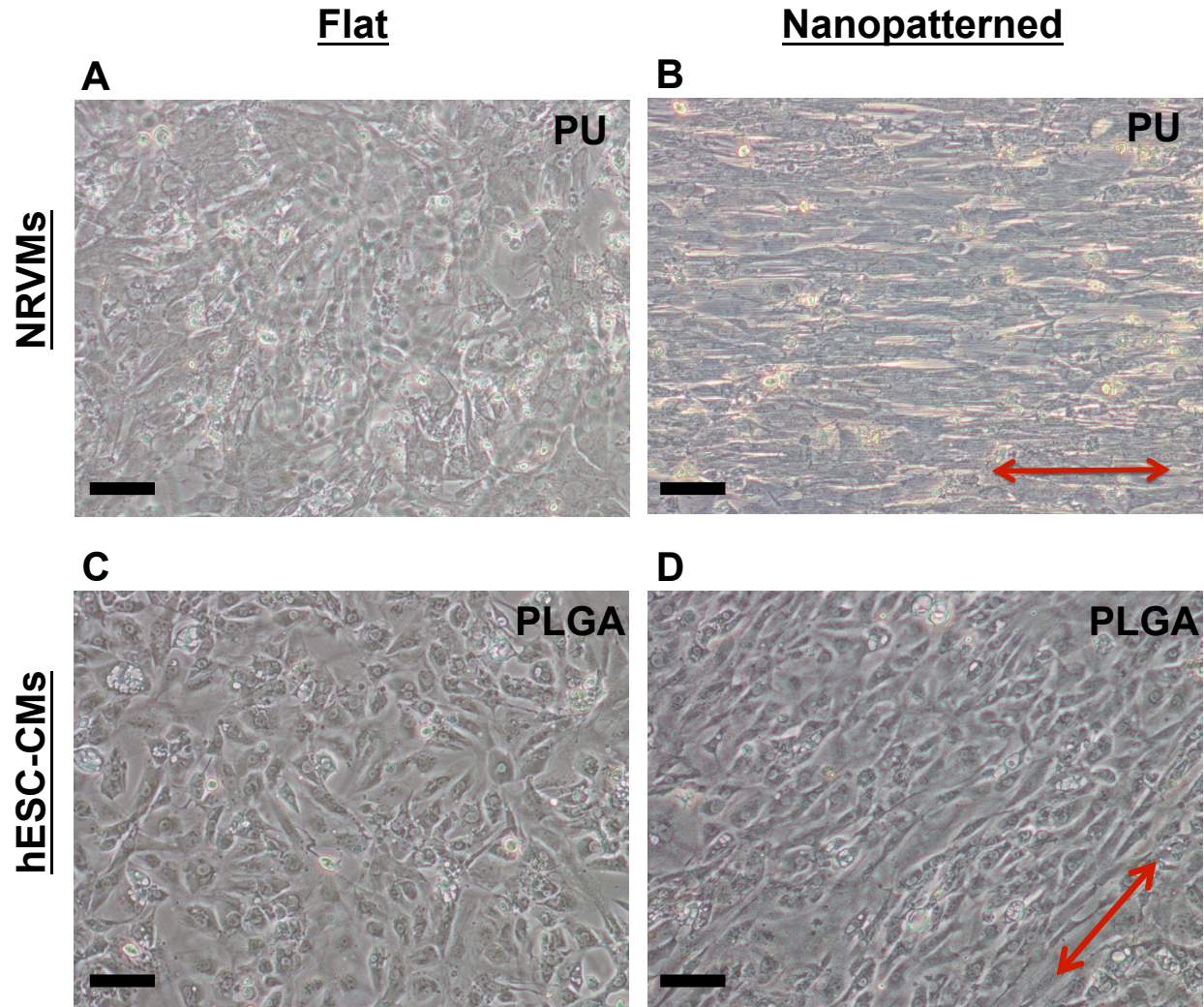


Figure 2.3. Aligned cardiomyocytes. 10x bright field images of NRVMs after 7 days of culture (A,B) and hESC-CMs after 2 days of culture (C,D). NRVMs cultured on NP-PU substrates (B) exhibit obvious structural alignment while NRVMs on flat PU substrates (A) are randomly aligned. Arrows in (B) and (D) indicates direction of NP anisotropy. Scale bar = 100 μ m.

Immunohistochemical analysis highlights the impact of the NPs on cell cytoskeletal organization and alignment. Actin microfilaments (F-actin) and α -sarcomeric actinin in cells cultured on the NPs become aligned along the nano-ridges but remain randomly distributed on flat surfaces (**Fig. 2.4**). Cardiac cells exhibit spontaneous beating after 24-48 hours on the NPs.

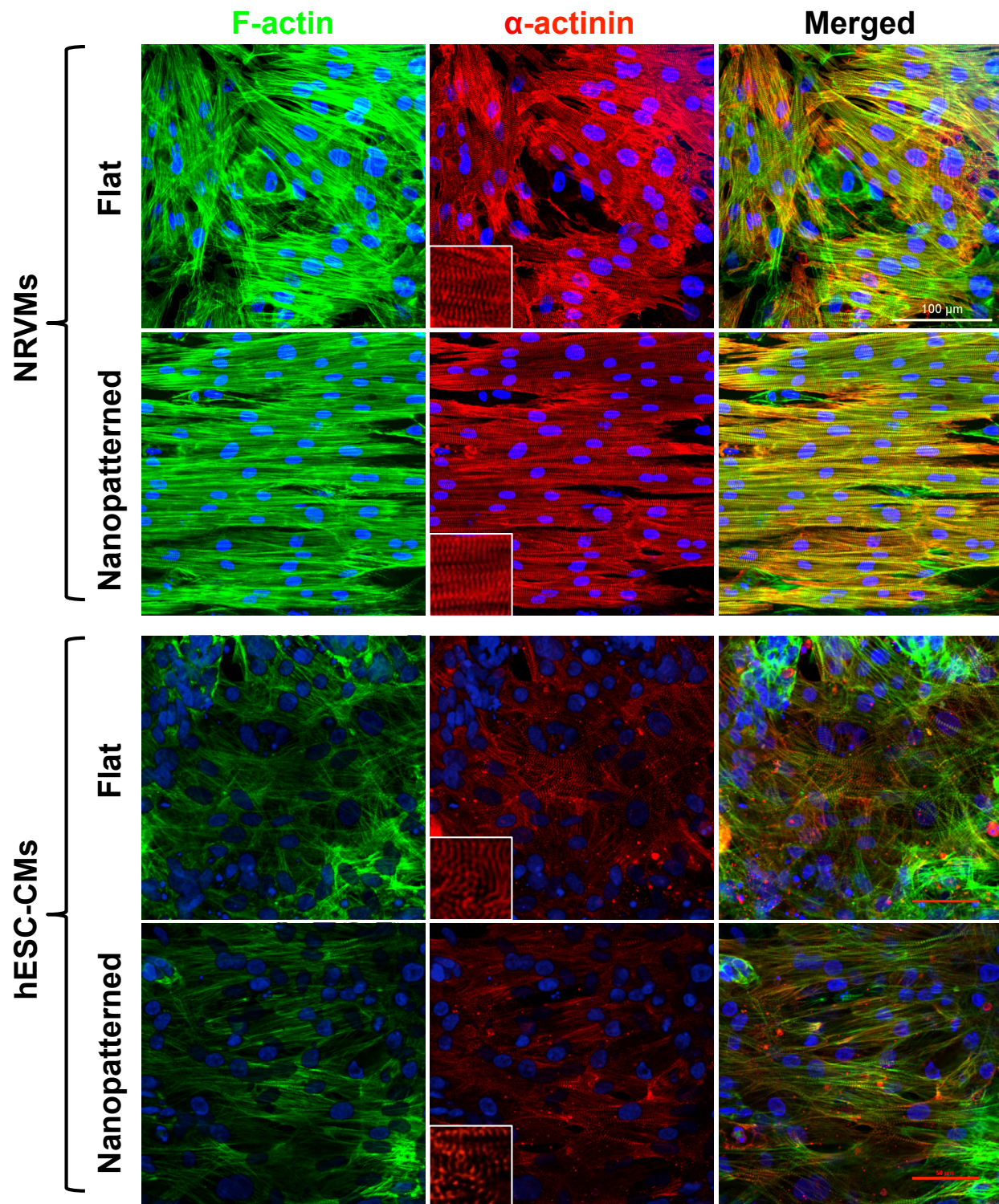


Figure 2.4. Immunofluorescent confocal imaging. Cell cytoskeletal alignment and striations; α -sarcomeric actinin (red), cell nuclei (blue), and F-actin (green). Cells cultured on NP substrates have aligned α -sarcomeric actinin and F-actin fibers while cells cultured on flat substrata have randomly oriented fibers. Inset on red α -sarcomeric actinin channel clearly show striated sarcomeres.

2.4.3 Anisotropic Nanopatterned Surfaces Exert a Topographic Size-Dependent Effect on Human Pluripotent Stem Cell-Derived Cardiomyocytes

Kim et al. showed that NRVMs cultured on NPs of various dimension sizes had variable connexin-43 protein expression levels, with higher expression on 800 nm wide ridges and grooves¹⁰³. It was not known, however, if hPSC-CMs would respond in a similar nanotopography size-dependent manner. We therefore cultured Rockefeller University human embryonic stem cell-derived cardiomyocytes (RUES2-CMs) on NPs of various dimensions ranging from the nano- to the micron-scale for two weeks (**Fig. 2.5A**). Compared to the flat condition, RUES2-CMs on NPs with dimensions of 400-1600 nm were significantly larger and more elongated, while RUES2-CMs on 200 nm NPs responded similarly to flat (**Fig. 2.5B**). We also found that cell attachment and monolayer formation was significantly hindered on 5000 nm NPs resulting in many balled up cells (**Fig. 2.5A,B**) and a lower cell density at the end of two weeks (**Fig. 2.5C**). RUES2-CMs exhibited a graded cytoskeletal alignment, with greater actin fiber alignment on NPs with larger dimensions (**Fig. 2.5D**).

Since monolayer formation was inhibited on micron-scale topographies and the largest, most anisotropic cells were found on NPs around 800 nm in dimension, we next examined functional and biochemical measures of cardiac maturation on the optimum 800 nm NPs compared to flat. RUES-CMs exhibited faster Ca^{2+} release kinetics on 800 nm NPs compared to flat as measured by the time to peak Ca^{2+} signal (**Fig. 2.5E,F**). Additionally, genes associated with lower diastolic membrane potentials (KCNJ2) and hypertrophy (TNNT2) were significantly upregulated in RUES2-CMs on 800 nm NPs compared to flat. Although not significantly downregulated, there was also a trend

towards lower expression of the gene associated with the pacemaker funny current (HCN4).

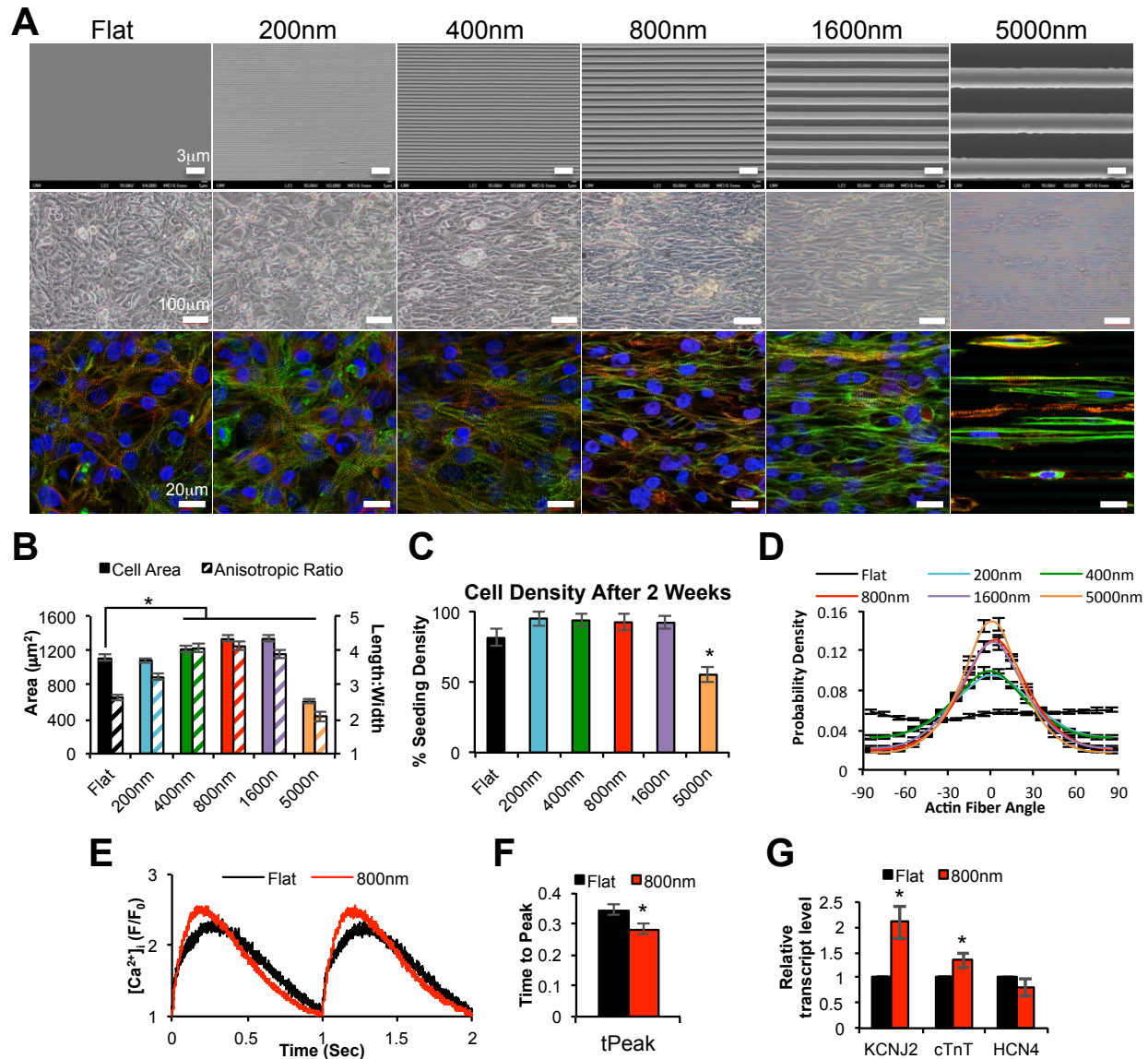


Figure 2.5. Nanotopographic size-dependent structural response. (A) Top row: scanning electron micrographs of NP surfaces with various dimensions. Middle row: bright field images of hPSC-CMs on NP surfaces of various dimensions. Bottom row: confocal immunofluorescent images of hPSC-CMs cultured on NPs of various dimensions. Red = α -actinin, Green = F-actin, Blue = DAPI. (B) Cell area and cellular anisotropic ratio of hPSC-CMs cultured on the labeled topographic dimensions. (C) Cell monolayer density relative to initial seeding density of hPSC-CMs on the different topographic dimensions. (D) Relative cytoskeletal fiber alignment of hPSC-CMs on various topographies. (E-F) Fluo-4 calcium imaging and time-to-peak Ca^{2+} measurements of hPSC-CMs between flat and 800 nm NPs. (G) RT-PCR expression levels of cardiac maturation markers of hPSC-CMs on 800 nm NPs compared to flat.

2.4.4 Long-Term Culture on Anisotropic Nanotopography Promotes Myofibril Development and T-Tubule Formation

hiPSC-CMs were generated from cells collected either from urine⁶⁵ or skin fibroblasts using directed differentiation of high-density monolayers with a combination of activin-A and BMP-4, along with small molecules that sequentially activate and repress Wnt/ β -catenin signaling²²³ (**Fig. 2.6A,B**). At 15-20 days post-differentiation (p.d.), single hiPSC-CMs were replated at low density onto fibronectin-coated nanopatterned surfaces fabricated with parallel grooves and ridges of 800 nm width and spacing to promote cell spreading¹⁰³. Just after replating, 15-20 days p.d. hiPSC-CMs were round-shaped and demonstrated sporadic signs of myofibrillogenesis with sparse expression of sarcomeric elements and assembly in fieri (**Fig. 2.6C**). Obtaining mechanical measurements from these pre-myofibrils is quite challenging due to their short length and fragility. Therefore, to achieve myofibril lengths ($>50+ \mu\text{m}$) and stability that would enable us to obtain mechanical measurements on our experimental platform, hiPSC-CMs were cultured for an additional 60 days on nanopatterned surfaces (80-100 days p.d. total). This resulting in cells that were elongated (**Fig. 2.6D**), and many had dimensions similar to those found for cultured adult cardiomyocytes (**Fig. 2.6E**). Cells were rod-shaped with average lengths of $127.3 \pm 8.8 \mu\text{m}$ and average widths of $14.7 \pm 0.9 \mu\text{m}$. In all hiPSC-CMs, myofibril alignment followed the nanotopographical pattern.

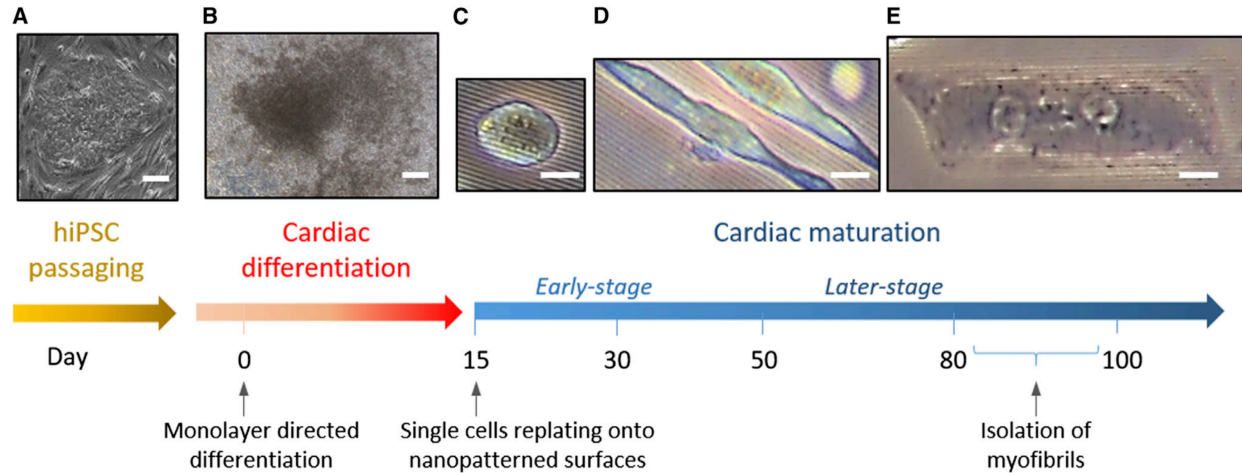


Figure 2.6. Protocol for cardiac maturation of human pluripotent stem cells on nanopatterned surfaces in long-term cultures. (A–E) Phase-contrast images of hiPSCs at different stages of differentiation and cardiac maturation. (A) hiPSC passaging before differentiation. (B) At day 0, hiPSCs were differentiated into cardiomyocytes with a monolayer-directed differentiation protocol. Beating areas were visible from day 8 post-differentiation (p.d.). (C) At 15 p.d, single hiPSC-CMs were replated onto nanopatterned surfaces until day 80–100 in low density for cardiac maturation. Early-stage hiPSC-CMs were round-shaped and lacked of aligned myofibrils. (D) After replating, hiPSC-CMs showed early progressive elongation and increased myofibril density/alignment. (E) Later-stage hiPSC-CMs were rod-shaped and myofibrils were highly aligned. These cells had length of $127.3 \pm 8.8 \mu\text{m}$ and width of $14.7 \pm 0.9 \mu\text{m}$ ($n = 44$ from controls). Scale bars in (A) and (B) represent $200 \mu\text{m}$; scale bars in (C), (D), and (E) represent $20 \mu\text{m}$.

Later-stage hiPSC-CMs (80–100 p.d. total; **Fig. 2.6E**) were stained for Z-bands (anti-actinin). As observed by phase-contrast imaging (**Fig. 2.7A**), quantitative immunocytochemical analysis demonstrated later-stage control-hiPSC-CMs were characterized by aligned myofibrils and clearly defined Z-bands spanning the width of the cells. We used fast Fourier transform analysis to quantify the myofibril alignment index based on the regularity of Z-bands. Later-stage hiPSC-CMs (**Fig. 2.7**) has an extended cell area (control = $7,696 \pm 519 \mu\text{m}^2$ and perimeter (control = $219 \pm 11 \mu\text{m}$ with an elevated index of myofibril alignment (control = 0.014 ± 0.002), indicating a high impact on myofibril organization with this combinatorial approach for cardiomyocyte maturation.

We further investigated the cell structure by staining for caveolin-3 (CAV3), a scaffolding protein of the sarcolemma, which is associated with the formation of caveolae and T tubules during the development of striated muscle²²⁴. CAV3 is also implicated in several electromechanical regulation pathways²²⁵. Top and side views of confocal images (**Fig. 2.7B**) display the organization of cells co-stained for CAV3 and Z bands. In a 3D reconstruction of the confocal images, the cells had a homogeneous distribution of CAV3 over the entire sarcolemma. CAV3 staining revealed finger-like invaginations tightly associated with Z bands, likely representing transverse (T)-tubule-like structures during development²²⁶. This finding provides evidence that transverse-axial tubular system (TATS) formation may occur together with myofibrillogenesis and suggests a strict inter-play between tubules and Z bands during cardiac development on nanopatterned surfaces.

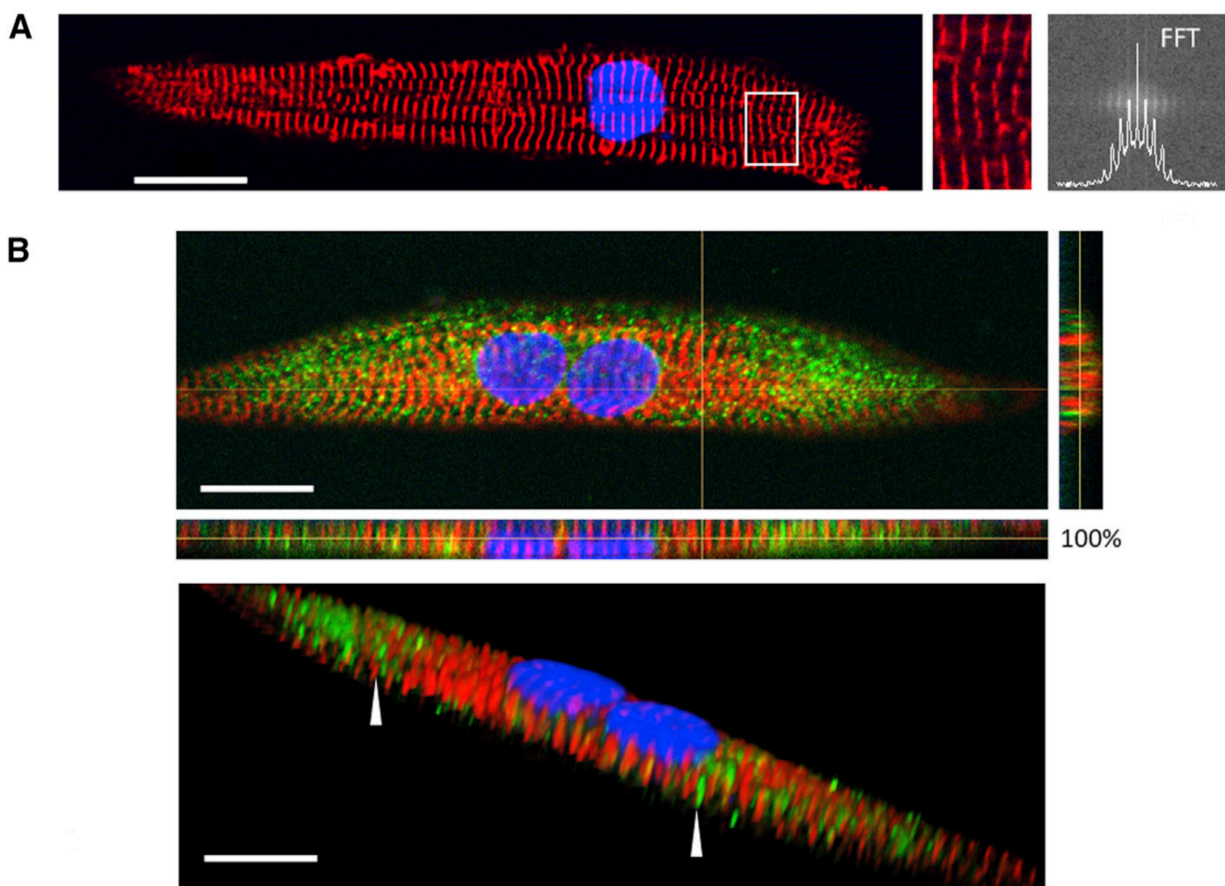


Figure 2.7. Later-stage hiPSC-CMs grown on a nanopatterned surface promote myofibril alignment and T-tubule formation (A) Confocal images of later-stage hiPSC- CMs grown on nanopatterned surfaces. Cells were stained for Z bands (α -actinin, red) and for nuclei (DAPI, blue). hiPSC-CMs exhibited mature morphology with rod-shaped, aligned myofibrils and clearly defined Z bands (enlargement of the area within the white rectangle) as quantified by fast Fourier transform (FFT) analysis. (B) Confocal images of hiPSC-CMs stained with antibody against caveolin-3 (green), α actinin (red), and nuclei (blue). Top: hiPSC-CMs displayed development of a transverse-axial tubular system (TATS), with both transverse and axial elements. Bottom: representative 3D picture of TATS in hiPSC-CMs. Arrowheads in the 3D reconstruction highlight transverse tubular elements (green), running in parallel to a Z line (red). Axial tubules occurred perpendicularly to the Z lines and were evident in the side view. Scale bars represent 20 μm .

2.5 Discussion

Structurally and functionally mature cardiac tissues are lacking for *in vitro* applications of cardiac tissue engineering. The CFL nanofabrication methods technique described here are robust techniques for achieving cellular alignment, influencing macroscopic tissue function, and maturing single cell cardiomyocyte characteristics due to the scalability of

the system. Large areas can easily be patterned and used for cell culture. Macroscopic cellular alignment is essential in cardiac tissue engineering in order to create biomimetic, functional tissue as it influences both mechanical and electrical properties of the myocardium²²⁷.

Various other methods, such as micro-contact printing, electrospinning, and microtopography, have previously been employed to control the structure of engineered cardiac tissue on the microscale²²⁸⁻²³⁰. While these techniques have proven successful in gaining cellular alignment, it is likely that the structure and function of *in vivo* cardiac tissue is governed by the much smaller, nanotopographical cues of the ECM and thus our NP substrate should prove advantageous. Additionally, these micro-scale patterning techniques have inherent disadvantages compared to our anisotropically nanofabricated substrates. For instance, our nanopatterning method is more cost-effective and controllable than electrospinning. Micro-contact printing, on the other hand, relies on cells adhering to specific lanes to gain structural anisotropy. In order to create a functional monolayer, cells must then either proliferate or migrate out of these lanes to form tight junctions. This is much more difficult for cells with little to no proliferative capacity such as neonatal cardiomyocytes. Microtopography is also limited by the inability to create tightly coupled cell monolayers. Due to the scale of the cells compared to the microtopography, cells reside on top of or inside the microridges. This limits the amount of cell-cell interaction and decreases cell-cell coupling. With our method, cells become aligned via the bioinspired nano-scale topography and can freely interact with neighboring cells as the feature sizes are at least an order of magnitude

smaller than the cells themselves. This allows for more biomimetic, tightly coupled cells monolayers to be created.

Morphological analysis of hPSC-CMs cultured on NPs for 21 days showed the optimal structural phenotype occurred on NPs ~800 nm in dimension with peak cell alignment, anisotropic ratio, monolayer formation/maintenance, and cytoskeletal alignment. Interestingly, we also observed an increase in the rate of Ca^{2+} entry into the cytosol when hPSC-CMs were cultured on 800 nm NPs. This functional change may be a result of tighter excitation contraction couple (ECC) between the L-type Ca^{2+} channels and the sarcoplasmic reticulum. The development of a primitive T-tubules in hPSC-CMs cultured long-term on the NPs also suggests a more advanced development of ECC mechanisms.

Beyond imparting advanced structural and functional features to hPSC-CMs, the biomimetic nanotopography also enables the development of advanced bioengineering platforms such as single myofiber mechanical testing of hPSC-CMs. Without an elongated morphology, myofibers from hPSC-CMs cultured on traditional flat cell culture surfaces are too short and often very fragile. The NP surfaces promote robust, in register sarcomere formation that results in myofibers capable of being handled and loaded into a custom-built apparatus required to measure single myofiber mechanical properties¹⁸⁰.

Taken together, these data highlight the benefit of biomimetic cell culture substrata for hPSC-CM cardiac tissue engineering. Not only do such platforms create more mature cells for preclinical screening applications but important developmental processes such as ECC and sarcomerogenesis can also be studied in greater detail.

Chapter 3. Nanopatterned Human iPSC-based Model of a Dystrophin-Null Cardiomyopathic Phenotype

This chapter has been published in the following co-first authored manuscript¹¹¹:

- **Macadangdang J***, Guan X*, Smith AST, Lucero R, Czerniecki S, Childers MK, Mack DL, Kim D-H. Nanopatterned Human iPSC-Based Model of a Dystrophin-Null Cardiomyopathic Phenotype. *Cellular and Molecular Bioengineering*. 2015; 8(3): 320–332.

3.1 Abstract

Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) offer unprecedented opportunities to study inherited heart conditions *in vitro*, but are phenotypically immature, limiting their ability to effectively model adult-onset diseases. Cardiomyopathy is becoming the leading cause of death in patients with Duchenne muscular dystrophy (DMD), but the pathogenesis of this disease phenotype is not fully understood. Therefore, we aimed to test whether biomimetic nanotopography could further stratify the disease phenotype of DMD hiPSC-CMs to create more translationally relevant cardiomyocytes for disease modeling applications. We found that anisotropic nanotopography was necessary to distinguish structural differences between normal and DMD hiPSC-CMs, as these differences were masked on conventional flat substrates. DMD hiPSC-CMs exhibited a diminished structural and functional response to the underlying nanotopography compared to normal cardiomyocytes at both the macroscopic and subcellular levels. This blunted response may be due to a lower level of actin cytoskeleton turnover as measured by fluorescence recovery after

photobleaching. Taken together these data suggest that DMD hiPSC-CMs are less adaptable to changes in their extracellular environment, and highlight the utility of nanotopographic substrates for effectively stratifying normal and structural cardiac disease phenotypes *in vitro*.

3.2 Rationale

Human induced pluripotent stem cells (hiPSCs) offer the means to generate a potentially limitless supply of a diverse array of human cell types for both basic science and clinical applications²³¹⁻²³³. Moreover, isolation of cells from patients with rare genetic conditions now holds the potential to generate disease specific human cells with which to further our understanding of underlying pathologies²³². Integration of these cells with bioengineering technologies has great promise for enabling the development of culture systems that can accurately recapitulate specific inherited disease phenotypes and assess symptomatic progression⁶⁴.

Given the prevalence of heart disease in society¹⁹⁴, models of cardiomyopathic conditions using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have become one of the most intensely investigated tissues by bioengineers seeking to synthesize biomimetic tissues for drug development and cell replacement applications^{23,33,51,126,234-236}. One of the major goals of cardiac tissue engineering using human pluripotent stem cell technology is to create a functional piece of human myocardial tissue to serve as a high fidelity *in vitro* model of the heart for drug screening applications and the study of specific disease mechanisms^{9,10}. Unlike animal models, human pluripotent stem cell derived cardiomyocytes (hPSC-CMs) theoretically express the full array of ion channels, sarcomeric proteins, and metabolic machinery found in

patient's cells. To date, there have been a number of different iPSC models of inherited cardiac diseases that have been published, including ion channelopathies⁴⁸⁻⁵⁰, dilated cardiomyopathy⁶⁸, and Barth syndrome⁶⁴. In addition, our group has previously developed a urine cell-derived model of Duchenne muscular dystrophy (DMD). These dystrophin-null hiPSC-CMs exhibit aberrant metabolic activity, altered calcium (Ca^{2+}) handling, and are more prone to hypotonic mechanical stress⁶⁵.

DMD is an X-linked genetic disorder affecting ~1:3500 live male births. Frameshift mutations in the dystrophin gene result in the translation of a truncated dystrophin protein, which is non-functional and essentially unexpressed in cells²³⁷. Under normal conditions, dystrophin is an integral part of the dystrophin-glycoprotein complex (DGC), a major structural and signaling hub that helps link the actin cytoskeleton to the extracellular matrix (ECM) and provide cellular stability²³⁸. With the loss of dystrophin, the DGC is disrupted, leaving myocytes particularly prone to mechanical stress and rupture. Over time this results in muscle degeneration and the deposition of fibrotic tissue, eventually leading to premature death. However, with improved palliative treatment leading to prolonged lifespans, cardiomyopathy is becoming the leading cause of death in patients with DMD. While it is known that patients with DMD exhibit diastolic dysfunction and arrhythmias, the pathogenesis of this disease phenotype in cardiac tissue is not fully understood. The *mdx* mouse model of DMD has provided valuable insights into the disease pathology in skeletal muscle, but, paradoxically, *mdx* mice exhibit minimal cardiac dysfunction, limiting their utility for screening translational cardiac therapies⁷⁸. In fact, a recent clinical trial with sildenafil, which showed promise in alleviating cardiac dysfunction in the *mdx* mouse⁷⁹, was

actually found to exacerbate cardiac symptoms in DMD patients and had to be terminated early⁸⁰. Therefore, the need for an accurate human cardiac model of dystrophin cardiomyopathies has never been greater.

In order to be of practical relevance, *in vitro* cardiac tissue screening assays utilizing hiPSC-CMs must be able to comprehensively stratify healthy and disease states in order to quantify the ability for novel treatments to rescue the disease phenotype. Here we present a simple biomimetic engineered platform for culturing hiPSC-CMs that enhances the structure and morphology of cardiomyocytes so that aberrant disease-specific characteristics relating to cell structure and contractile function in DMD can be detected. Our novel anisotropically nanofabricated substrata (ANFS) consist of 800 nm parallel arrays of grooves and ridges, mimicking the ordered structure of the myocardial extracellular matrix. We have shown previously that these patterned substrates promote the alignment of cultured cardiomyocytes, leading to the formation of anisotropic monolayers closely resembling the *in vivo* structure of myocardial tissue¹⁰³. In this system, both cellular action potential propagation and contractility are highly anisotropic and consistent with the underlying nanotopographic cues, suggesting that ECM-like nanotopographic substrates provide a powerful guidance cue regulating cardiomyocyte alignment and function *in vitro*^{96,239}.

In this aim, cardiomyocyte structural development on ANFS was compared between DMD hiPSC-CMs and normal controls. In this manner, the capacity for dystrophin null cardiomyocytes to respond to exogenous mechanical cues was evaluated. These presented data demonstrate that ANFS mimicking the native ECM microenvironment help to stratify the DMD disease phenotype *in vitro* when compared

to traditional flat culturing substrates. As such, the described system provides a more accurate and comprehensive model of DMD cardiomyopathy-in-a-dish for use in subsequent mechanistic studies and drug screening applications.

3.3 Scientific Methods

3.3.1 Capillary Force Lithography

ANFS with 800 nm topographic features were fabricated via UV-assisted capillary force lithography as previously described²⁴⁰. Briefly, first liquid polyurethane acrylate (PUA) prepolymer was drop dispensed onto a silicon master mold of the desired nanotopographic dimensions. A transparent polyester film (PET) was then placed on top of the dispensed PUA. After exposure to UV radiation ($\lambda = 250\text{-}400\text{ nm}$), the film was peeled away from the silicon master, creating a PUA mold. A polyurethane-based prepolymer (NOA76, Norland Products, Inc.) was then drop dispensed onto standard glass coverslips and the PUA mold was placed on top. The mold was then exposed to UV radiation for curing. After curing, the PUA mold was peeled off, leaving behind an ANFS for cell culture. **Figure 3.1** depicts a schematic representation of this process. Flat polymer control surfaces were fabricated in the same manner, only with a bare PET film instead of a PUA mold. Prior to cell seeding, the ANFS are UV sterilized and coated with human fibronectin at 5mg/mL (Life Technologies).

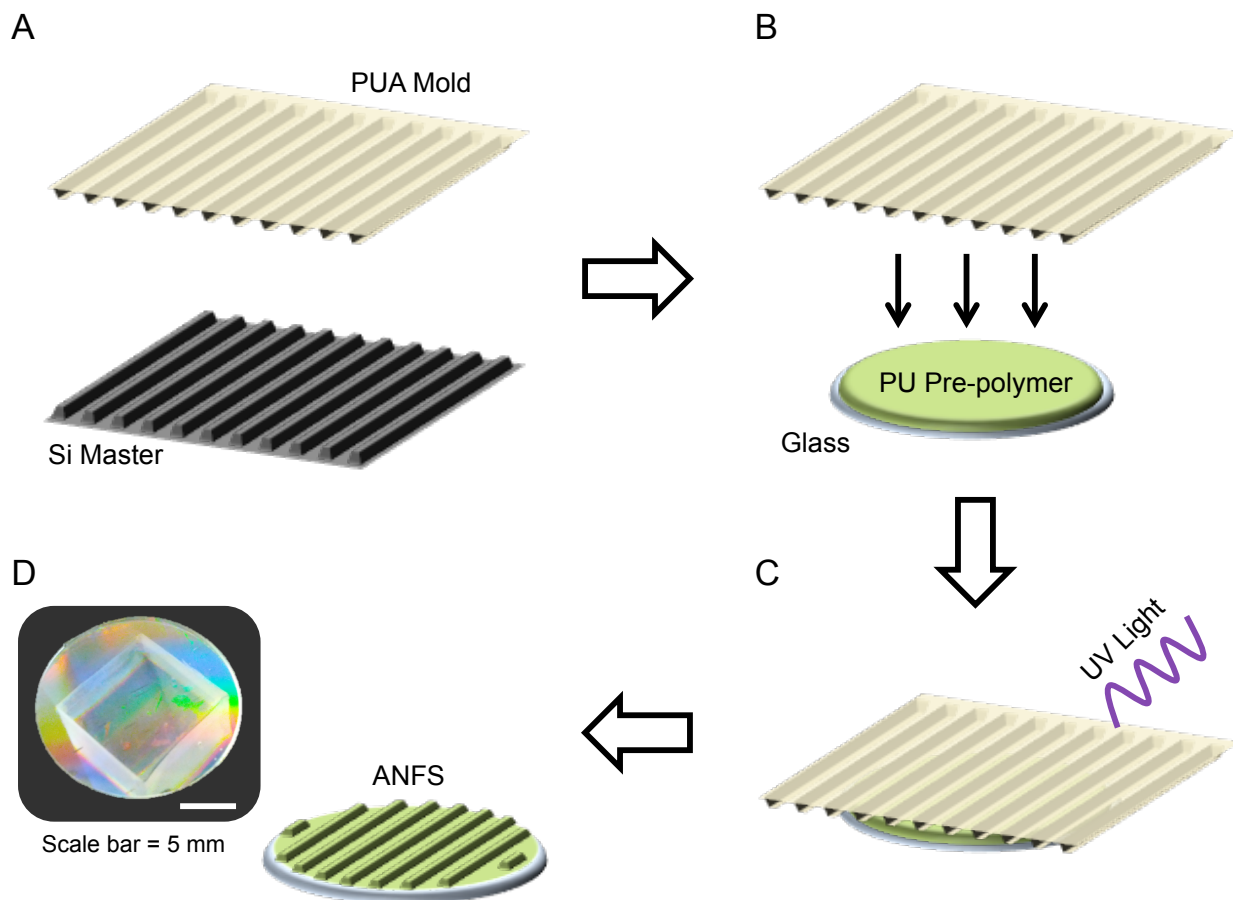


Figure 3.1. Schematic representation of anisotropically nanofabricated substrata (ANFS) fabrication process. (A) A polyurethane-acrylate (PUA) replica mold is created from a silicon (Si) master with the desired 800 nm topographic dimensions. (B) This PUA mold is then used to mass-produce ANFS by placing the PUA mold on top of a liquid polyurethane (PU) photo-curable pre-polymer. (C) The system is then exposed to UV light to polymerize the PU. (D) The PUA mold can then be peeled away, leaving behind the ANFS. The photograph depicts the iridescent surface of the ANFS caused by light diffraction at the nanotopographic surface.

3.3.2 Induced PSC Production and Cardiac Differentiation

Informed consent was obtained from all patients as directed by the Institutional Review Board (IRB) policies. Urine cells were isolated and expanded from a single healthy male participant (normal) and a DMD patient harboring a large dystrophin mutation as previously described^{65,241}. A polycistronic lentiviral vector encoding human Oct3/4, Sox2, Klf4, and c-Myc²⁴² was used to reprogram the urine cells into iPSCs⁶⁵. The

derivative normal and DMD hiPSC lines were karyotyped and shown to be normal 46, XY karyotypes, and were subsequently used for differentiation. A monolayer-based directed differentiation was used as previously published⁶⁵. **Figure 3.2A** details the specific timing and reagents used in our cardiac differentiation protocol. Briefly, hiPSCs are plated into a monolayer and fed serum-free media supplemented with specific morphogen proteins and small molecule Wnt modulators to guide the cells down the cardiac lineage. At day 20 post-induction, hiPSC-CMs were dissociated with 0.05% Trypsin-EDTA, plated onto flat or ANFS at 175,000 cells/cm², and cultured for an additional 14 days post-replating before further analysis, unless otherwise stated. The purity of cardiomyocyte populations plated onto experimental surfaces was confirmed by flow cytometry (**Fig. 3.2B**). In all cell populations used, cardiomyocyte purity was found to lie in the range of 70 to 90% (data not shown).

The stated cell density was established based on preliminary investigation as optimal for producing confluent cardiomyocyte monolayers. Preliminary analysis with hiPSC-CMs revealed not significant structural differences between day 14 and day 100 cultures on ANFS (data not shown). As such, 14 days was selected as a suitable endpoint for these analyses as a sufficient length of time for cells to develop stable structural phenotypes. Cultures were fed every other day with serum-free RPMI-B27 plus L-glutamine.

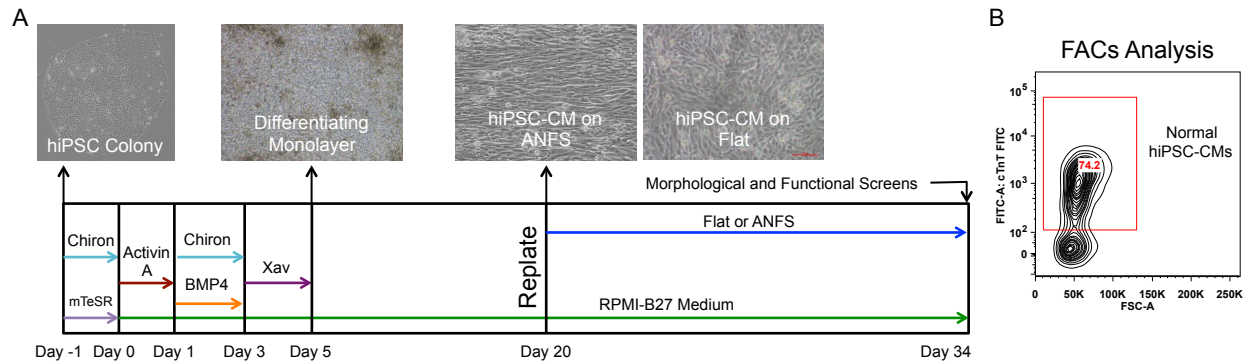


Figure 3.2. Protocol of hiPSC cardiac differentiation. (A) Timeline and representative images of the monolayer-based cardiac differentiation protocol and plating regimen for these studies. The timing and duration of specific morphogens (Activin-A and Bone Morphogenic Protein-4 [BMP4]) and small molecule Wnt modulators (Chiron and Xav939) specify cardiomyocyte development. (B) Representative flow cytometry result of cardiac purity for a cardiac differentiation prep. Differentiation runs with cardiac purity >70% were used for subsequent analysis.

3.3.3 CRISPR Knockout of Dystrophin

Guide sequences targeting the 3'-end of the first muscle exon of human dystrophin locus were designed using the online CRISPR design tool²⁴³. Forward and reverse single strand oligodeoxynucleotides (ssODN) were synthesized with added BbsI restriction enzyme site at 5'-end (Eurofins Genomics, Huntsville AL). Validated guide sequence was then cloned into the plasmid hSpCas9 (BB)-2A-Puro (px459, addgene # 48139) to construct the targeting plasmid, according to the method described previously²⁴⁴. Normal male iPSC UC3-4 was electroporated with the target plasmid using the Neon transfection system (Life technologies) according to the manufacturer's protocol. After electroporation, cells were gently plated in 24-well-plate with 500ul of mTeSR1 medium (Stemcell technologies) and 10 µg/mL of ROCK inhibitor Y-26732 (Selleckchem). Medium was refreshed every day and 0.8 µg/mL puromycin selection was initiated 48 hours post transfection for 2 days. Individual colonies survived puromycin selection were manually picked and passaged into 96-well-plate for further

expansion. Genomic mutation of the dystrophin locus was confirmed by Sanger's sequencing (Genewiz, Seattle). Dystrophin protein knockout in the iPSCs was confirmed via Western blot (data not shown).

3.3.4 Cell Morphological Analysis

Normal and DMD hiPSC-CMs were cultured on flat or ANFS for two weeks and then observed using a Nikon Eclipse TS100 microscope. Phase contrast images were taken from three random fields of view per substrate (N = 5 biological replicates per condition). Morphological parameters such as cell length, width, and area were measured using ImageJ (National Institutes of Health). Once outlined, ImageJ processing tools were used to fit an ellipse to the outlined region, and the major and minor axes of each cell were determined from that shape. Cell morphology was measured by manually outlining clearly defined cell borders of cells that showed rhythmic beating in videos of the same field of view¹⁰³. For all examined conditions, at least 250 cells were measured.

3.3.5 Immunohistochemistry

Cells were fixed in 4% paraformaldehyde for 15 minutes and blocked with 5% goat serum in PBS for 1 hour at room temperature. Cells were then incubated with mouse anti- α -sarcomeric actinin monoclonal antibody (1:500, Sigma-Aldrich) diluted in 1% goat serum in PBS overnight at 4°C. The next day, cells were washed with PBS followed by staining with Alexa-594 conjugated goat-anti-mouse secondary antibody (1:200, Invitrogen) and Alexafluor 488 phalloidin (1:200, Invitrogen). Counterstaining was performed with Vectashield containing DAPI (Vector Labs). Images were captured with a Nikon A1R confocal microscope using a Nikon CFI Plan Apo VC 60X water immersion

objective. For each condition, at least 4 biological specimens were prepared and imaged, with at least 4 images taken per specimen.

3.3.6 Cytoskeletal Alignment Measurements

Fluorescent images of F-actin fibers of cardiomyocytes on unpatterned and patterned substrates were analyzed for alignment using a custom MATLAB script as described previously²⁴⁵. A 2D convolution was performed on each image and a Sobel edge-emphasizing filter was applied to extract the horizontal and vertical edges within the image. These edges were then combined to calculate the gradient magnitude of each pixel. A magnitude threshold was applied to select the major F-actin fibers. Next, the gradient orientation was calculated by determining the angle of the gradient with the x- and y-axes. Finally, the angle that the orthogonal of the gradient makes with the x- and y-axes was calculated. This orthogonal angle (from -90° to $+90^\circ$ with respect to the x-axis) is the principle orientation of the individual F-actin fibers. A probability density histogram of the fiber angle distribution was calculated for each image and averaged per condition.

3.3.7 Sarcomere Length Analysis

The α -actinin confocal images were analyzed using standard analysis plugins in ImageJ. To calculate sarcomere length, a straight line was drawn perpendicular to at least ten consecutive well-defined α -actinin-positive bands. An intensity profile was then plotted across the entire length of the line and the distance between intensity peaks was calculated. Between ten and fifteen measurements were made per image and averaged per condition.

3.3.8 Correlation-based Contraction Quantification

Our CCQ method utilizes Particle Image Velocimetry (PIV) and Digital Image Correlation (DIC) algorithms²⁴⁶ to provide relevant contractile endpoints from bright field video recordings. Briefly, a reference video frame is divided into a grid of windows of a set size. Each window is run through a correlation scheme with a second frame, providing the new location for that window in the second frame. This displacement is converted into a vector map, which provides contraction angles and, when spatially averaged, contraction magnitudes and velocities. The correlation equation used provides a Gaussian correlation peak with a probabilistic nature that provides sub-pixel accuracy. The videos used to perform this analysis were taken using a Nikon TS100 microscope at 12 frames per second (fps).

3.3.9 Fluorescent Recovery After Photobleaching (FRAP)

Briefly, cardiomyocytes were differentiated as previously described⁶⁵. Cells were replated on BD matrigel-coated glass bottom dishes as single cells. Cellular actin was labeled with Life Technologies CellLight® Actin-RFP (BacMam 2.0). Imaging was performed on a Nikon A1R confocal using a Nikon CFI Plan Apo VC 60X water immersion objective, and cells were maintained at 37°C using a Tokai Hit Stagetop Incubator. Cardiomyocytes were identified by rhythmic beating. A region of sarcomere was bleached using a 561nm laser at 100% power, and recovery of fluorescence was measured every second for 60 seconds, followed by every 8 seconds until 10 minutes after bleaching. The size of the bleaching area was identical for all experiments.

Fluorescence recovery was calculated using pre-bleach fluorescence intensity in the bleached area. Statistics were performed using Graphpad's Prism software.

3.3.10 Statistical Analysis

Statistical significance between flat and nanopatterned, as well as between normal vs DMD vs KO DMD cultures, was determined using Two-Way ANOVA with Tukey's pairwise *post hoc* analysis using SigmaPlot software unless otherwise stated. For the contraction angle analysis, a chi-square test run at 5% significance was utilized to quantify uniformity in alignment distributions. This test was calculated using MATLAB. For all statistical analyses, a p value less than 0.05 was considered significant. Error bars represent standard error of the mean (SEM).

3.3.11 Ethical Approval

Procedures performed in studies involving human subjects were approved by the University of Washington Institutional Review Board, approval number 45581. Human subject activities were performed in accordance with the regulatory requirements laid down in U.S. Code of Federal Regulations, Title 45 Department of Health and Human Services Part 46, Protection of Human Subjects.

3.4 Results

3.4.1 DMD hiPSC-CMs Display a Blunted Response to Nanotopography Compared to Normal Control

Our previous data with NRVMs demonstrated that 800 nm ANFS can be used to promote *in vivo*-like structure and function in cultured cardiomyocytes¹⁰³. We therefore

set out to answer whether ANFS could be used to enhance the structure of hiPSC-CMs in order to further stratify cardiomyocyte phenotypes in a disease model of DMD cardiomyopathy. Phase contrast images of synchronously beating monolayers of normal hiPSC-CMs (**Fig. 3.3A,B**) demonstrate that under healthy conditions hiPSC-CMs align very well with the underlying anisotropic nanotopographic cues (ANFS are oriented horizontally in all images). Both patient-derived DMD hiPSC-CMs and isogenic dystrophin knockout (KO DMD) hiPSC-CMs, on the other hand, demonstrate a less drastic macroscopic alignment on the ANFS compared to normal cardiomyocytes (**Fig. 3.3C-F**).

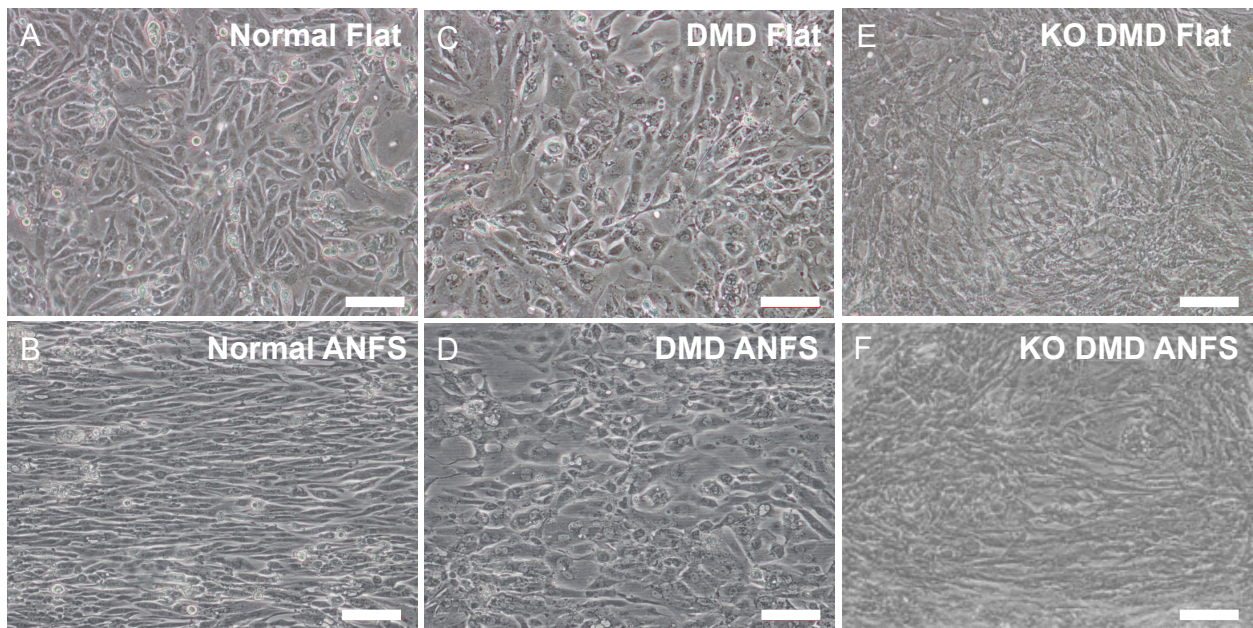


Figure 3.3. Bright field images of normal (A and B), patient-derived DMD (C and D), and KO DMD hiPSC-CMs (E and F) on flat or ANFS after two weeks of culture. Cells on flat substrata (A,C, and E) are randomly oriented while cells on ANFS (B,D, and F) have a more anisotropic and aligned structure. ANFS are oriented horizontally in the images. Scale bar: 100mm.

Looking at the subcellular structure with immunocytochemistry, again we found that qualitatively, normal hiPSC-CMs showed a higher level of anisotropy and alignment on the ANFS compared to flat control (**Fig. 3.4A,B**). The actin cytoskeleton in normal

cardiomyocytes exhibit local alignment in the flat condition but overall showed no preferential directionality. Conversely, actin cytoskeleton of normal hiPSC-CMs on ANFS were highly aligned in the direction of the ANFS. DMD and KO DMD hiPSC-CMs on flat substrates (**Fig. 3.4C,E**) were randomly organized similar to normal, but showed much less directionality than normal cardiomyocytes on ANFS (**Fig. 3.4D,F**).

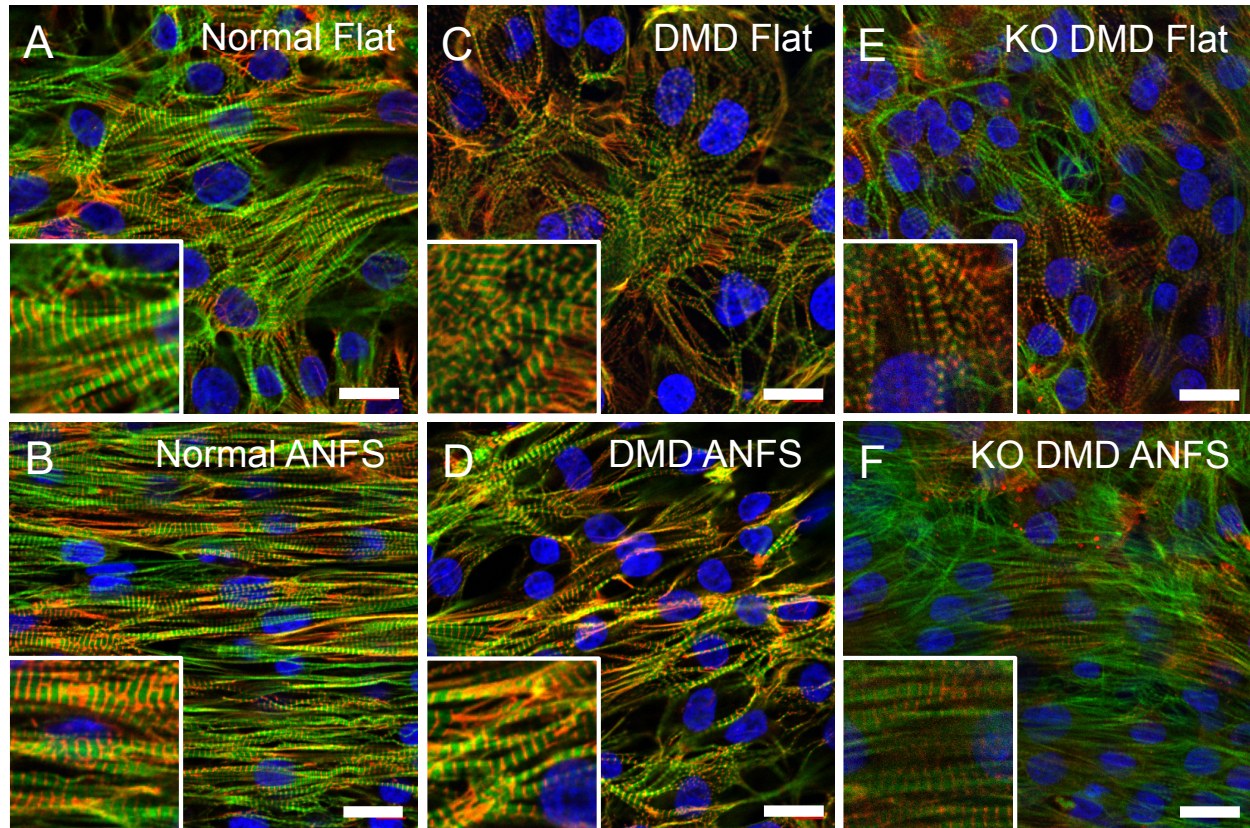


Figure 3.4. Immunofluorescent confocal images of sarcomere structure in normal (A and B), patient-derived DMD (C and D), and KO DMD hiPSC-CMs (E and F) on flat or ANFS after two weeks of culture. Cells have highly aligned cytoskeletal and sarcomere structures on ANFS compared to flat substrata, but DMD hiPSC-CMs show less alignment than normal hiPSC-CMs. Insets show close-ups of striated sarcomeres. Green = F-actin, Red = α -actinin, Blue = DAPI. Scale bar: 20 μ m.

In order to quantify the cellular response to the nanotopography, we began by measuring cell morphology and anisotropy from the bright field images. We found that hiPSC-CMs from all the cell lines (normal, DMD, and KO DMD) had a higher anisotropic ratio (cell length:width ratio) on ANFS compared to flat substrates (**Fig. 3.5A**, $p <$

0.001). Patient-derived and KO DMD hiPSC-CMs, however, had a lower anisotropic ratio on ANFS (3.73 ± 0.12 and 3.92 ± 0.24 , respectively) compared to normal on ANFS (4.83 ± 0.09 , $p < 0.001$ for both comparisons). Next, we measured the distribution of actin fiber angles from the confocal images. hiPSC-CMs from all cell lines exhibited random cytoskeletal alignment as evident by the relatively flat actin fiber angle distribution in **Figure 3.5B** (solid lines). Normal hiPSC-CMs on ANFS, on the other hand, were much more aligned based on the peaked distribution of actin fiber angles (indicating most actin fibers were preferentially aligned in a single direction). As the qualitative analysis suggests, however, patient-derived and KO DMD hiPSC-CMs were less aligned and had an actin fiber distribution that fell between the flat and highly aligned distributions of normal control. Taken together these data suggest that DMD hiPSC-CMs have a diminished capacity to respond to topographic cues compared to normal hiPSC-CMs. Cell density was assessed for each examined condition by counting the total cells per field of view during morphological assessment. No significant difference was observed in cell density across all examined conditions (data not shown), indicating that the observed differences in cell structure was not due to variance in population density.

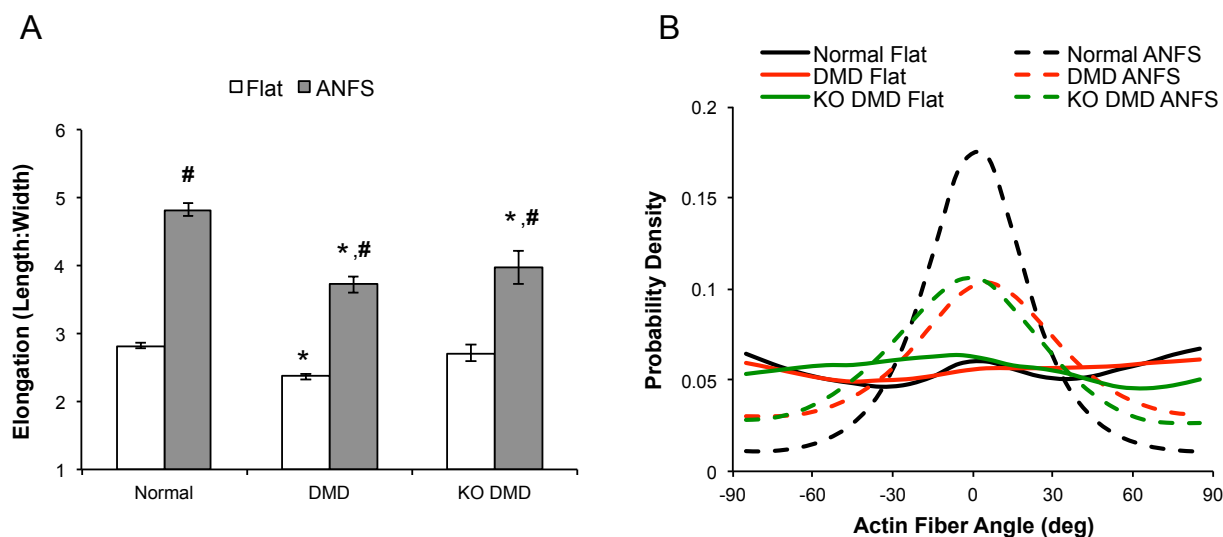


Figure 3.5. Morphological analysis of cardiomyocytes show that DMD hiPSC-CMs are less responsive to substrate nanotopographical cues than normal control hiPSC-CMs. (A) Cell elongation was measured by taking the ratio of the major and minor axes of the cell in ImageJ. Patient-derived and KO DMD hiPSC-CMs are less elongated on ANFS compared to normal control. * $p < 0.05$ via Two-Way ANOVA with Tukey's Post-Hoc analysis compared to normal within topographic group. # $p < 0.05$ compared to flat within cell type. (B) Probability density histogram of F-actin fiber orientations highlight that cells on traditional flat substrata have randomly oriented cytoskeletons as evident by the flat distributions (solid lines). Cells on ANFS (dotted lines) are more preferentially aligned in the direction of the nanotopography and have a peaked distribution of F-actin fibers.

3.4.2 DMD hiPSC-CMs Exhibit a Cardiomyopathy-Like Phenotype

The clinical manifestation of the cardiac involvement in DMD is generally described as cardiomyopathy complicated by arrhythmias²⁴⁷. We measured cell area from bright field images to test whether this phenotype was apparent in our DMD cardiomyopathy models. While normal hiPSC-CMs on ANFS had larger cell areas ($591 \pm 12.2 \text{ mm}^2$) than on flat substrates ($465 \pm 9.07 \text{ mm}^2$), suggesting a greater degree of cellular hypertrophy and/or cell spreading induced by the ANFS ($p < 0.001$), patient-derived DMD hiPSC-CMs were significantly larger than normal on both flat ($796 \pm 15.5 \text{ mm}^2$, $p < 0.001$) and ANFS ($719 \pm 19.0 \text{ mm}^2$, $p < 0.001$) (**Fig. 3.6A**). In fact, DMD hiPSC-CMs were moderately smaller on ANFS than flat substrates. KO DMD hiPSC-CMs displayed a

similar trend on flat ($695 \pm 19.5 \text{ mm}^2$) and ANFS ($653 \pm 30.6 \text{ mm}^2$) as the patient-derived DMD hiPSC-CMs and were significantly larger than normal hiPSC-CMs ($p < 0.001$) (but on average slightly smaller than the patient-derived hiPSC-CMs, $p = 0.003$).

Next we measured sarcomere length from the confocal images of the cells. Similar to the results with cell area, patient-derived DMD hiPSC-CMs had significantly longer resting sarcomere lengths (flat = $1.95 \pm 0.021 \text{ mm}$, ANFS = $1.93 \pm 0.017 \text{ mm}$) than normal on both flat ($1.78 \pm 0.013 \text{ mm}$) and ANFS ($1.78 \pm 0.014 \text{ mm}$, $p < 0.001$) (**Fig. 3.6B**). Again, this disease trend held true in the KO DMD hiPSC-CMs as cells on both flat and ANFS had significantly longer sarcomere lengths than normal hiPSC-CMs (flat = $1.84 \pm 0.018 \text{ mm}$, ANFS = $1.83 \pm 0.018 \text{ mm}$, $p = 0.011$). This abnormally long resting sarcomere length for patient-derived and KO DMD hiPSC-CMs, combined with the increased cell area, indicates that DMD hiPSC-CMs appear to recapitulate some of the structural characteristics of DMD cardiomyopathy.

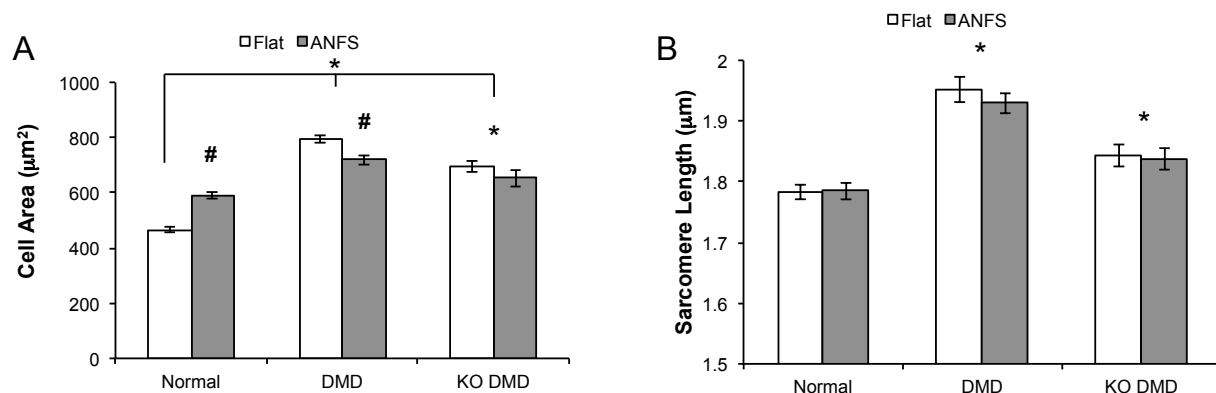


Figure 3.6. DMD hiPSC-CMs have a cardiomyopathy-like phenotype. (A) Cell area measurements show that patient-derived and KO DMD hiPSC-CMs are larger on both flat and ANFS compared to normal control. * $p < 0.05$ via Two-Way ANOVA with Tukey's Post-Hoc analysis compared to normal. # $p < 0.05$ compared to flat within cell type. (B) Sarcomere analysis highlights that patient-derived and KO DMD hiPSC-CMs have atypically long resting sarcomere lengths compared to normal control. * $p < 0.05$ compared to normal hiPSC-CMs.

3.4.3 DMD hiPSC-CMs Demonstrate Alterations in Contractile Properties

Based on our CCQ contraction analysis, patient-derived DMD hiPSC-CMs showed greater variability and lower alignment in contraction angles on nanopatterned substrates compared to normal hiPSC-CMs (**Fig. 3.7A-D**). Cells on ANFS had slower overall contraction velocities than on flat substrates in all conditions. Interestingly, the patient-derived and KO DMD hiPSC-CMs had faster contraction velocities on both flat and ANFS compared to normal hiPSC-CMs (**Fig. 3.7G**, $*p < 0.05$). The chi-square tests showed that each ANFS group showed significantly lower variation in contraction angle than flat with the same cell condition at the 5% significance level. The chi-square test also showed that DMD hiPSC-CMs on ANFS showed higher variation in contraction angle than normal hiPSC-CMs on flat. This can be interpreted as the flat substrates showing less alignment than the ANFS overall, and the DMD hiPSC-CM on ANFS showing less alignment than the normal controls.

When contraction in parallel with the nanotopographic cues (longitudinal velocity) was isolated and analyzed independently, it was found that no significant difference between normal and DMD cells was observed (**Fig 3.7H**, $p = 0.65$). Normal cells on ANFS exhibited highly anisotropic contraction parameters (**Fig. 3.7I**). As such, the isolated transverse contraction velocities were significantly smaller than those in parallel with the topography (**Fig. 3.7I**, $p = 0.03$). A greater level of variability in contraction direction was observed in DMD cells (**Fig. 3.7D**). No significant difference was observed in longitudinal and transverse contraction velocities in KO DMD cells (data not shown, $p = 0.48$) or patient-derived DMD cells on ANFS, (data not shown, $p = 0.08$). Taken together, although the contraction velocities are greater in DMD hiPSC-CMs compared

to normal, contraction longitudinal to the ANFS is not different. Since contraction is also highly anisotropic on the ANFS for normal hiPSC-CMs and more variable in direction for DMD cells, these results could lead to larger overall contraction velocities.

No significant differences in spontaneous contraction frequencies were observed between any experimental condition, suggesting the differences observed were due to fundamental changes in cell structure, rather than variation in beat rhythmicity.

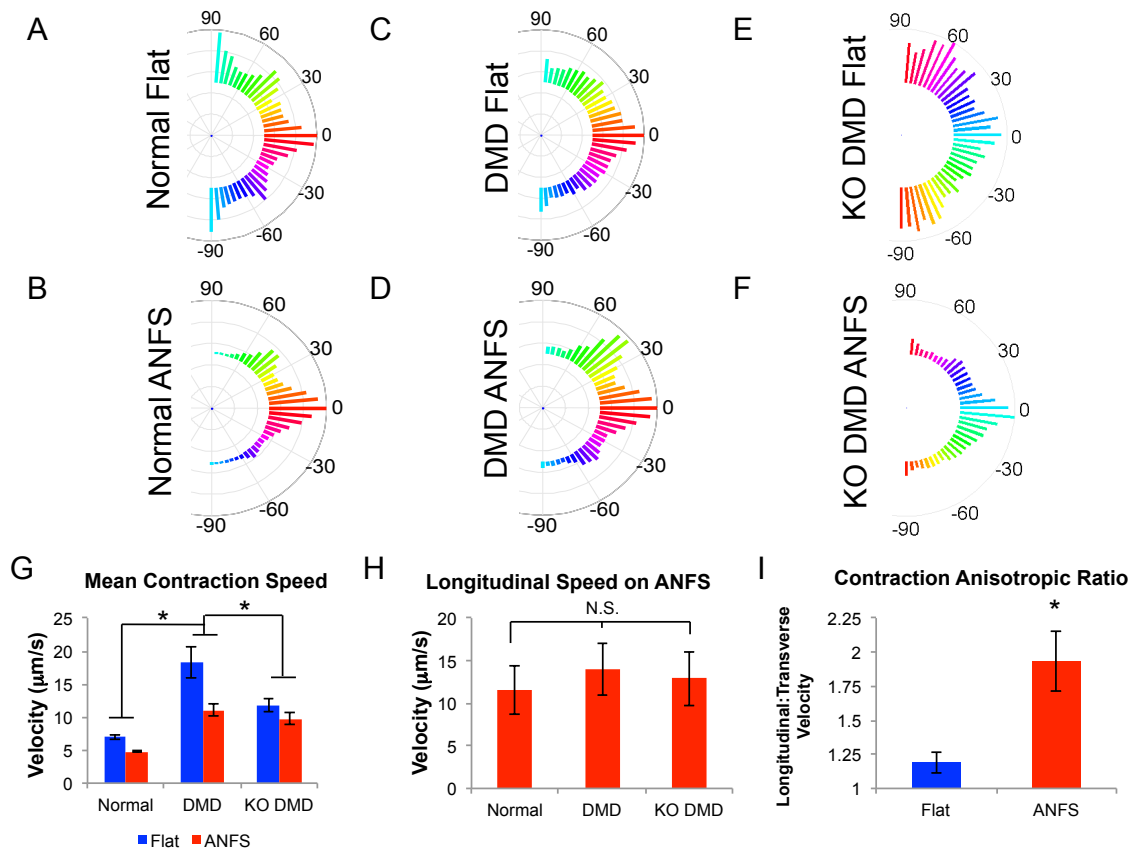


Figure 3.7. Correlation-based Contraction Quantification (CCQ) analysis of beating monolayers. (A-F) Average contraction angle histograms of normal (A and B), patient-derived DMD (C and D), and KO DMD hiPSC-CMs (E and F) on flat and ANFS. Patient-derived DMD hiPSC-CMs have more random contraction angles on ANFS than normal hiPSC-CMs. (G) Maximum contraction speeds of normal, patient-derived DMD, and KO DMD hiPSC-CMs. In general, DMD hiPSC-CMs have faster contraction speeds than normal hiPSC-CMs (* $p < 0.05$). (H) Isolated longitudinal contraction velocities in the direction of cell alignment and nanotopography show no significant difference between groups ($p = 0.65$). (I) Contraction velocity anisotropic ratio (Longitudinal:Transverse Velocity) of normal hiPSC-CMs on flat and ANFS. Normal hiPSC-CMs are highly functionally anisotropic on ANFS. These results, together with the contraction angle histograms, suggest there is greater variability in the DMD hiPSC-CM contractile directions, leading to the larger overall contraction speeds in (G).

3.4.4 Slower Cytoskeletal Turnover May Explain Blunted DMD hiPSC-CM Response to ECM Cues

Dystrophin is a vital component connecting the actin cytoskeleton to the extracellular matrix. Therefore, it is reasonable to predict that DMD hiPSC-CMs would have a blunted response to ECM cues such as nanotopography. However, just as the mechanism behind increased membrane fragility in DMD is debated and not fully understood²³⁷, the reasoning why DMD hiPSC-CMs should be less organized on nanotopography than normal is not straightforward. Thus, to further investigate how the loss of dystrophin leads to this diminished contact guidance, we performed FRAP measurements of sarcomeric actin turnover in normal and DMD hiPSC-CMs (**Fig. 3.8**). We found that compared to normal, DMD hiPSC-CMs had significantly lower actin turnover in the sarcomere region as measured by lower FRAP levels after 10 minutes. This result is in agreement with our results of cytoskeletal alignment of normal and DMD hiPSC-CMs on ANFS (**Fig. 3.5B**). With a lower rate cytoskeletal turnover, the cells would have less ability to reorganize their cytoskeleton in response to the contact guidance cues imparted by the nanotopography.

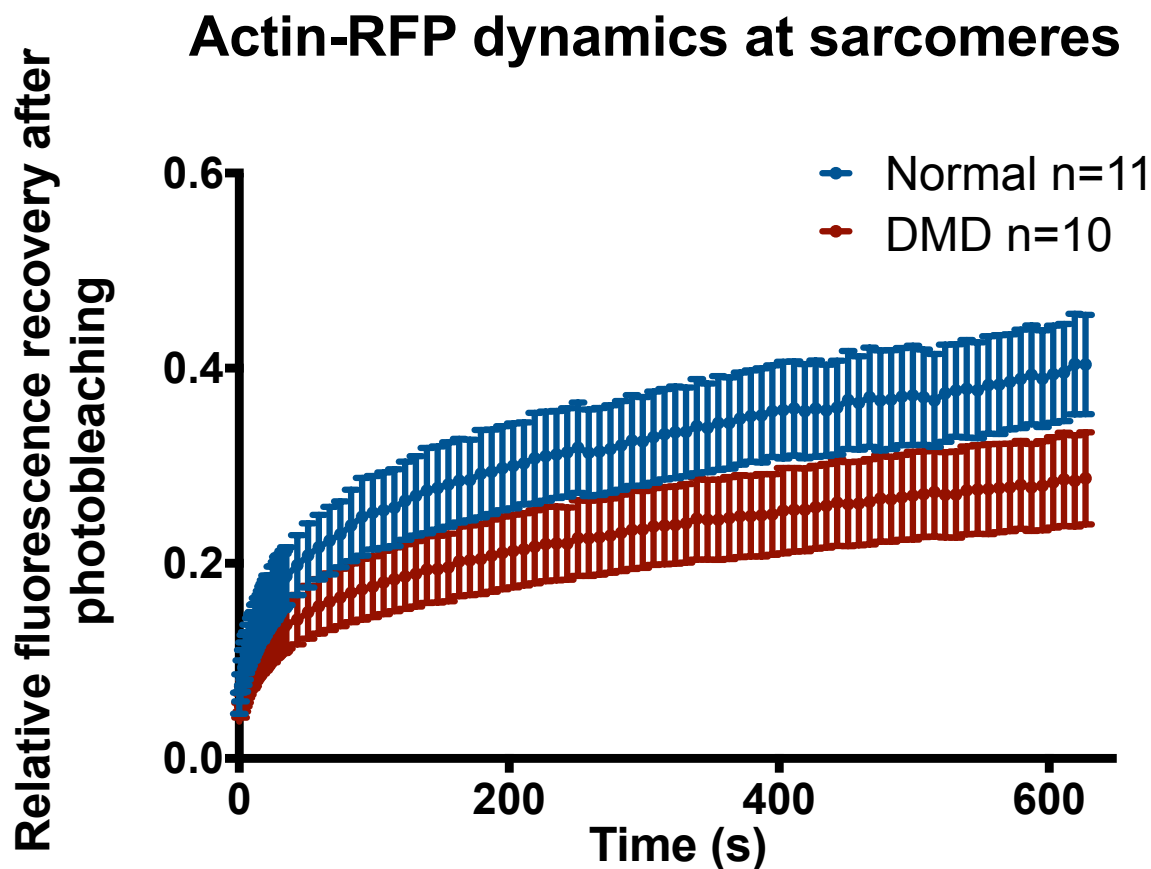


Figure 3.8. Fluorescence recovery after photobleaching (FRAP) measurements of sarcomeric actin in normal and DMD hiPSC-CMs demonstrate that DMD hiPSC-CMs have significantly slower actin turnover than normal control. Slower actin turnover may explain why DMD hiPSC-CMs are less responsive to extracellular matrix cues such as nanotopography, as the cells are less able to adapt to changes in their environment.

3.5 Discussion

Once cardiomyopathy has developed in patients, there is no known cure outside of heart transplantation, a procedure rarely performed in DMD patients due to weak respiratory muscle function, respiratory insufficiency, and short supplies of donor hearts²⁴⁸. Therefore, it is imperative to discover new therapies that can slow the progression of diseases (such as DMD) that cause cardiomyopathy, to improve the quality of life for these patients until a more definitive, long-term cure is found²⁴⁹. Human

iPSC disease models have an incredible potential to elucidate important molecular mechanisms driving diseases such as DMD, and their availability opens the door to developing new methods for developing and testing novel therapies. In this study, we aimed to assess the ability of biomimetic nanotopographic cell culture substrates to exacerbate the disease phenotype in an *in vitro* model of DMD cardiomyopathy so that the diseased state is more distinct from the healthy one. Such a platform could be used to identify simple, high-throughput endpoints with which to screen novel therapeutics for their capacity to ameliorate the cardiac pathology of DMD.

We hypothesized that a biomimetic microenvironment would further stratify the disease phenotype in our dystrophin-null hiPSC-CM model of DMD cardiomyopathy by adding more *in vivo*-like ECM cues that would necessitate cell-ECM interactions and activation of the DGC. In addition, while single cell analyses often constitute effective *in vitro* systems for studying cell structure, monolayer cultures were chosen for this study to provide a more accurate representation of myocardial tissue. Eventual application of this technology for drug screening and functional studies will benefit from analysis of whole tissue mechanics and their breakdown in disease.

When normal control, patient-derived DMD, and KO DMD hiPSC-CMs were cultured on traditional flat substrates, there was very little difference between their structures. There was no difference in their actin cytoskeletal alignment and minimal differences in cell shape (**Fig. 3.5**). When the cells were cultured on the biomimetic ANFS, however, a striking difference in the structure of the DMD cells became apparent compared with normal controls. Both patient-derived and KO DMD hiPSC-CMs failed to align as robustly as normal cells on the ANFS, and there was a larger reduction in

elongation between normal and DMD hiPSC-CMs on ANFS than flat substrates. Comparing the isogenic controls (normal and KO DMD), we found that the cell elongation was only significantly different on the ANFS, while the cells on the traditional flat surfaces showed no difference in their elongation. Therefore the ANFS did indeed help to bring out a more severe “disease phenotype” and helped to stratify healthy from diseased hiPSC-CMs.

In this study we measured sarcomere length as a surrogate for sarcomere organization. While we found that patient-derived and KO DMD hiPSC-CMs had longer sarcomere lengths compared to normal control cells, we interpret this data to suggest that this increased sarcomere length did not result from increased maturation, because there did not appear to be a concomitant increase in cellular organization (as evident from **Figs. 3.3-3.5**). Unbiased measurements, such as organization order parameter (OOP)²⁵⁰, may elucidate this increased sarcomere organization in our cultures and are under current investigation by our group. Dilated cardiomyopathy, such as the type observed with DMD patients however, is associated with lower myofilament calcium sensitivity^{251,252}. In accordance with this calcium insensitivity, the resting sarcomere length of dilated cardiomyopathic cardiomyocytes has been found to be longer than WT control²⁵³. Thus, our findings of increased cell area along with longer resting sarcomere lengths in DMD hiPSC-CMs suggests an accurate model of dilated cardiomyopathy-in-a-dish. Further investigation into the Ca^{2+} sensitivity of DMD hiPSC-CM myofibrils is necessary.

The most prominent finding of this work was that both patient-derived and KO DMD hiPSC-CMs displayed a blunted cellular response to the topographic cues

imparted by the ANFS. The fact that structural phenotypes in patient derived and KO DMD hiPSC-CMs were not significantly different from each other, but were different from normal controls, supports the hypothesis that the abnormal structural phenotype observed in DMD patient cells directly results from dystrophin deficiency, and is not simply due to cell line variability between patient cells and normal controls. Taken together, findings presented here from data gathered in cell lines derived from normal controls, engineered dystrophin-null, and naturally occurring dystrophin-mutants, support the overall hypothesis that dystrophin plays a direct role in regulating cell mechanotransduction and responding to biomimetic nanotopography *in vitro*.

DMD hiPSC-CMs were less organized both macroscopically and subcellularly on ANFS compared to normal control cardiomyocytes. Although a mechanistic explanation is not known, our findings of lower actin turnover in DMD hiPSC-CMs provide an interesting possibility. The loss of dystrophin is known to disrupt the DGC, but this disruption is not lethal and does not interfere with cardiac differentiation. Therefore, compensatory mechanisms may help to offset the loss of dystrophin. Other groups have postulated that utrophin may play an important roll in compensating for the loss of dystrophin due to its homology with dystrophin²⁵⁴. Other proteins such as integrins and focal adhesion proteins might also be differentially expressed in DMD cardiomyocytes that help to maintain connection with the ECM²⁵⁵. These compensatory mechanisms, however, may be less dynamic than the DGC, and thus inhibit actin cytoskeletal remodeling. Thus, when the DMD hiPSC-CMs establish their connections with the ECM, they may be less able to respond to changes to the microenvironment, such as dissociation and replating. The assay system developed in this study represents a

useful tool to further test this theory, and our group is working to answer this question in more rigorous detail.

In this study, we focused on the structural changes of DMD patient hiPSC-CMs. However, to utilize hiPSC-CMs for effective and comprehensive disease modeling, we must also be able to measure the functional output of cells directly. To that end, we performed video-based contraction mapping of the synchronously beating monolayers. We found that patient-derived DMD hiPSC-CMs display faster overall contraction and relaxation velocities compared to normal controls ($p < 0.001$). Additionally, both normal and patient-derived DMD cells on flat substrates manifest faster contraction and relaxation velocities than cells on ANFS ($p = 0.01$). Although one might assume DMD cells would have slower, weaker contraction kinetics than normal cells, the increase in overall contraction speed may be due to the loss of anisotropic contraction properties. When contraction in parallel with the topographies was isolated, no significant differences between normal and DMD cells was observed (**Fig. 3.7H**). The variability in transverse contraction levels across the different conditions examined could therefore explain why overall contraction was higher in DMD cultures. The very low transverse contraction speeds in normal cells likely reduced overall measurements in these cultures, whereas the variable level in DMD cultures led to larger overall contraction velocity measurements.

While we are unable to definitively explain this increase in overall contractile performance in DMD hiPSC-CMs, an alternative hypothesis for the observed data is that greater contraction and relaxation kinetics in DMD cells is a result of their less stable attachment to the underlying substrate. Breakdown of the DGC in DMD cells may

result in cultures that are less tightly bound to the substrate, and are better able to contract against these rigid surfaces, leading to apparent improvements in function compared with normal cells. Overall, our contractility results indicate that the DMD hiPSC-CMs have less organized beating on ANFS compared to normal controls (F-test showed higher variation in DMD cells than normal at the 5% significance level), but that DMD hiPSC-CMs have inherently faster contractility under the conditions tested (**Fig. 3.7**).

3.5.1 Conclusions

In this chapter, we demonstrate that engineered nanotopographies enable clearer distinction between DMD and normal structural and functional phenotypes in cultured hiPSC-CMs. Specifically, the differences in the observed responses of diseased and normal cardiomyocytes to ANFS are suggestive of a structural cardiomyopathy-like phenotype in DMD cells. Moreover, the collected data accentuate that ANFS can be used to stratify the structural and functional differences between normal and DMD hiPSC-CMs in a way not possible on conventional flat substrates. The developed platform also offers a simple, reliable, and reproducible method for screening phenotypic changes in these cells in response to novel therapeutic treatment. Such technology could be invaluable to the development of a new DMD test bed for drug discovery, enabling the provision of better care to patients suffering from this debilitating condition in the near future.

Chapter 4. Combinatorial Maturation (ComboMat) Strategy for hPSC-CMs

4.1 Abstract

Current cardiac differentiation protocols produce immature cardiomyocytes with structure and function that resembles fetal hearts. These immature cardiomyocytes are suboptimal for cardiac disease modeling of adult-onset diseases and preclinical drug cardiotoxicity screening for adult patients. Here we describe methods for inducing the structural, molecular, metabolic, and functional maturation of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) *in vitro*, to more closely mimic cardiomyocytes in the adult heart. Our combinatorial maturation (ComboMat) approach utilizes nanotopographic substrate cues, Let-7 miRNAs, and thyroid hormone T3 at specific time-points during the culture period to induce advanced phenotypic development. Nanopatterned substrates can be fabricated via capillary force lithography using polyurethane, Nafion, or polydimethylsiloxane. Nanoscale topographic features are fabricated at physiologically-relevant length scales ($> 25\text{cm}^2$) with high fidelity. Let-7 over-expression is achieved by lentiviral transduction of the miRNA into cultured cardiomyocytes. The custom Let-7 lentiviral vector consists of a pLKO backbone using an RNA polymerase III promoter, U6, to drive transcription of the mature form of microRNA Let7i. Furthermore, the pLKO backbone endows antibiotic resistance for purification of successfully transduced cardiomyocytes. To mimic the spike in thyroid hormone post-birth, elevated T3 hormone is applied directly to the feeding medium. Each cue was selected and developed based on its specific method of action on

cultured cardiomyocytes; namely structural organization of the cytoskeleton through controlled interaction of focal adhesions with nanopatterned substrates, thyroid-receptor mediated outside-in signaling by T3, and alterations in metabolic pathways via alteration of gene expression patterns by Let-7 over-expression. Application of this combinatorial strategy promotes greater cardiomyocyte maturation than is achievable in experimental controls or in cells exposed to any one cue in isolation, supporting the importance of these multiple cues acting synergistically to improve development. Observed improvements include greater contraction velocity, advanced sarcomeric development, enhanced mitochondrial activity, improved fatty acid metabolism, more adult-like genetic profile, and enhanced electrophysiological properties. Although each of these methods has been shown previously to enhance cardiomyocyte maturation, the innovation lies in carefully establishing optimal timing for application of these cues in concert, and developing suitable strategies for combining these protocols to leverage significant advantages in terms of improving cellular phenotype. Given the importance of achieving “adult-like” cells for preclinical screening applications, this novel technique could have a significant impact on next generation drug-development protocols.

4.2 Rationale

Cardiovascular disease remains the leading cause of death for both men and woman worldwide, with a rapidly growing impact on developing nations¹. Inherited cardiomyopathies are a major cause of heart disease in all age groups, including children and young, otherwise healthy adults⁴. Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) are a promising tool for studying cardiomyopathy because they vastly increase the accessibility to human cardiomyocytes expressing the full array

of ion channels, protein isoforms, and genetic and metabolic machinery found in the human heart²⁵⁶. A major goal of cardiac disease modeling is to gain insight into the onset and progression of cardiomyopathies in order to effectively design therapeutic strategies. Many patients with inherited cardiomyopathies, however, do not present with cardiac symptoms until late adolescence or early adulthood⁴. However, the potential of hPSC-CM disease modeling is limited since current differentiation protocols produce immature cardiomyocytes with structural, functional, and biochemical properties that most closely resemble the fetal heart²⁵⁷.

With this in mind, developmental maturation is one of the most prominent, yet often neglected, shortcoming of hPSC-CMs in cardiac disease models. To date, immature hPSC-CMs have been used to study channelopathies^{47,62,258}, metabolic syndromes⁷⁰, hypertrophic and dilated cardiomyopathies^{67,68}, as well as other delayed-onset cardiac diseases such as Duchenne Muscular Dystrophy (DMD) cardiomyopathy^{65,81}. While such reports have certainly been groundbreaking and valuable²⁵⁹, the majority of the findings are based on the performance of immature, underdeveloped cardiomyocytes. If the building blocks of a model are suboptimal, making predictions for future development is much more difficult and potentially less accurate.

Toward this end, we developed a novel, combinatorial bioengineering strategy, termed the ComboMat protocol, aimed at accelerating the maturing of hPSC-CMs. The ComboMat protocol utilizes nanotopographic substrate cues, thyroid hormone T3, and Let-7i microRNA (miR) overexpression at specific time-points during the culture period to induce advanced structural and phenotypic development of hPSC-CMs. Each cue

was carefully chosen to beneficially impact specific aspects of hPSC-CM development. The structure-function relationship of cells and tissues has been well-established and anisotropic nanotopographic substrate cues have been shown to promote the structural maturation of primary and hPSC-CMs^{101,103}. These biophysical cues, however, have minimal impact on the metabolic development of hPSC-CMs. Therefore, thyroid hormone T3 was introduced as a biochemical cue to boost the metabolic, calcium handling, and contractile performance of the hPSC-CMs⁸³. Let7i helps to improve metabolic capacity, hypertrophy, and the genetic profile¹⁶². When combined together into the ComboMat protocol, these three combinatorial cues work in a synergistic fashion to markedly enhance the structure and function of hPSC-CMs to more mature levels. Cardiomyocytes exposed to the ComboMat protocol displayed enhanced sarcomere development, improved electrophysiological and contractile function, improved mitochondrial respiratory capacity, and a transcriptome upregulated for metabolic and muscle development.

4.3 Scientific Methods

4.3.1 Experimental Groups

RNAseq was performed on cells exposed to all different combinations of the three maturation cues: NPs, T3, and Let7i. We also included an empty vector (EV) negative control for virus exposure. For all other assays, we compared the full ComboMat protocol (Let7i+NP+T3) to a viral vector Control (EV+Flat+No T3) and each maturation cue in isolation: NP (EV+NP+No T3), T3 (EV+Flat+T3), and Let7i (Let7i+Flat+No T3).

4.3.2 hiPSC Cell Line Generation and Maintenance

Normal urine-derived human induced pluripotent stem cells (hiPSCs) were obtained from an IRB-approved protocol as previously described⁶⁵. Briefly, a polycistronic lentiviral vector encoding human Oct3/4, Sox2, Klf4, and c-Myc²⁴² was used to reprogram cells collected from clean-catch urine samples into iPSCs⁶⁵. The derivative hiPSC line (UC3-4) was karyotyped and shown to be normal 46, XY karyotype. hiPSCs were fed mTeSR culture media (StemCell Technologies) and passaged using 1.9 U/mL Dispase (ThermoFisher). Cells were cultured in hypoxic conditions at 37°C, 5% CO₂, and 5% O₂.

4.3.3 Cardiac Directed Differentiation and Cell Culture

A monolayer-based directed differentiation protocol was used as previously described¹¹¹. hiPSCs were dissociated into single cells using Versene (ThermoFisher) and plated at a high density (250,000 cells/cm²) in mTeSR onto Matrigel-coated plates. Once a monolayer had formed, cells were treated with 1μM Chiron 99021 (Stemgent) in mTeSR until induction with 100nM Activin-A (R&D Systems) in RPMI 1640 (ThermoFisher) supplemented with B27 minus insulin (RPMI/B27/Ins-) and a switch to normoxic conditions (37°C, 5% CO₂, ambient O₂) at day 0. After 18 hours, cells were fed 5 ng/mL BMP4 (R&D Systems) plus 1μM Chiron 99021 in RPMI/B27/Ins- media. At day 3 post-induction, cells were fed 1μM Xav 939 (Tocris) in RPMI/B27/Ins- media. At day 5 post-induction, cells were fed fresh RPMI/B27/Ins- media. At day 7 post-induction, cells were switched to RPMI-B27 media containing insulin and media (RPMI/B27/Ins+) was refreshed every other day.

4.3.4 Lentiviral Transduction of Let7i miRNA and Purification of Cardiomyocytes

The full description of the creation of the Let7i overexpression (OE) construct can be found in Kuppusamy et al.¹⁶². The control vector to Let7i was an eGFP expression pLKO.1 TRC vector (Addgene) under the U6 promoter. An eGFP construct was cloned between AgeI and EcoRI sites of the pLKO.1 TRC vector. To generate lentiviral particles, 293FT cells were plated one day before transfection. On the day of transfection, 293FT cells were co-transfected with psPAX2 (Addgene #12260), pMD2.G (Addgene #12259), vector and polyethylenimine (PEI). Medium was changed 24 hours later and the lentivirus particles were then harvested 24 and 48 hours later. Lentiviral particles were concentrated using PEG-it Virus Precipitation Solution (5x) (System Biosciences).

At day 15 post-induction, beating monolayers of hiPSC-CMs were dissociated with 10 U/mL collagenase type I in DPBS containing calcium and 10 U/mL DNase for 1 hour at 37°C. Cells were then collected, spun down, and resuspended in TrypLE (ThermoFisher) containing 10 U/mL DNase for 5 minutes. A P1000 micropipette was used to triturate clumps of hiPSC-CMs in the TrypLE until single cells were obtained. Cells were then plated onto Matrigel-coated plates at 100,000 cells/cm². The following day, viral transduction of the cells was performed by diluting virus in the presence of hexadimethrine bromide (Polybrene, 6µg/ml) in RPMI/B27/Ins+ media overnight. Cells were washed with PBS the following day and RPMI/B27/Ins+ was replaced.

Cardiomyocytes were then purified via metabolic challenge²⁶⁰ by feeding cells DMEM without glucose, sodium pyruvate, or glutamine (ThermoFisher) containing 2% horse serum and 4 mM lactate for three days without media change. Afterward, hiPSC-

CMs were switched back to RPMI/B27/Ins+ media containing 2 $\mu\text{g/mL}$ puromycin dihydrochloride for three days to select for successfully transduced hiPSC-CMs.

4.3.5 Nanofabrication of Nanopatterns and NanoMEAs

Nanopatterned substrates were fabricated via capillary force lithography as previously described²⁴⁰. Polyurethane acrylate (PUA) master molds were fabricated by drop dispensing liquid PUA pre-polymer onto a deep-ion etched silicon masters with the intended nanotopographic geometries (800nm wide ridges and grooves, with 500nm depth) and placing a transparent polyester (PET) film on top. After curing under a high-powered UV light source, the PUA and PET film were peeled from the silicon master to create a PUA master mold. To create the nanopatterned cell culture substrates, a polyurethane-based UV-curable polymer (NOA76, Norland Optical Adhesive) was drop dispensed onto glass primer treated 18mm, #1 coverslips (Fisher Scientific) and the PUA master mold was placed on top. After curing with a UV light source, the PUA master mold was peeled away, leaving behind the nanopatterned surface (NPs). Control flat substrates were fabricated using the same process but replacing the nanopatterned PUA master with an unpatterned PET film.

To prepare surfaces for cell culture, PU-based NPs and control flat substrates were washed with 70% ethanol, oxygen plasma treated at 50 W for 5 minutes, placed under a UV-C light source for 1 hour to sterilize, then incubated for 24 hours at 37°C with 5 $\mu\text{g/cm}^2$ human fibronectin (Life Technologies). hPSC-CMs were plated at a density of 150,000 cells/cm².

Nanopatterned MEAs (NanoMEAs) were fabricated as described in a manuscript currently under revision. Briefly, the ion permeable polymer Nafion (Sigma-Aldrich) was patterned onto commercial multi-well MEA plates (Axion Biosystems) via solvent-mediated capillary force lithography. Prior to cell seeding, control flat or NanoMEA Nafion surfaces were sterilized with UV-C light and then incubated for 6 hours at 37°C with 5 $\mu\text{g}/\text{cm}^2$ human fibronectin (Life Technologies). hPSC-CMs were plated in droplet fashion at 25,000 cells per well of the MEA plate.

4.3.6 Immunofluorescence Imaging and Morphological Analysis

After three weeks of the ComboMat protocol, cells were prepared for immunofluorescent analysis by fixing in 4% paraformaldehyde (Affymetrix) for 15 minutes at room temperature. Samples were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS and blocked with 5% goat serum. Cells were then incubated with mouse anti- α -actinin monoclonal antibody (1:1000, Sigma-Aldrich) in 1% goat serum overnight at 4°C. After washing with PBS, the samples were stained with Alexa-594 conjugated goat-anti-mouse secondary antibody (1:200, Invitrogen) as well as Alexafluor 488 labeled Phalloidin (1:200, Invitrogen). Nuclei counterstaining was performed with Vectashield containing DAPI (Vector Labs). A Nikon A1R confocal microscope with a Nikon CFI Plan Apo VC 60x water immersion objective was used to capture detailed immunofluorescent images for sarcomere analysis while a 20x air objective was used to obtain low-powered images of cells for measuring cell binucleation percentage. Cells from both objective powers were used to measure cell size. For morphological and sarcomere analysis, hPSC-CMs were plated at 10,000

cells/cm². For each condition, at least three biological specimens were plated with at least 7 cells imaged per specimen.

4.3.7 Electron Microscopy and Ultrastructural Analysis

After three weeks under the ComboMat protocol, hiPSC-CMs were fixed in 4% glutaraldehyde in sodium cacodylate buffer for 2 hours at room temperature for transmission electron microscopy (TEM) analysis. Samples were then washed in buffer and stained in buffered 1% osmium tetroxide for 30 minutes on ice. Next, samples were washed in water and dehydrated in a graded series of ethanol. Samples were then treated by infiltration of 1:1 ethanol:Epon-Araldite epoxy resin followed by two changes of pure Epon-Araldite. At this point, a second coverslip was placed on top of the samples in a 60°C oven for polymerization. The following day, the coverslips were dissolved using hydrofluoric acid and the samples were mounted onto blocks for sectioning. Ultra-thin (70-80 nm) axial sections of single cells were contrasted with lead citrate prior to imaging with a JEOL JEM 1200EXII at 80 kV. ImageJ (National Institutes of Health) was used to measure Z-band width as defined as the width of contiguous electron-dense bands within a sarcomere structure.

4.3.8 Sarcomere Patterning Quantification

Immunofluorescent images were quantified for sarcomere alignment, pattern strength, and sarcomere length using a scanning gradient / Fourier transform script in MATLAB (Mathworks). Each image was broken into several small segments which were individually analyzed. Using a directional derivative, the image gradient for each segment was calculated to determine the local alignment of sarcomeres. The pattern

strength was then determined by calculating the maximum peaks of one-dimensional Fourier transforms in the direction of the gradient. The lengths of sarcomeres were calculated by measuring the intensity profiles of the sarcomeres along this same gradient direction. The frequency at which the intensity profiles crossed their mean allowed an accurate calculation of local sarcomere length within each image segment. Once completed, this analysis allowed for unbiased subcellular-resolution mapping of sarcomere patterning, even in cells lacking the alignment cues provided by nanotopography.

4.3.9 MEA Electrophysiological Analysis

Electrophysiological analysis of spontaneously beating cardiomyocytes was collected for 2 minutes using the AxIS software (Axion Biosystems). After raw data collection, the signal was filtered using a Butterworth band-pass filter and a 90 μ V spike detection threshold. Field potential duration was automatically determined using a polynomial fit T-wave detected algorithm.

4.3.10 Mitochondrial Functional Assay

Cellular metabolic function was measured using the Seahorse XF96 extracellular flux analyzer as previously published¹⁶². After three weeks of treatment with the ComboMat protocol, cells were trypsinized and replated onto fibronectin coated (5 μ g/cm²) XF96 plates at a density of 30,000 cells per well. The MitoStress and Palmitate assays were performed three days post-replating onto the XF96 plates. One hour prior to experiments, RPMI/B27/Ins+ media was replaced with Seahorse XF Assay media supplemented with sodium pyruvate (Gibco/Invitrogen, 1mM) and 25mM glucose for the

MitoStress assay or 25mM glucose with 0.5mM Carnitine for the Palmitate assay. For the MitoStress assay, injection of substrates and inhibitors were applied during the measurements to achieve final concentrations of 4-(trifluoromethoxy) phenylhydrazine at 1 μ M (FCCP; Seahorse Biosciences), oligomycin (2.5 μ M), antimycin (2.5 μ M) and rotenone (2.5 μ M). For the Palmitate assay, injection of substrates and inhibitors were applied during the measurements to achieve final concentrations of 200mM palmitate or 33 μ M BSA. The OCR values were further normalized to the number of cells present in each well, quantified by the Hoechst staining (Hoechst 33342; Sigma-Aldrich) as measured using fluorescence at 355 nm excitation and 460 nm emission. Basal respiration in the MitoStress assay was defined as the OCR prior to oligomycin addition while maximum respiratory capacity was defined as the change in OCR in response to FCCP from the OCR after the addition of oligomycin. Exogenous fatty acid utilization was defined as the maximum change in OCR after the addition of palmitate.

4.3.11 Contraction Analysis

Our video-based contraction analysis algorithm, termed Correlation-based Contraction Quantification (CCQ), utilizes particle image velocimetry and digital image correlation algorithms²⁴⁶ to track the motion of cells from bright field video recordings as previously published¹¹¹. Functional outputs such as contraction magnitude, velocity, and angle are output as a vector field for each video frame and can be averaged spatially or temporally. In brief, a reference frame is divided into a grid of windows of a set size. To track motion, each window is run through a correlation scheme comparing a second frame, providing the location of that window in the second frame. The correlation equation used provided a Gaussian correlation peak with a probabilistic nature that

provides sub-pixel accuracy. The videos used for this analysis were taken with a Nikon camera at 20x magnification and 30 frames per second.

4.3.12 RNAseq Analysis

Total RNA was extracted using trizol (Thermo Fisher Scientific) from RUES2 hESC-CMs. RNA-seq samples were aligned to hg19 using Tophat (PMID: 19289445, version 2.0.13). Gene-level read counts were quantified using htseq-count (PMID: 25260700) using Ensembl GRCh37 gene annotations. Genes with total expression above 3 RPKM summed across RNA-seq samples were kept for further analysis. *princomp* function from R was used to for Principal Component Analysis. DESeq (PMID: 20979621) was used for differential gene expression analysis. Genes with fold change >1.5 were considered differentially expressed. topGO R package (PMID: 16606683) was used for Gene Ontology enrichment analysis.

For the heat map of pathway enrichment, each condition was compared against the Control (EV-Flat) condition, and up-regulated genes (>1.5 fold change) and down-regulated genes were identified (< -1.5 fold change). Hypergeometric test was performed on up- and down-regulated genes separately for enrichment against a curated set of pathways that are beneficial for cardiac maturation, resulting in a m by n matrix, where m is the number of pathways (m=7) and n is the number of conditions (n=10). The negative log₁₀ of the ratio between enrichment p-value for up- and down-regulated genes were calculated to represent the overall net “benefit” of a treatment: large positive value (>0) means the treatment results in more up-regulation of genes in cardiac maturation pathways than down-regulation of these genes, and more negative

values means the treatment results in more down-regulation of genes in cardiac maturation pathways.

4.3.13 qRT-PCR

Total RNA was extracted using trizol (Thermo Fisher Scientific) from RUES2 hESC-CMs. RNA was DNase treated with DNA-free kit (Ambion). For mRNA qPCR reverse transcription reaction, iScript Reverse Transcription Supermix for RT-qPCR (BioRad) was used. qPCR was performed using SYBR green chemistry and ABI 7300 Real time PCR system. Samples were normalized using GAPDH as a house keeping gene. All primers can be found below.

Table 4.1. PCR Primers for Cardiac Maturation Markers

Primer Name	Sequence 5' to 3'
MYOM3 Forward	AATGACACACGGATTGATCCC
MYOM3 Reverse	CGCATCTCCTAATCTCCAGGG
DES Forward	GACGTGGATGCAGCTACTCTA
DES Reverse	GGAACGCGATCTCCTCGTTG
COX6A2 Forward	CCTTCAACTCCTATCTCCACTCG
COX6A2 Reverse	GTTGGTAGGGACGGAACTCG
LPL Forward	ACAAGAGAGAACCAGACTCCAA
LPL Reverse	GCGGACACTGGGTAATGCT
GAPDH Forward	CTGGGCTACACTGAGCACC
GAPDH Reverse	AAGTGGTCGTTGAGGGCAATG

4.3.14 Flow Cytometry

Cells were dissociated and prepared for flow cytometry before and after lactate purification and puromycin selection to determine selection efficiency. Cells were first fixed in 4% paraformaldehyde for 15 minutes. Cells were then permeabilized with 0.75% Saponin and stained in PBS containing 5% FBS with either 1:100 mouse anti-cTnT or isotype control mouse IgG antibodies. Alexa-488 conjugated goat-anti-mouse

secondary antibody (1:200, Invitrogen) was used for visualization. Samples were run on a BD Sciences FACS Canto flow cytometer and analyzed with FlowJo software.

4.3.15 Statistical Analysis

Statistical significance between groups was determined using either a One-Way or Two-Way ANOVA with Tukey's pairwise *post hoc* analysis from SigmaPlot software unless otherwise stated. For all statistical analyses, a p-value less than 0.05 was considered significant. Error bars represent standard error of the mean (SEM).

4.4 Results

4.4.1 Transcriptional Regulation of Cardiomyocyte Maturation

In lieu of a single universal maturation factor, we investigated the combinatorial impact of three distinct cues, biomimetic nanopatterned topography (NP), thyroid hormone T3 (T3), and Let7i miRNA overexpression (Let7i), incorporated into a single procedure termed the ComboMat protocol (schematically depicted in **Figure 4.1a**). To do so, we performed RNA-sequencing (RNAseq) to look at the whole transcriptome of hPSC-CMs exposed to different combinations of the three maturation cues. Using principal component analysis (**Fig. 4.1b**), we found that the ComboMat group (all three factors combined together) separated well from all other conditions. Additionally, T3 exerted a pronounced effect on the transcriptome as all T3 treated groups cluster along PC2.

To better understand which pathways associated with cardiomyocyte maturation were being influenced by the different combination of maturation cues, we generated an enrichment heat map that consists of seven hallmark pathways of cardiomyocyte maturation, (**Fig. 4.1c**). We found that as more maturation conditions were layered on

top of one another, the cardiomyocytes appeared to be more mature. This culminated in the ComboMat group showing the most robust maturation with up-regulation in six of the pathways and down-regulation in cell cycle.

Since the heat map showed the ComboMat group had the highest degree of maturation based on our chosen seven pathways, we next decided to determine which processes, in general, were being most changed in the ComboMat group compared to Control (EV-Flat). **Figure 4.1d** shows 8 up-regulated gene ontology (GO) terms based on *P*-value. Pathways related to metabolism such as pyruvate and glucose metabolic processes, long-chain fatty acid import, and glycolytic process were up-regulated as well as GO terms associated with muscle processes including muscle tissue development, muscle contraction, and striated muscle cell differentiation.

To assess which genes were contributing the most in the separation of the ComboMat group in the PC analysis, we generated a bubble plot (**Fig. 4.1e**) to compare the ComboMat and Control groups along PC1. The gene that was most up-regulated along PC1 in the ComboMat group was Hair Growth Associated gene (HR), a thyroid hormone co-repressor. Krupel-like factor (KLF9), a transcription factor, was also significantly up-regulated. KLF9 has been reported to bind to the PPAR γ promoter, a critical gene in fatty acid metabolism²⁶¹. Cytochrome c oxidase subunit 6A2 (COX6A2), another gene associated with cardiac metabolism, was also up-regulated in the ComboMat group. Furthermore, myosin light chain 2 (MYL2), the ventricular isoform and a hallmark of cardiomyocyte maturation, was also up-regulated. For comparison, we have also created a bubble plot comparing human fetal and adult cardiomyocyte gene expression (**Fig. 4.1f**) with the same genes highlighted in **Figure 4.1e**.

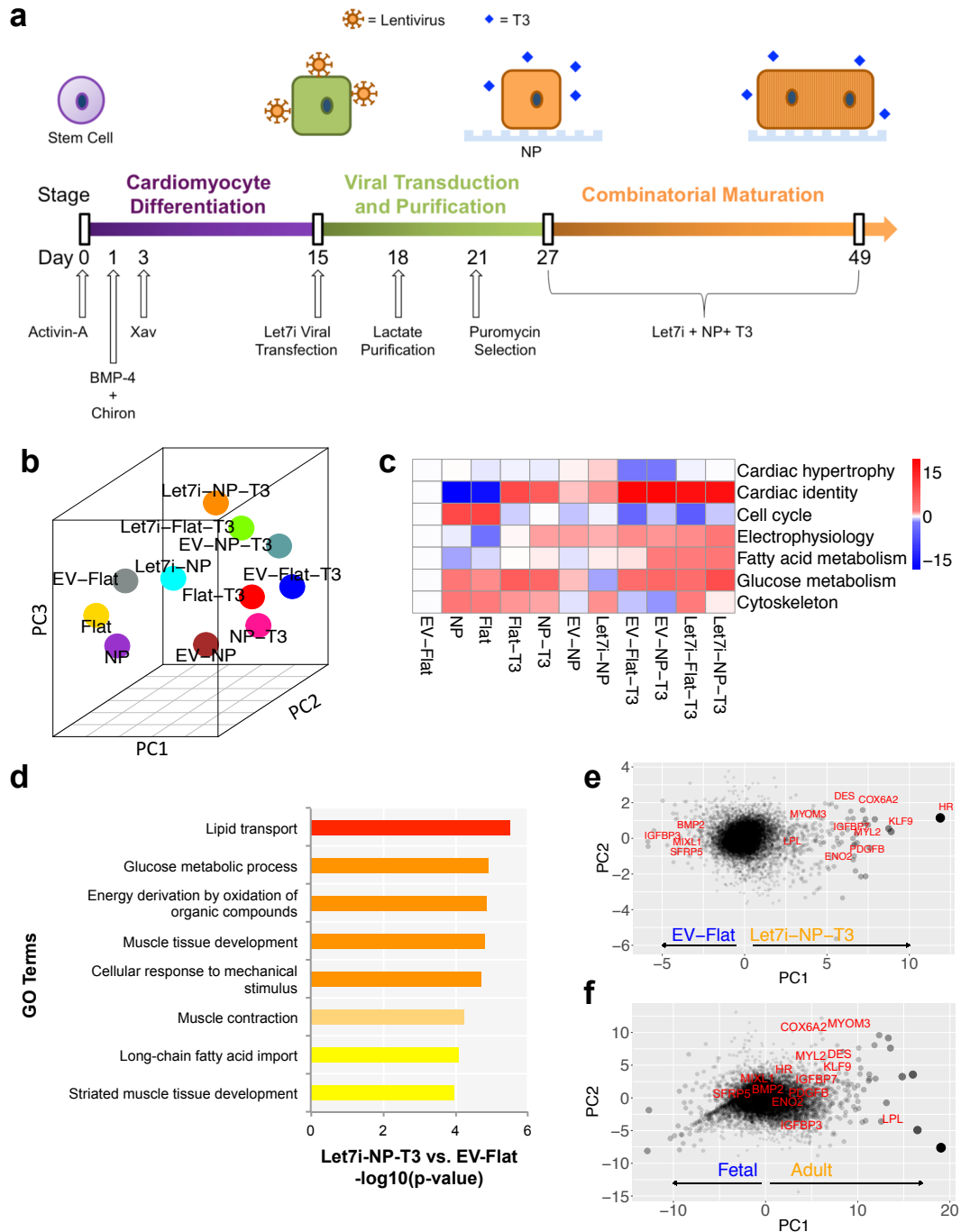


Figure 4.1. ComboMat Protocol Promotes Transcriptional Maturation. (A) Schematic timeline of the ComboMat protocol. (B) Visualization of the Principle Component Analysis in three-dimensions. (C) Net enrichment in cardiac maturation pathways of differentially expressed genes as a result of different treatments. Red indicates genes up-regulated by the treatment show more significant enrichment in the pathway than genes downregulated by the treatment; blue means the opposite. (D) Gene ontology enrichment results of up-regulated genes in Let7i-NP-T3 combination treatment compared to EV-Flat control. Color gradient and bar lengths represent significance of enrichment. (E-F) Bubble plot of PCA loadings showing genes' contributions to the separation of RNAseq samples comparing EV-Flat to Let7i-NP-T3 (E) and fetal to adult cardiac cardiomyocytes (F).

4.4.2 Structural Maturation and Sarcomere Development

Immunofluorescence imaging (**Fig. 4.2a**) confirmed that hPSC-CMs exposed to NPs elongate in the direction of the nanotopography with a more anisotropic, rod-shaped morphologies. Sarcomeres also develop with a higher degree of directionality and order on NPs, resulting in more sarcomeres in register with one another (**Fig. 4.2b**). Compared to Control, hPSC-CMs exposed to just T3 or Let7i were significantly larger (**Fig. 4.2f**) but exhibited rounded or irregularly morphologies. When exposed to all three cues in the ComboMat protocol, hPSC-CMs were rod-shaped as well as significantly larger than each cue in isolation (**Fig. 4.2f**). hPSC-CMs exposed to the ComboMat protocol also displayed a repetitive sarcomere banding pattern along the length of the cell in contrast to the circumferential banding found in the Control group, corresponding to a longer resting sarcomere length around 1.8 μm (**Fig. 4.2c**). hPSC-CMs in the ComboMat group also exhibited a higher binucleation percentage (**Fig. 4.2e**) in the range of what is found in the adult human heart²⁶².

Investigating the ultrastructure of the hPSC-CMs via transmission electron microscopy (TEM), we found that hPSC-CMs exposed to Control conditions exhibited low density, disorganized myofibrils and only punctate Z-body formation (**Fig. 4.2b**). The application of NPs, T3, or Let7i by themselves improved the development of more organized, wider Z-bands (**Fig. 4.2d**) but overall sarcomeres remained rather disorganized and at a low density. In contrast, hPSC-CMs exposed to the ComboMat protocol developed much more ordered sarcomeres with the emergence of Z-bands and H-zones (**Fig. 4.2b**). Z-band width was also significantly wider in the ComboMat group than Control or each cue in isolation.

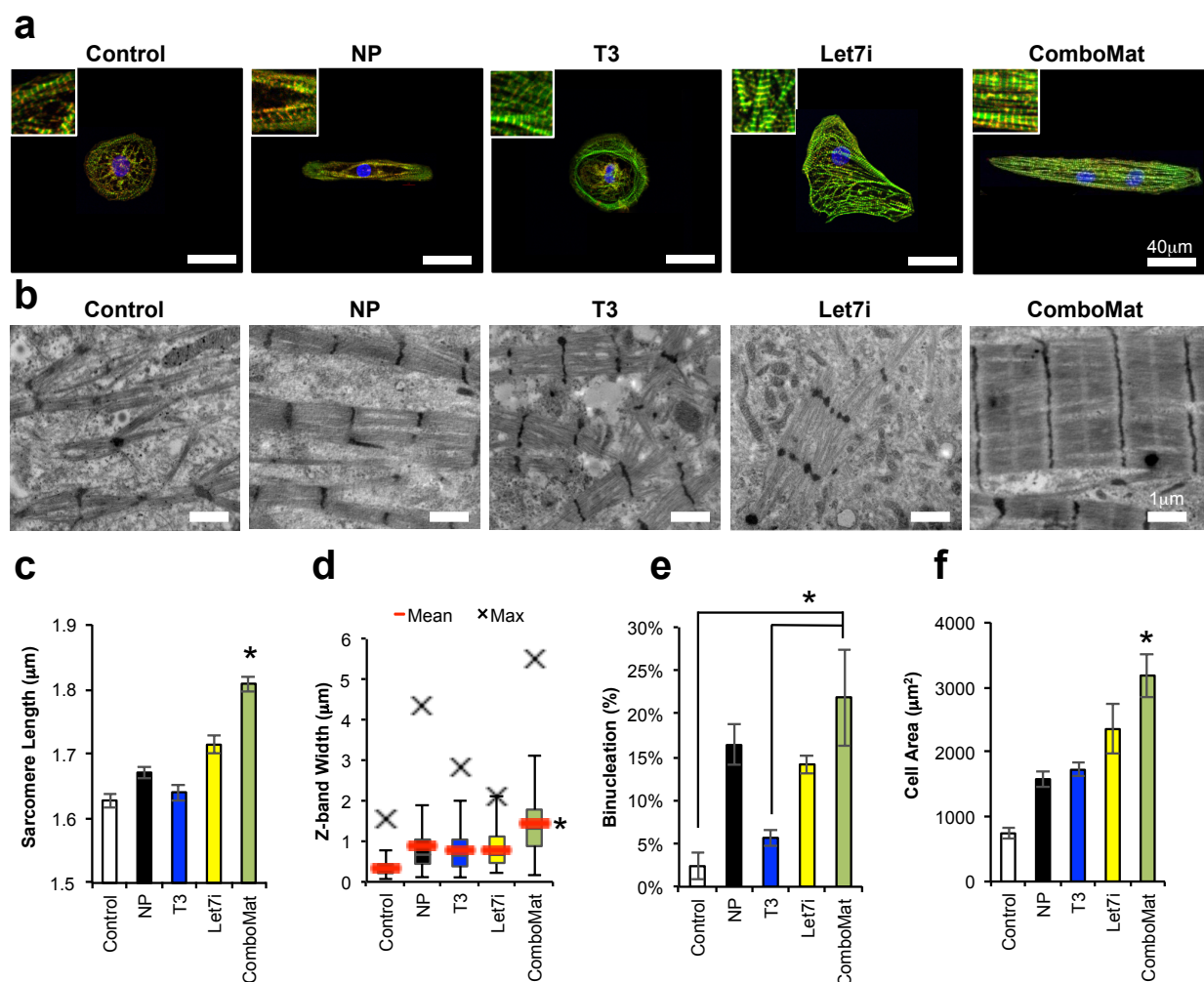


Figure 4.2. Structural Maturation of hPSC-CMs. (A) Immunofluorescent images of hPSC-CMs exposed to the ComboMat protocol or each cue in isolation. Red = α -actinin, Green = F-actin, Blue = DAPI. (B) TEM images of sarcomeres from hPSC-CMs exposed to the labeled cues of the ComboMat protocol. Quantification of sarcomere length (C), Z-band width (D), binucleation percentage (E), and cell area (F) for hPSC-CMs exposed to the ComboMat protocol or each cue in isolation. Bars represent averages $\pm$ S.E.M. * $P < 0.05$.

4.4.3 Electromechanical Functional Improvements

To test if the genetic and structural changes imparted by the ComboMat protocol manifested into enhanced functional performance of the hPSC-CMs, we employed two noninvasive measurements of electromechanical function. Using Correlation-based

Contraction Quantification (CCQ)¹¹¹, we measured contractions of hPSC-CM monolayers paced at 1 Hz and filmed at 30 frames per second. By spatially averaging these contraction vectors together, we measured maximum contraction and relaxation velocities (annotated as red and blue marks in **Fig. 4.3a**) for the Control, NO, T3, Let7i, and ComboMat condition and found that cells exposed to the ComboMat protocol had significantly faster contractions compared to Control or any maturation cue in isolation (**Fig. 4.3b**). To help explain this, we examined the angles of displacement during contractions and found that hPSC-CMs cultured on NPs contract in a more uniform direction than cells cultured on flat substrates (**Fig. 4.3c**). To quantify this preferred directionality, we measured the contraction anisotropic ratio (AR) and found that cells on NPs, such as those in the ComboMat protocol, had a contraction magnitude over two times greater in the direction parallel with the underlying nanotopography than perpendicular to it (ComboMat AR = 2.39 ± 0.01) (**Fig. 4.3d**). This was in contrast with cells grown on traditionally flat substrates, where contractions are randomly oriented, which contracted with the same magnitude in all directions (Control AR = 1.04 ± 0.02).

In addition to contraction dynamics, we also sought to measure cardiomyocyte electrical activity using multielectrode arrays (MEAs). **Figure 4.3e** shows temporally averaged field potential recordings from Control and ComboMat cultures. Measuring spontaneous electrical activity of cardiac monolayers, we found that hPSC-CMs exposed to the ComboMat protocol had significantly longer field potential durations than the Control, NP, and T3 groups (**Fig. 4.3f**). Additionally, the ComboMat protocol significantly slowed the beat rate of the cardiomyocytes compared to Control or each maturation cue in isolation (**Fig. 4.3g**).

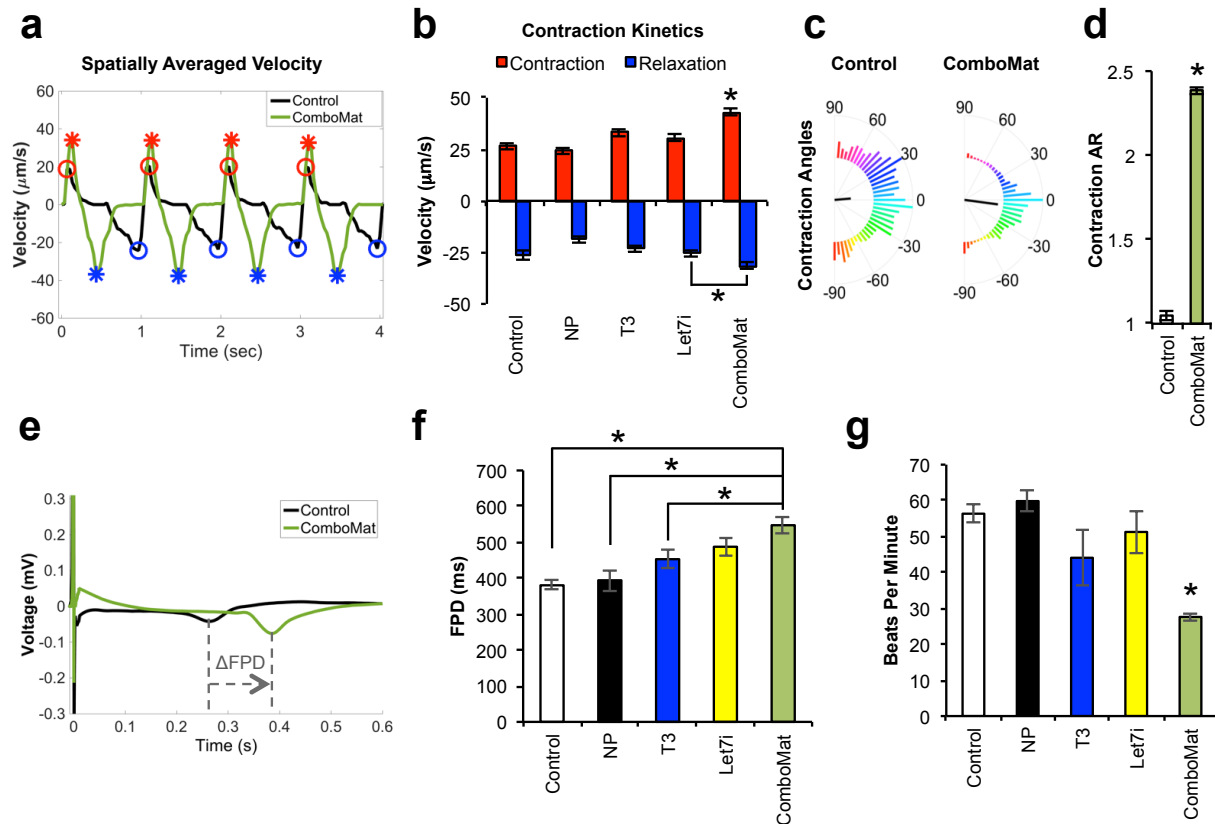


Figure 4.3. Electromechanical Maturation. (A) Representative traces of contraction velocities averaged across an entire video frame for Control or ComboMat hPSC-CMs. Red marks indicate peak contraction velocity, while blue demark peak relaxation velocity. (B) Quantification of peak contraction and relaxation velocities. (C) Contraction angle distribution histogram for Control or ComboMat hPSC-CMs. (D) Contraction anisotropic ratio of the magnitude of contraction in the longitudinal and transverse directions. (E) Representative field potential recording denoting the change in FPD between Control and ComboMat hPSC-CMs. Quantification of FPD (F) and beat rate expressed as Beats Per Minute (G) for hPSC-CMs exposed to the CombMat protocol of each cue in isolation. Bars represent averages $\pm$ S.E.M. * $P < 0.05$.

4.4.4 Metabolic Shift and Maturation

Our RNAseq analysis presented above suggested significant changes to cardiac metabolism imparted by the ComboMat protocol. Thus we probed the overall metabolic capacity as well as fatty acid utilization of the cardiomyocytes using a Seahorse mitochondrial flux analyzer. Using the “MitoStress assay” (**Fig. 4.4a**), we found that all groups had similar basal respiration levels but cells exposed to the ComboMat protocol

had significantly larger changes in OCR after FCCP addition than Control (**Fig. 4.4b**). Interestingly, we also found that NPs had a significant impact on maximum respiratory capacity even after being re-plated onto the flat plastic surfaces of the Seahorse micro-chambers for three days prior to measurements. We also explored the ability of the cardiomyocytes to utilize exogenous fatty acids via the “Palmitate assay” (**Fig. 4.4c**) in both an hiPS (UC3-4) and hES (RUES2) cell line. For UC3-4 hPSC-CMs, this fatty acid stress test revealed that cells exposed to just T3 or the full ComboMat protocol had greatest change in OCR after palmitate addition (**Fig. 4.4d,e**). For RUES2 hPSC-CMs, we found the ComboMat group alone had the greatest change in OCR after palmitate exposure (**Fig. 4.4e**).

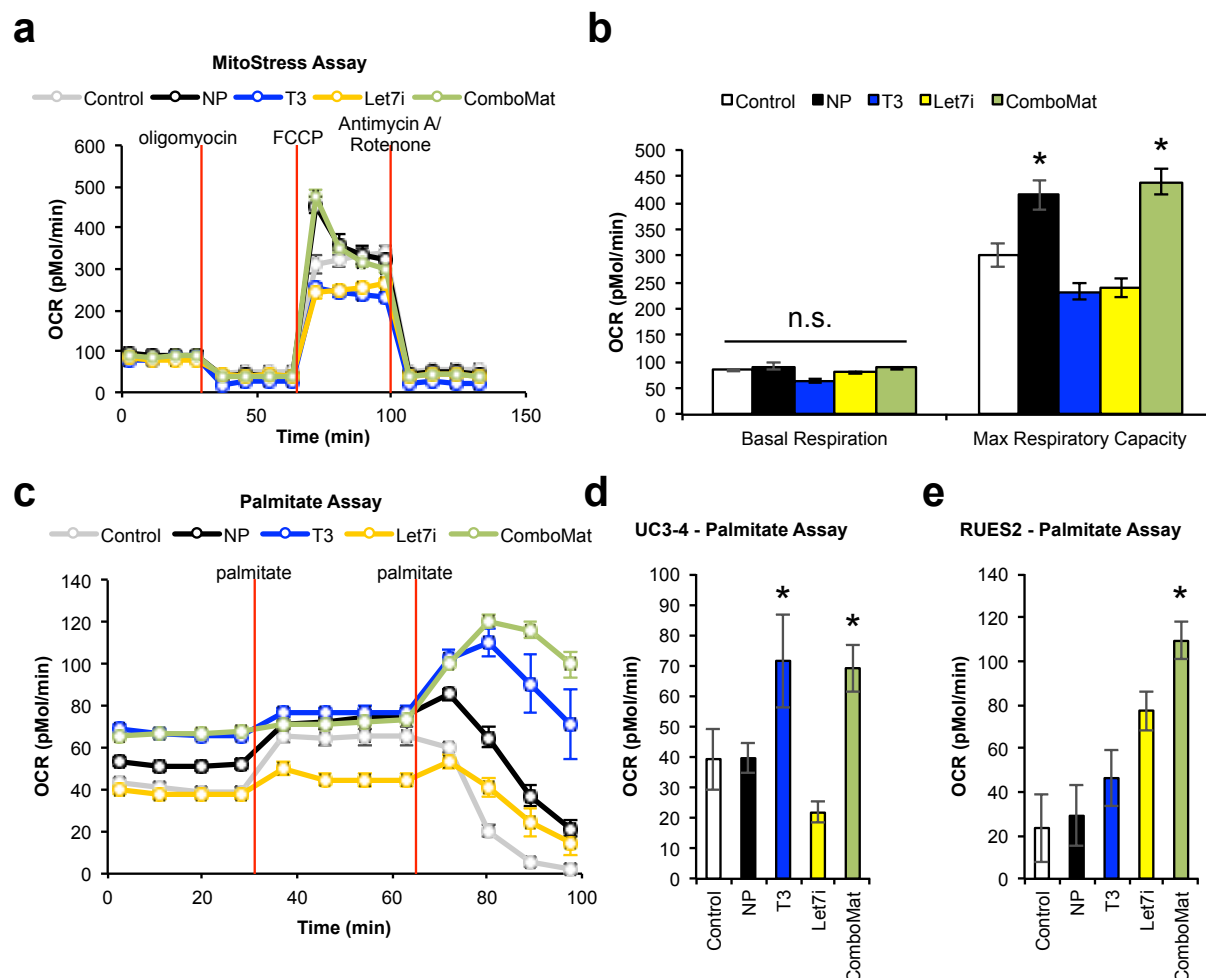


Figure 4.4. Seahorse Metabolic Assays. (A) Representative OCR profile in response to ATP synthase inhibitor oligomycin, uncoupler of electron transport and oxidative phosphorylation (FCCP), and electron transport chain blockers rotenone and antimycin during MitoStree assay. (B) Quantification of basal and maximum respiratory capacity. (C) Representative OCR trace for fatty acid stress test using palmitate. (D-E) Quantification of OCR change in response to palmitate for UC3-4 hiPSC-CMs (D) and RUES2 hESC-CMs (E). Bars represent averages $\pm$ S.E.M. * P <0.05.

4.5 Discussion

Building functional heart muscle *in vitro* from hPSC-CMs is an attractive alternative to animal models because hPSC-CMs theoretically express the full array of ion channels, contractile proteins, and metabolic machinery found in the human heart. In order to be adopted, *in vitro* screening assays must predict the response of the

human heart with high fidelity. Functional immaturity, however, remains one of the greatest limitations facing hPSC-CM translational efforts, particularly in preclinical screening application. Due to the complexity of cardiac development, it is unlikely there exists a single “silver bullet” method or factor for producing truly mature cardiomyocytes. The ComboMat protocol utilizes nanotopographic substrate cues, thyroid hormone T3, and Let-7i microRNA (miR) overexpression at specific time-points during the culture period to induce advanced structural and phenotypic development of hPSC-CMs. Each cue was chosen to impact specific aspects of hPSC-CM development. Nanotopographic substrate cues have been shown to promote the structural maturation of primary and hPSC-CMs^{101,103}. These biophysical cues, however, have minimal impact on the metabolic development of hPSC-CMs. Therefore, thyroid hormone T3 was introduced as a biochemical cue to boost the metabolic, calcium handling, and contractile performance of the hPSC-CMs⁸³. Preliminary results found, though, that long-term T3 exposure had a negative impact on sarcomere development and beat rate variability (**Fig. 4.5**), reminiscent of complications from hyperthyroidism. To counteract these detrimental effects, we sought to manipulate the transcriptome using miRs such as the Let7 family. Along with improving metabolic capacity, hypertrophy, and the genetic profile¹⁶², Let7i helps to regulate the expression of the Hair Growth Associated gene that acts as a transcriptional co-repressor of many nuclear receptors, including the thyroid hormone receptor.

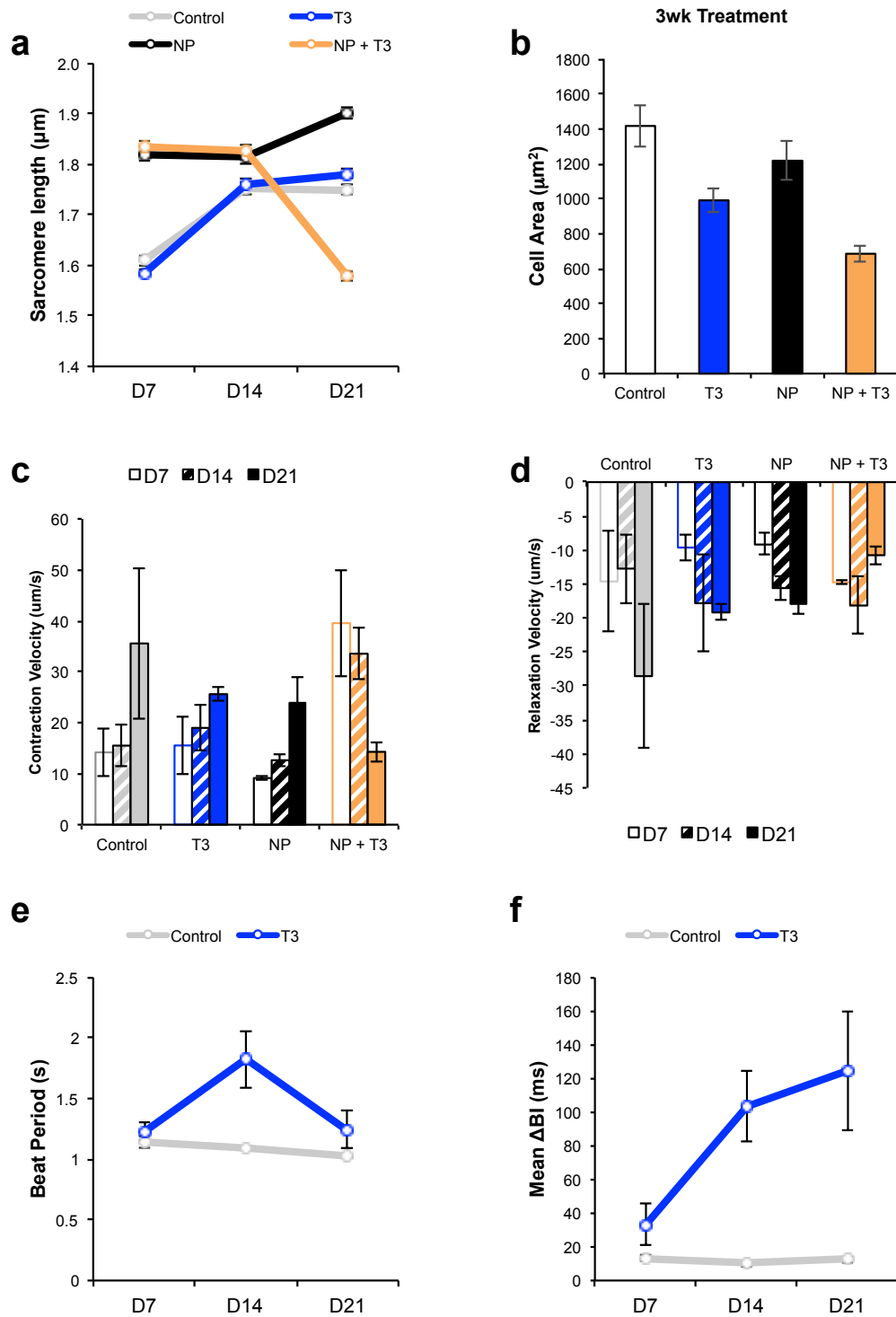


Figure 4.5. Long-Term T3 Exposure Without Let7i. Quantification of sarcomere length (A), cell area (B), contraction and relaxation velocities (C-D), beat period (E) and mean change in beat interval (F) for hPSC-CMs exposed to control or T3 conditions. Extended T3 exposure resulted in an overall decrease in structure and performance compared to control conditions. hPSC-CMs displayed a biphasic response to T3, with an initial benefit but a reduction in structure in function after 3 weeks of exposure. This differed from hPSC-CMs exposed to Let7i, which seemed to help mitigate these long-term negative impacts of T3.

The ComboMat protocol proved to be a powerful tool in modulating cardiomyocyte development *in vitro*, increasing cell area, producing longer resting sarcomere lengths, and achieving more physiologically relevant binucleation percentages. Many of the functional enhancements that we observed from the ComboMat protocol were predicted by our RNAseq analysis. GO terms for muscle development and contraction were upregulated in the ComboMat group, which resulted in faster contraction kinetics as measured by our CCQ contraction mapping. Metabolic GO terms such as fatty acid import were also upregulated in the ComboMat group and subsequently we found that cells exposed to the ComboMat group had a larger spare respiratory capacity and ability to utilize exogenous fatty acids. Thus, there was agreement between the transcriptional and functional changes imparted by the ComboMat protocol providing validation of our methods.

It is of note that we also observed deviations from previous findings^{83,162} in both the MitoStress and Palmitate assays with UC3-4 hPSC-CMs. In the MitoStress assay, the T3 and Let7i groups unexpectedly had lower maximum respiratory capacities than Control (**Fig. 4.4b**). In the Palmitate assay, the Let7i group also unexpectedly had a lower response to palmitate addition than Control. To validate our methods, we also performed the Palmitate assay with RUES2 hESC-CMs and found a trend that confirmed previous findings¹⁶². Thus we attribute the deviation from previous findings to cell line variability and inherent genetic and functional differences. What was most promising was that regardless of which cell line used, the ComboMat protocol imparted

the highest degree of metabolic functional benefit, increasing the protocol's applicability to other cell sources.

Chapter 5. Mature Cardiac DMD Model and Phenotypic Drug Screen

*Note: Chapters 4 and 5 are being submitted as a single manuscript for publication.

5.1 Abstract

Cardiac differentiation of human pluripotent stem cells (hPSCs) typically produces cardiomyocytes with structural, functional, and biochemical properties that most closely resemble the fetal heart. Previously we presented a combinatorial method (ComboMat) that produces hPSC-CMs with enhanced sarcomere development, improved electrophysiological and contractile function, increased mitochondrial respiratory capacity, and a transcriptome upregulated for metabolic and muscle development. When the ComboMat protocol is applied to a CRISPR-edited dystrophin knockout (KO) model of DMD cardiomyopathy, a distinctive, endogenously occurring disease phenotype emerges. Mature dystrophin KO hPSC-CMs exhibit greater propensity for arrhythmia with a higher resting cytosolic calcium content compared to hPSC-CM controls. A phenotypic drug screen of dystrophin KO hPSC-CMs using the ComboMat protocol identified compounds that mitigated arrhythmogenic behavior. The ComboMat protocol can be applied to other cardiac disease models, cardiotoxicity studies, or cardiac tissue engineering applications.

5.2 Rationale

Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disorder resulting from a loss of dystrophin protein expression in skeletal and cardiac muscle. Cardiomyopathy has replaced respiratory failure as the leading cause of death in DMD patients as

greater attention to supportive ventilation emerged as a standard of care²⁴⁷. Animal models of DMD, such as the *mdx* mouse, have shown to be poor predictors of patients' response in the clinic^{78,80}. Our previous reports demonstrated that DMD patient-derived and CRISPR-mediated dystrophin knockout hPSC-CMs display phenotypic differences compared to healthy controls but these differences were mild or were induced by exogenous acute stress such as hypotonic challenge^{65,111}. Other models, developed by Lin et al.⁸¹ and Young et al.¹⁹³, provide valuable insight into the DMD-specific disease mechanism and potential therapeutic treatment options, but both reports were based on fetal-like hPSC-CMs. We hypothesize that combinatorial protocols to advance the developmental program and maturation hPSC-CMs will improve the predictive model of cardiomyopathy found in patients with DMD.

To test this hypothesis, we developed a bioengineering strategy, termed the ComboMat protocol (Chapter 4), aimed at accelerating the maturation of hPSC-CMs to exacerbate adult-onset cardiac disease phenotypes *in vitro*. When matured using our combinatorial approach, CRISPR-generated dystrophin knockout (KO DMD) hPSC-CMs exhibit a much greater propensity for arrhythmia on a multi-electrode array (MEA) cardiac screening platform and have a higher resting cytosolic calcium content. Without maturation, we could not distinguish KO DMD hPSC-CMs from healthy isogenic control cells. This exacerbated disease state enables the ability to more easily screen potential therapeutic compounds, such as Ca^{2+} channel blockers, while effectively screening out known false positives such as sildenafil⁸⁰. Thus, the ComboMat protocol produced more physiologically relevant hPSC-CMs for disease modeling and drug screening applications.

5.3 Scientific Methods

Cardiac differentiation, cell culture, ComboMat treatment, nanofabrication of nanopatterns and NanoMEAs, immunofluorescent imaging, mitochondrial functional assay, MEA electrophysiological analysis, and statistical analyses were all performed in the same manner described in the Chapter 4 scientific methods section.

5.3.1 hiPSC Cell Line Generation and Maintenance

KO DMD hiPSCs (UC1015-6) were generated using CRISPR-Cas9 technology to create an isogenic pair from the Normal parental line UC3-4. Briefly, guide sequences targeting the first muscle specific exon of human dystrophin were used to delete a single G base at position 263 of the dystrophin gene to create a null allele. KO of dystrophin was confirmed via RT-PCR. KO DMD hiSPCs were maintained in the same manner as the Normal UC3-4 parental line.

5.3.2 MEA Electrophysiological Analysis

Electrophysiological analysis of spontaneously beating cardiomyocytes was collected for 2 minutes using the AxIS software (Axion Biosystems). After raw data collection, the signal was filtered using a Butterworth band-pass filter and a 90 μ V spike detection threshold. Field potential duration was automatically determined using a polynomial fit T-wave detected algorithm. Beat interval analysis on 30 consecutive beats was performed as previously published⁴⁴ to output the following 6 parameters:

Table 5.1. MEA-based Beat Interval Arrhythmia Parameters

Parameter	Description
StDev BI	The standard deviation of beat intervals.
Mean Δ BI	The mean absolute value of the difference between consecutive beats.
Median Δ BI	The median absolute value of the differences between consecutive beats.
StDev Δ BI	The standard deviation of the absolute value of the difference between consecutive beats.
% Δ BI > 100ms	The percentage of absolute Δ BI values exceeding 100 ms.
% Δ BI > 250ms	The percentage of absolute Δ BI values exceeding 250 ms.

5.3.3 Calcium Imaging

Intracellular calcium content was measured using the ratiometric indicator dye fura2-AM as described previously⁸⁶. In brief, cells were incubated in 0.2 μ M fura2-AM dye in Tyrode's solution for 20 minutes at 37°C and washed with PBS. Spontaneous Ca²⁺ transients were then monitored with the Ionoptix Stepper Switch system coupled to a Nikon inverted fluorescence microscope. The fluorescence signal was acquired using a 40x Olympus objective and passed through a 510 nm filter, and the signal was quantified using a photomultiplier tube. Diastolic resting calcium concentration was quantified in the IonOptix software IonWizard using the formula:

$$[Ca^{2+}] = K_d * \frac{R - R_{min}}{R_{max} - R} * \frac{Sf2}{Sb2}, \quad \text{where}$$

K_d = fura2 calcium dissociation constant = 225nM

$R_{\max}$ and $R_{\min}$ = ratio values measured under saturating calcium levels and in the absence of calcium, respectively.

Sf2/Sb2 = ratio between the background subtracted wavelength 2 excited fluorescence in zero and saturating calcium solutions, respectively.

5.3.4 High Throughput Screening

Cardiomyocytes were dissociated 14 days post differentiation into a single cell suspension by 5 minute incubation in TrypLE (Life Technologies) and plated at 10,000 cells per well on opaque-bottom 384-well plates (Nunc) pre-coated with 1 mg/mL Matrigel (Corning) for one hour at 37°C. Cells were then cultured on the 384-well plates for an additional 16 days, with media exchanges every 72 hours. After 15 days, compounds dissolved in DMSO were distributed to the plates using the CyBi Well Vario 384/25 liquid handler (Cybio, Germany) to achieve concentrations of 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M in duplicate. After 18 hours of incubation, medium was aspirated and pure water was injected to reach 12.5% normal osmolarity using BioTek EL406 Washer Dispenser (BioTek, Winooski, VT). After 30 minutes incubation, supernatants were removed and plates were assayed by adding CellTiter Glo (Promega) according to the manufacturer's instructions. After 5 minutes, the luminescence was measured using EnVision Multilable Resaer (PerkinElmer). All data were processed and visualized by Tibco Spotfire (Tibco Spotfire, Boston, MA). Percentage visibility was calculated by comparing signal from each well to the average of the control wells treated with DMSO alone (32 control wells per plate).

5.3.5 Phenotypic Drug Screen

Cardiomyocytes were dissociated and plated onto NanoMEA platforms as described above. After four days post-replating, baseline electrophysiological measurements were made. Cells were then exposed to drugs ranging four orders of magnitude and MEA recording were taken every seven days after drug exposure. For Ca^{2+} imaging, the drug concentration resulting in the largest reduction in beat rate variability was chosen and cells were exposed to that concentration for seven days prior to imaging.

5.4 Results

5.4.1 ComboMat Protocol Matures KO DMD hPSC-CMs to a Similar Level as Normal Controls

To begin, we tested whether dystrophin knockout (KO DMD) hPSC-CMs responded in a similar manner to the ComboMat protocol as their Normal (UC3-4) isogenic counterparts described above. Using the Seahorse MitoStress assay (**Fig. 5.1a**), we found that KO DMD hiPSC-CMs had an increased maximum respiratory capacity when exposed to the ComboMat protocol compared to Control conditions (**Fig. 5.1b**). Within the Control or ComboMat conditions, however, there was no statistical difference between the Normal and KO DMD groups. We also measured the baseline electrophysiological characteristics of the KO DMD cells using our custom MEA platform. The beat rate of the KO DMD hiPSC-CMs decreased in response to the ComboMat protocol to the same extent as the Normal hPSC-CMs (**Fig. 5.1c**). Additionally, the FPD of KO DMD hPSC-CMs increased when exposed to ComboMat compared to Control similarly as the Normal hPSC-CMs (**Fig. 5.1d**). From a structural

standpoint, the KO DMD hiPSC-CMs exhibited the characteristic elongated morphology with in-register sarcomeres when exposed to the ComboMat protocol, whereas KO DMD hiPSC-CMs exposed to Control conditions were more rounded with more disorganized sarcomeres (Fig. 5.1e).

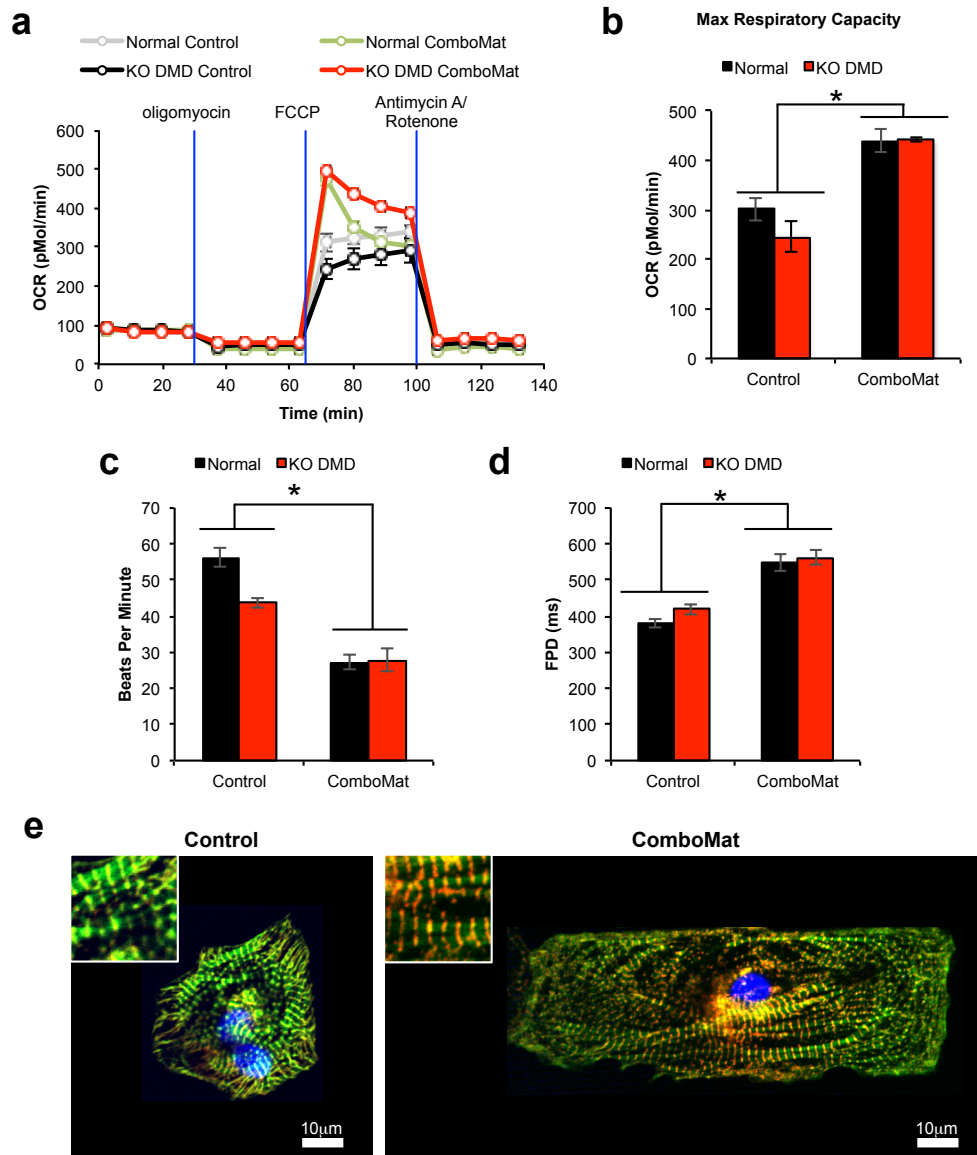


Figure 5.1. KO DMD hiPSC-CMs Respond to ComboMat Protocol Similarly as Normal Control. (A-B) Seahorse metabolic profile of KO DMD hiPSC-CMs exposed to Control or ComboMat conditions compared to Normal hiPSC-CMs. (C-D) MEA-based measurements of electrophysiology. (E) Immunofluorescent images of KO DMD hiPSC-CMs exposed to Control (left) and ComboMat (right) conditions. Red = α -actinin, Green = F-actin, Blue = DAPI. Bars represent averages $\pm$ S.E.M. *P<0.05.

5.4.2 Endogenous Disease Phenotype in Dystrophin-Null hPSC-CMs

We hypothesized that by inducing hypertrophy and overall cardiomyocyte maturation, DMD hiPSC-CMs would inflict greater mechanical stress and cellular damage, resulting in irregular electrical activity and arrhythmias, which would be absent in immature cells. We used the NanoMEA platform to record the electrical activity of spontaneously beating monolayers of the isogenic pair of Normal and KO DMD hPSC-CMs exposed to Control or ComboMat conditions. Using a method of arrhythmia detection relying on beat rate variability⁴⁴, we found that KO DMD hPSC-CMs exposed to the ComboMat protocol were significantly more arrhythmogenic than less mature KO DMD hPSC-CMs in the Control condition. **Figure 5.2a** shows representative field potential traces from KO DMD hPSC-CMs exposed to Control (black) or ComboMat (red) conditions with individual beat intervals annotated. By measuring the difference in time of the beat interval from one beat to the next (ΔBI) and plotting this change for 30 consecutive beats (**Fig. 5.2b**), we found that matured KO DMD hPSC-CMs had a striking instability in beat rate. From one beat to the next, we saw relatively large changes in beat intervals indicative of a more arrhythmogenic state. Without an autonomous nervous system in the dish, hPSC-CMs should develop a steady beating pattern without much variation from beat to beat. This steady behavior is apparent in the Control conditions and healthy Normal cells (**Fig. 5.2b**). To quantify the level of beat interval variability, we calculated the percentage of beats with a $\Delta BI > 250$ ms (**Fig. 5.2c**) and the arithmetic mean for ΔBI over 30 consecutive beats (**Fig. 5.2d**).

To identify a potential mechanism for this beat rate variability, we measured intracellular calcium (Ca^{2+}) concentrations using the ratiometric Ca^{2+} indicator Fura-2 on

an IonOptix imaging setup calibrated to calculate nanomolar intracellular Ca^{2+} concentrations. **Figure 5.2e** depicts representative Ca^{2+} traces from KO DMD hPSC-CMs exposed to Control or ComboMat conditions. Matured KO DMD hPSC-CMs have an elevated baseline, or diastolic resting Ca^{2+} level, compared to the less mature cells in the Control group. Quantifying this diastolic Ca^{2+} level, we found a similar trend to the electrical instability presented previously. Normal and KO DMD hPSC-CMs in the Control groups had cytosolic Ca^{2+} levels that were not statistically different from one another (**Fig. 5.2e**). KO DMD hPSC-CMs matured using the ComboMat protocol, however, have an elevated cytosolic Ca^{2+} concentration compared to Normal hPSC-CMs.

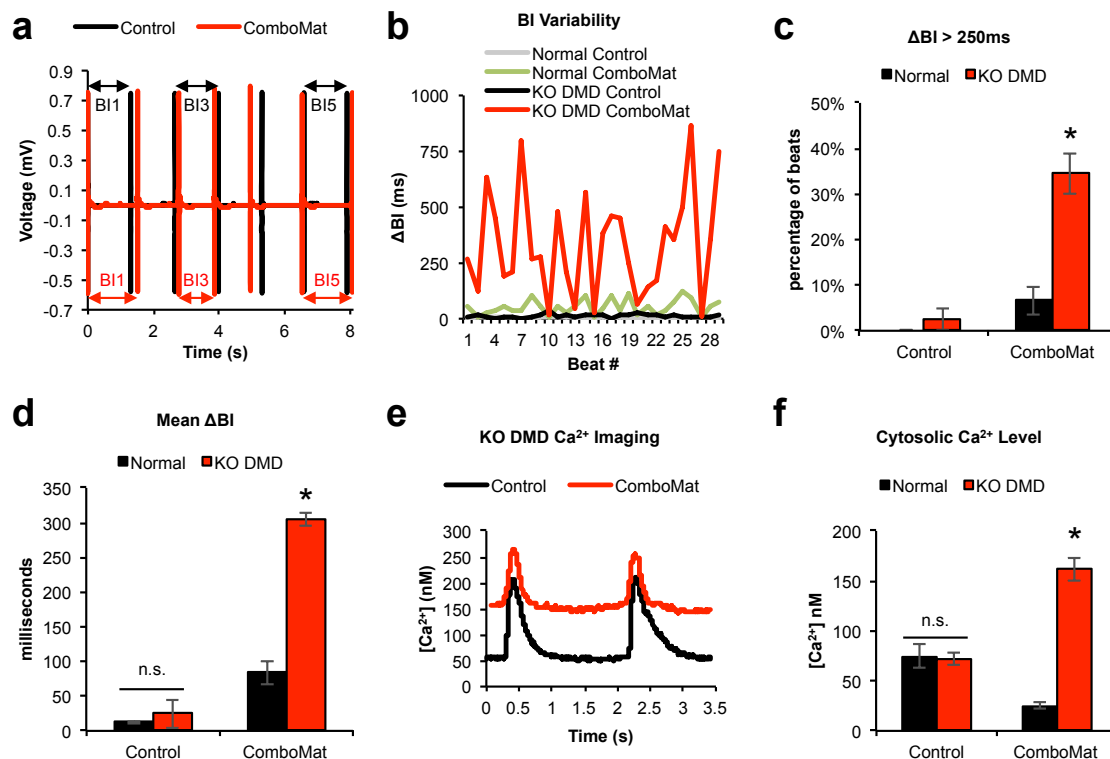


Figure 5.2. Endogenous DMD Disease Phenotype Exposed. (A) Representative field potential recordings with beat intervals annotated. (B) Change in beat interval (ΔBI) plotted for 30 consecutive beats. (C) Average percentage of beats with a $\Delta\text{BI} > 250\text{ms}$. (D) The mean ΔBI for entire recording. (E) Representative Fura-2 Ca^{2+} imaging traces. (F) Quantification of diastolic Ca^{2+} content in the cytosol. Bars represent averages $\pm$ S.E.M. * $P < 0.05$.

Importantly, when comparing Normal cells to the KO DMD hPSC-CMs, it is only when the cells are matured using the ComboMat protocol that a separation can be easily identified. When the maturation cues are applied in isolation there is no separation between the healthy Normal cells and the KO DMD hPSC-CMs (**Fig. 5.3**). In the Control condition where the cells are less mature, Normal and KO DMD hPSC-CMs have indistinguishable electrical characteristics. This disease phenotype (increase arrhythmogenic behavior) is therefore maturation-dependent.

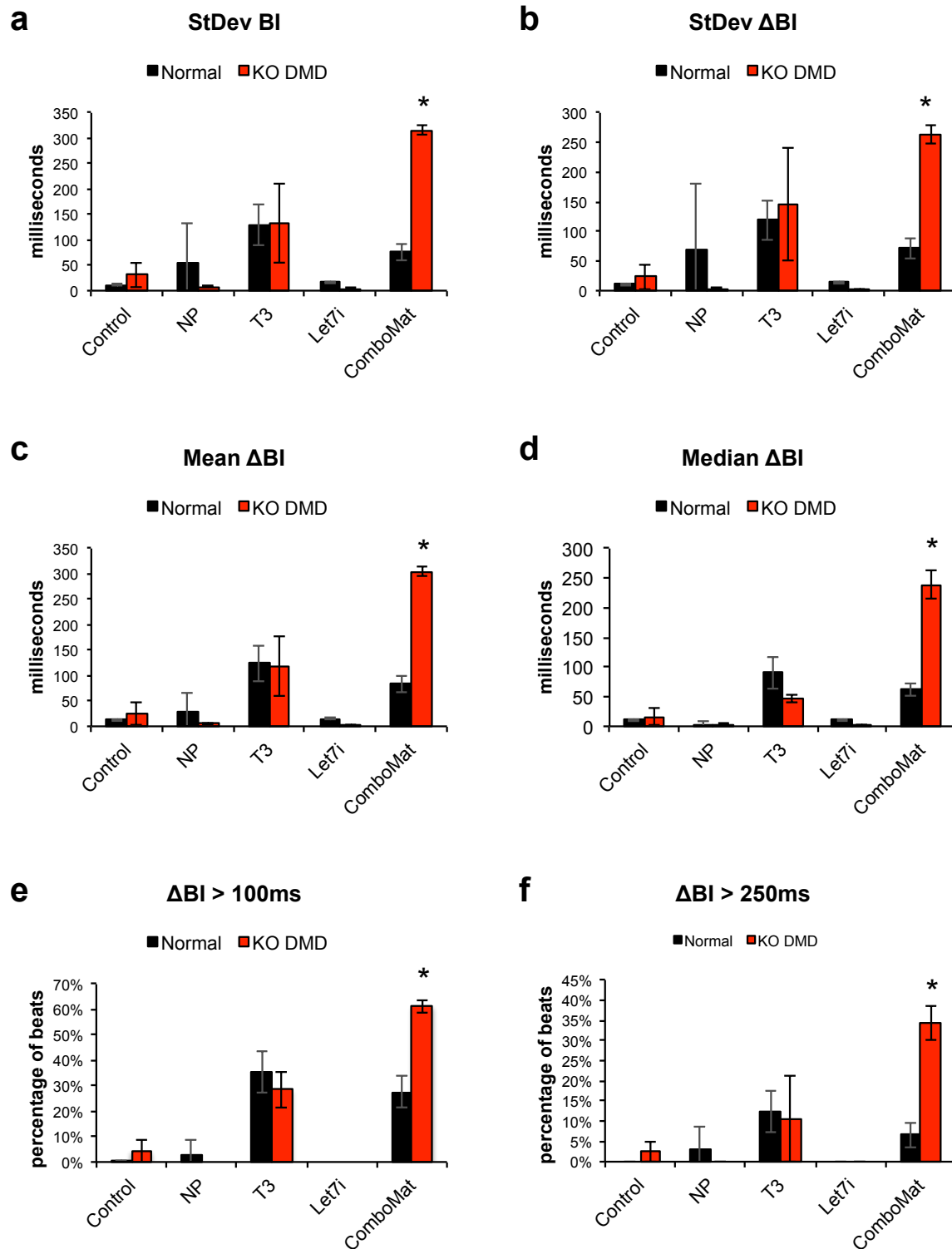


Figure 5.3. Full ComboMat Necessary for Disease State Stratification. (A-F) Arrhythmia metrics as outlined by Gilchrist et al.⁴⁴ There is no statistical difference between Normal and KO DMD hPSC-CMs when the maturation cues are employed in isolation but when combined into the ComboMat protocol, there is a separation between the healthy Normals and diseased KO DMD hPSC-CMs.

5.4.3 Phenotypic Drug Screen Using Mature hPSC-CMs

In order to identify potential drug targets or classes of drugs for a more detailed phenotypic drug screen, we first performed a preliminary medium throughput, semi-automated screen with a small library of 2,000 diverse molecules with various mechanisms of action. Intracellular ATP (a widely used assay of cell viability in high throughput screens) was measured in KO DMD hPSC-CMs after hypotonic stress. Overall the Z-prime of the assay was 0.71. The 8,000 data points (2,000 compounds at 4 concentrations), were ranked and plotted based on the Z-score (**Fig. 5.4a**). Taking into consideration the Z-score ranking (>3), standard deviation of replicates, and escalating dose-ranging response, 39 hits were identified from the 2000 input compounds (~2% hit rate) (**Table 5.1**). Of these hits, 9 (~23%) were classified as Ca^{2+} channel blockers.

Table 5.2. Primary High Throughput Screening Hits

Compound	Average of % Viability	Z score	Mechanism of action
ACEPROMAZINE MALEATE	239.1±38.37	6.57	Sedative
ACYCLOVIR	258.33±14.16	7.44	Antiviral
AKLOMIDE	276.60±12.37	8.26	Antiprotozoal, Coccidiostat
ALTRETAMINE	188.22±2.87	4.27	Antineoplastic
AMINOHIPURIC ACID	207.49±7.09	5.14	Renal function diagnosis
AMIODARONE HYDROCHLORIDE	168.14±8.45	3.37	Adrenergic agonist, coronary vasodilator, Ca channel blocker
ANTIMYCIN A	180.04±1.89	3.90	Antifungal, antiviral, interferes in cytochrome oxidation
ASCORBIC ACID	181.48±23.68	3.97	Antiscorbutic, antiviral
ATENOLOL	171.27±1.77	3.51	Beta adrenergic blocker
BENZALKONIUM CHLORIDE	194.64±23.10	4.56	Anti-infective (topical)
BEPRIDIL HYDROCHLORIDE	178.65±3.48	3.84	Antiarrhythmic
BIOTIN	315.95±51.89	10.04	Vitamin B complex
CANTHARIDIN	239.59±14.65	6.59	Blister agent (terpenoid)
CEFDINIR	185.70±45.76	4.16	Antibacterial
CHLORCYCLIZINE HYDROCHLORIDE	238.84±35.23	6.56	H1-antihistamine
CINCHOPHEN	168.44±44.63	3.38	Analgesic, antipyretic,

			antiinflammatory
CLOPIDOGREL SULFATE	194.84±50.3	4.57	Platelet aggregation inhibitor
CORTISONE ACETATE	205.92±12.32	5.07	Glucocorticoid
COTININE	188.79±56.0	4.30	Antidepressant
CROTAMITON	178.25±27.22	3.82	Antipruritic, Scabicide
DACARBAZINE	177.63±45.35	3.79	Antineoplastic
DOXORUBICIN	207.98±34.29	5.16	Antineoplastic
ESTROPIPATE	208.65±30.77	5.19	Estrogen
GITOXIGENIN DIACETATE	177.20±1.17	3.77	Cardiac glycoside
HALOPERIDOL	197.30±19.03	4.68	Antidyskinetic, antipsychotic
HYDROXYUREA	187.65±49.61	4.25	Antineoplastic, inhibits ribonucleoside diphosphate reductase
MEBHYDROLIN			
NAPHTHALENESULFONATE	172.81±39.86	3.58	H1 antihistamine
NAPROXOL	168.76±2.61	3.39	Antinflammatory, analgesic, antipyretic
NICOTINYL ALCOHOL TARTRATE	262.12±57.89	7.61	Vasodilator
NIMODIPINE	292.38±56.17	8.97	Calcium channel blocker, vasodilator
NITRENDIPINE	275.48±52.16	8.21	Calcium channel blocker, vasodilator
OXYPHENBUTAZONE	176.96±42.53	3.76	Anti-inflammatory
PODOFILOX	168.46±28.98	3.38	Antineoplastic, inhibits microtubule assembly, and human DNA topoisomerase II; antimitotic agent
PROGLUMIDE	175.69±50.35	3.71	Anticholinergic
PUROMYCIN HYDROCHLORIDE	187.84±39.68	4.25	Antineoplastic, antiprotozoal
RETINYL PALMITATE	236.79±46.63	6.46	Provitamin, antixerophthalmic
SELAMECTIN	172.91±25.29	3.58	Antiparasitic, antimite
SULFADOXINE	195.32±23.52	4.59	Antibacterial
TERFENADINE	211.28±22.46	5.31	H1 antihistamine, nonsedating,
TILOPHONE	172.53±15.79	3.56	Antiviral

Incorporating this preliminary drug screen data with our findings of elevated intracellular Ca^{2+} content (**Fig. 5.3f**), we sought to utilize the NanoMEA platform to test whether nitrendipine (highlighted with blue box in **Fig. 5.4a**), a dihydropyridine Ca^{2+} channel blocker, could reduce the arrhythmogenic behavior of the mature KO DMD hiPSC-CMs. We also tested sildenafil, a known false positive in DMD patients, to see if our platform could correctly distinguish between the two classes of drugs. Even at an

early time point (four day ComboMat exposure) there was a significant difference in baseline beat rate variability between mature Normal hPSC-CMs and KO DMD hPSC-CMs, while immature cells were indistinguishable from each other (**Fig. 5.4b**).

We then exposed to cells to various concentrations of nitrendipine or sildenafil citrate for seven days and measured their beat rate variability. **Figure 5.4c,d** show the dose-response curves for nitrendipine and sildenafil, respectively. Nitrendipine significantly reduced the Mean ΔBI in mature KO DMD hPSC-CMs compared to DMSO carrier control. Doses up to 1 μM nitrendipine restored beat rate variability to levels within physiologically relevant ranges (<50 ms). Higher doses, however, ceased spontaneous beating (data not shown). In contrast, sildenafil had no effect on beat rate variability, regardless of the dose. Neither nitrendipine nor sildenafil had a significant impact on beat rate variability for cells exposed to Control condition or Normal hPSC-CMs exposed to the ComboMat protocol. The benefits of nitrendipine were washed out within 48 hours if removed from the cell culture media (**Fig. 5.4e**). Ca^{2+} imaging revealed that 100 nM nitrendipine reduced the diastolic Ca^{2+} content in the mature KO DMD hPSC-CMs compared to DMSO control (**Fig. 5.4f,g**). Nitrendipine had no impact on the diastolic Ca^{2+} content in cells exposed to the Control condition (**Fig. 5.4g**).

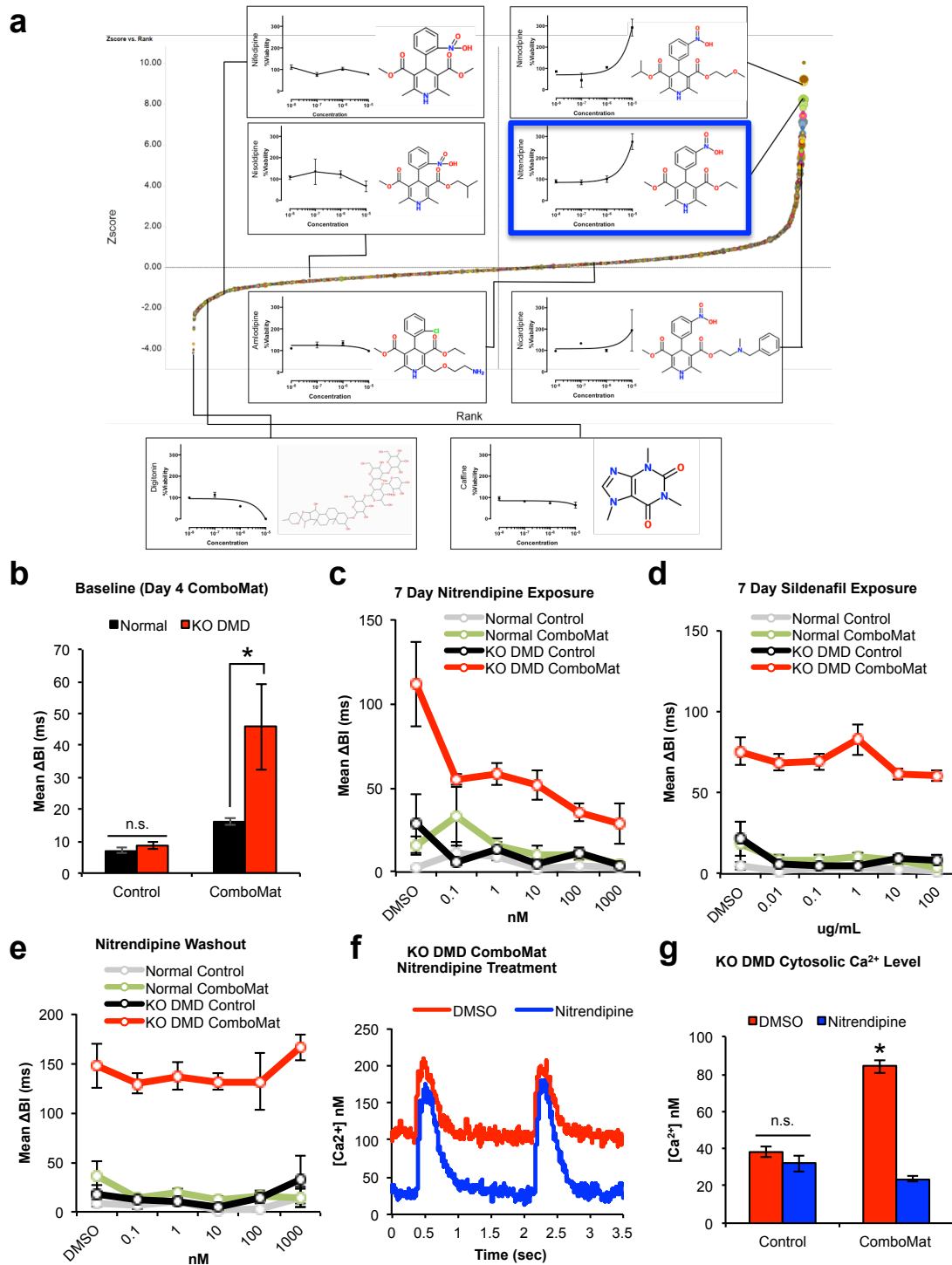


Figure 5.4. DMD Cardiomyopathy Phenotypic Drug Screen Using Mature hPSC-CMs. (A) Drug library ranked by Z-score for reduction in cell death in response to osmotic stress. Chemical structure of specific compounds also shown. (B) Quantification of mean ΔBI for Normal and KO DMD hPSC-CMs exposed to Control or ComboMat conditions. (C-D) Dose-response curve in response to nitrendipine and sildenafil after 7 days. (E) 48 hour washout of nitrendipine. (F) Representative Fura-2 Ca^{2+} traces and (G) quantification of diastolic Ca^{2+} content with and without nitrendipine. Bars represent averages $\pm$ S.E.M. * $P < 0.05$.

5.5 Discussion

The clinical manifestation of the cardiac involvement in DMD is generally described as cardiomyopathy complicated by arrhythmias²⁴⁷. Most patients, however, are asymptomatic in childhood and early teen years, even when skeletal muscle degeneration has set in. The likelihood of developing cardiomyopathy has been correlated with telomere length⁷⁸ but the mechanisms driving the cardiomyopathy seem to also coincide with cardiac growth, increased hemodynamic demands, and maturation. We hypothesized that by inducing maturation we could better model this disease progression in dystrophin-null hPSC-CMs.

Previous hPSC-CM models of DMD have used hypotonic challenge to bring out a measurable disease phenotype^{65,193}. While effective, hypotonic challenge is not physiologically accurate and is only a transient insult. Here we show that by maturing the dystrophin-null hPSC-CMs with the ComboMat protocol, cells present with electrical instability and Ca^{2+} overload. Along with its involvement in mediating cell injury through reactive oxygen species (ROS) and mitochondrial signaling pathways²⁶³, Ca^{2+} overload has long been known to mediate cardiac arrhythmias²⁶⁴ and has been implicated both *in vitro* and *in vivo* to be a driving force behind DMD pathophysiology²⁶⁵. Thus, we have demonstrated a maturation-dependent disease phenotype in dystrophin-null hPSC-CMs that recreates an early disease phenotype found in DMD patients.

This is an important distinction from previous DMD disease models and one that we believe represents a logical progression in the cardiac disease modeling field. Wen et al. also reported the need for maturation in their hPSC-CM model of arrhythmogenic right ventricular dysplasia in order to bring out a disease phenotype²⁶⁶ and Tiburcy et al.

demonstrated advanced maturation improved their ability to model heart failure via chronic catecholamine stimulation⁹². Together with our findings, we propose that other cardiomyopathies may be more faithfully modeled by using hPSC-CMs matured beyond the typical fetal stage of current directed differentiation methods.

Another major advancement of the present work over previous DMD disease models is the use of an isogenic control cell line. The KO DMD hPSC-CMs were generated via CRISPR-Cas9 genome editing tools targeting the 3'-end of the first muscle exon of the human dystrophin locus. Lin et al. were also able to demonstrate elevated cytosolic Ca^{2+} levels in their hPSC-CM model of DMD but comparisons were made to control cells with a different genetic background, limiting their conclusions⁸¹. We also contend that the relatively early stage of cardiac development that cell death was observed in previous hPSC-CM DMD models does not represent an accurate picture of the disease progression found in patients. Heart development is not noticeably impeded by a DMD dystrophin mutation. If cell death occurred at the fetal stage of cardiac development, one would expect gross morphological abnormalities to be present in DMD patients. Hearts of DMD patients, however, develop normally and cell death and fibrosis is only observed later in life²⁴⁷.

Finally, one of the most impactful aspects of this work is the development of a successful phenotypic drug screen. Methods such as the ComboMat protocol and NanoMEA platform can be scaled up for higher throughput screens of novel compounds. The noninvasive measurements can be used to monitor cell function longitudinally and the strong stratification of disease phenotype allows for easy comparison to healthy control to see if there is a restoration of function. We were able to

validate that Ca^{2+} channel blockers were indeed a true hit from our initial screen as well as correctly weed out a false positive drug hit from the *mdx* mouse model.

While it is known that patients with DMD exhibit diastolic dysfunction and arrhythmias, the pathogenesis of this disease phenotype is not fully understood. Ca^{2+} overload has long been postulated to play a central role in the pathophysiology of DMD cardiomyopathy²⁶⁵. Leaky Ca^{2+} channels on the cell membrane and sarcoplasmic reticulum, abnormal nitric oxide signaling, and tears in the sarcolemma have all been proposed as the means for Ca^{2+} entry into the cell but no consensus exists. The present model, with an endogenous electrophysiological disease phenotype in conjugation with elevated Ca^{2+} levels, could prove to be a valuable tool for addressing some of these questions. Taken together, the present data provide strong evidence that cardiac maturation is an important factor in accurately modeling adult onset disease such as DMD cardiomyopathy and that the ComboMat and NanoMEA platforms can be used to effectively screen potential therapeutic targets.

Chapter 6. Summary and Future Directions

The above work addressed key questions in the field of cardiac disease modeling regarding hPSC-CM maturation: Can adult-onset cardiomyopathies be modeled effectively with fetal-stage hPSC-CMs? Can bioengineering strategies be used to accelerate hPSC-CM maturation and build more adult-like cardiac tissues for disease modeling? In Aim 1, we explored the role of surface nanotopography on hPSC-CM development. Although we found that anisotropic nanotopography improved the structure and function of hPSC-CMs, nanotopography alone could not effectively stratify normal and dystrophin-null cardiomyocytes on a relevant functional level. Therefore in Aim 2, we developed a combinatorial maturation (ComboMat) approach incorporating nanotopography, thyroid hormone T3, and Let7i overexpression in order to significantly improved the structure and function of hPSC-CMs. In Aim 3, when we applied the ComboMat protocol to a CRISPR-edited dystrophin knockout (KO) model of DMD cardiomyopathy, a distinctive, relevant, and endogenously occurring disease phenotype emerged. Mature dystrophin KO hPSC-CMs exhibited a greater propensity for arrhythmia and had higher resting cytosolic calcium content. Without maturation, the dystrophin KO cells were indistinguishable from the healthy isogenic control cells. A phenotypic drug screen using these matured hPSC-CMs found that Ca^{2+} channel blockers alleviated this arrhythmogenic behavior while sildenafil, a known false positive, had no effect.

The research presented in this dissertation sets the stage for many immediate future directions that would have an immense impact in both the cardiac disease modeling and preclinical cardiotoxicity screening fields. The most pertinent next step of

this work would be to more deeply investigate the specific pathophysiology leading cardiac dysfunction in DMD patients. Ca^{2+} overload has long been postulated to be the root cause of muscle dysfunction and cell death in DMD patients²⁶⁵, but the pathway through which Ca^{2+} enters the cells is not fully understood. Our mature DMD cardiomyopathy model offers an excellent drug screening system to modulate different pathways such as NOX2 mediated oxidative stress²⁶⁷, β -adrenergic signaling²⁶⁸, stretch-activated channels²⁶⁹, membrane micro-tears^{270,271}, nitric oxide mitochondrial signaling²⁷², ryanodine receptor stabilizers²⁷³, and L-type Ca^{2+} channel blockers²⁷⁴. We observed that an L-type Ca^{2+} channel blocker, nitrendipine, helped reduce the Ca^{2+} load in the KO DMD cells but it is not known definitively whether that was the instigating source of Ca^{2+} . High throughput analysis of electrophysiological function using our NanoMEA screening platform, as well as in-depth analysis of Ca^{2+} entry into the cells through Ca^{2+} imaging and whole cell patch clamp, should provide a more complete picture of the DMD pathophysiology.

In addition to investigating the instigating pathology behind DMD cardiomyopathy, future studies could also look to employ truly corrective treatments, such as gene therapy²⁷⁵, to see if the DMD disease phenotype can be rescued. Such studies could be used as a baseline for translational efforts to test relative timing, dosing, and functional recovery for dystrophic heart muscle.

Beyond DMD cardiomyopathy, our ComboMat approach could also easily be applied to numerous other genetic cardiomyopathy models²⁷⁶. It would be interesting to note any differences between already reported disease phenotypes with those found with more mature hPSC-CMs. This would help to further answer the question of whether

information is being masked by the relative immature nature of most hPSC-CM disease models. Additionally, more mature phenotypes may also allow for more physiologically relevant measurements of function, such as single myofiber kinetics. Pioner et al. demonstrated that long-term culture on nanopatterns alone was insufficient to push myofiber development past the fetal stage¹⁸⁰. With the additional maturation boost from the ComboMat protocol, perhaps more adult-like myofibers could develop and the impact of various sarcomere-related mutations could be investigated in greater detail.

Finally, the ComboMat protocol could also be applied for hPSC-CMs used in preclinical cardiotoxicity screening. The question of how mature hPSC-CMs need to be in order to accurately predict *in vivo* behavior is still hotly debated. However, in combination with the NanoMEA platform, the ComboMat protocol could serve as a longitudinal test bed for drug responses of an array of known bad actors. As the cells progressively mature beyond what would be expected by time alone in standard culture, a more complete picture of how maturation impacts cardiotoxic drug responses could be developed. Overall, the work presented in this dissertation opens up the avenue for a new paradigm for stem cell-based disease modeling and preclinical drug screening.

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Appendix A

List of supplemental video (SV) files:

SV1. Video of beating differentiated human pluripotent stem cell-derived cardiomyocytes

SV2. Quiver video of Control (EV-Flat) contracting monolayer

SV3. Quiver video of ComboMat (Let7i-NP-T3) contracting monolayer

VITA

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