In Vitro Human Engineered Myocardium: A Study into both Pathological and Physiological Hypertrophy

by

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Abstract

The ability to generate cardiomyocytes from either embryonic stem cells or induced pluripotent stem cells provides an unprecedented opportunity to establish human in vitro models of cardiovascular disease as well as to develop platforms for the testing of novel cardiac therapeutics. We designed two different platforms, a biowire platform and post deflection platform, to generate engineered heart tissues (EHTs) to study a fundamental process in cardiomyocytes: hypertrophy. Both pathological and physiological hypertrophy was studied in order to garner a better understanding of each process. Physiological hypertrophy characteristics were observed using the biowire platform seen in improved myofibril alignment and downregulation of fetal genes. When electrical stimulation was added, a rate dependent effect on sarcomere maturation was observed by the increased frequency of I-bands and H-zones. Certain hallmark features of pathological hypertrophy, such as upregulation of brain natriuretic
peptide and sarcomere structure breakdown, were recapitulated when EHTs were treated with isoproterenol.
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1 Introduction

1.1 The need for in vitro models of human myocardium

Cardiovascular disease is the leading cause of death globally and is predicted to continue being the leading cause of death until at least 2030 [1]. While there have been many improvements to help relieve acute complications of cardiovascular disease there is no permanent solution to a failing heart other than a heart transplant. Consequently, the majority of patients suffering from chronic cardiovascular diseases, such as heart failure, are increasing to epidemic levels resulting in a serious burden on the medical system [2]. In Canada, heart failure affects more than 1% of the population and results in 9% of all deaths. Furthermore, heart failure is the most common cause of hospitalization of people over 65 years of age [3]. There are two main ways to overcome these issues. The first solution is reactive, more effective pharmaceuticals need to be created to treat the symptoms of heart failure but, need to be created and tested in an economical manner. The second solution is preventative. In order to ease the burden on the medical system, a better understanding of various cardiovascular diseases needs to be improved and better early detection methods need to be created [4].

The average time to produce a drug, as of 2002, was 12 years and 10 months with the associated costs reaching one billion USD [5]. While, this may seem like a small price to pay for pharmaceuticals that save many lives; unfortunately, this is not the case when there is an attrition rate of over 90% for the failure of drugs tested in clinical trials [6]. Consequently, it should not be a surprise to see that the number of new drugs being tested and approved over the past 15 years has decreased from 53 new molecular entities in 1996 to 16 new molecular entities approved in 2007 [7]. Consequently, this begs the questions of why is this happening and what can we do to improve this?
Modeling cardiac disease and testing potential pharmaceuticals has been primarily accomplished through the mouse model. Compared to other animals, mice are easy to house, breed, perform surgery upon, and genetically modify. However, recently it has become quite apparent that the mouse model, and in general animal models, cannot predict many severe outcomes that drugs, especially cardiac drugs, will have when administered to humans. Two examples of this are in the differences of a mouse’s metabolism and cardiac electrophysiology in comparison to a human’s.

The first example of how the mouse model diverges from humans is seen in lipoprotein metabolism and the mouse’s susceptibility to disorders of lipid metabolism. Mice do not have the enzyme cholesteryl ester transfer protein (CETP), which converts cholesterol from high density lipoprotein (HDL) to low density lipoprotein (LDL) particles [8]. Consequently, mice have very low levels of LDL cholesterol making mice resistant to the development of coronary atherosclerosis, even when placed on high fat diets. This makes mice unable to mimic the heart attack conditions that many humans experience unless an invasive surgical procedure of ligating a coronary artery is performed.

The second example is based on cardiac electrophysiology. A cardiomyocyte’s (CM) action potential is the blueprint that describes the fundamental currents that govern a cardiomyocyte’s function. It is the differences in electrophysiology between mouse and human cardiomyocytes that warrant concern about the fidelity of the mouse model. The action potential duration of a mouse ventricular cardiomyocyte is 50ms in comparison to the 250ms for a human [9]. While this difference can be explained away due to the differences in body size the shape differences of the action potentials cannot. What is concerning about this is that the differences in action potential are governed by various ion channels [10] which, if differ between species makes it difficult to determine and test arrhythmia based cardiac drugs. For example, the ventricular repolarization phase of the action potential (phase 3) in humans is governed mainly by slow and rapid delayed outwards potassium rectifiers. However, due to the faster action potential duration found in mice, this repolarization phase in mice is instead governed by different potassium
channels resulting in different currents such as: transient outwards potassium currents with a fast and slow recovery from inactivation ($I_{\text{to,f}}$ and $I_{\text{to,s}}$), a slowly inactivating potassium current ($I_{\text{K,slow1}}$ and $I_{\text{K,slow2}}$) and a steady state current ($I_{SS}$) [10-13]. Not only is the repolarization phase governed by different potassium channels but the $I_{\text{K,slow}}$ and $I_{SS}$ potassium channels found in mice have not had their equivalents detected in human ventricular myocytes [14]. However, while it is easy to find faults in the mouse model as a predictor of human cardiac diseases and as a platform to test potential cardiac drugs, it is still a valuable tool as we still do not have a replacement.

Since it has become apparent that the mouse model, and other animal models, are becoming inefficient in their abilities to predict human diseases and drug responses, new platforms need to be created that are human based. To do this, we look towards the field of stem cells and stem cell differentiation to provide the tools required to begin the creation of human based cardiac platforms.
2 Literature Review

2.1 hPSC-CMs: What can we do?

The ability to generate cardiomyocytes from either embryonic stem cells (ESCs) [15] or induced pluripotent stem cells (iPSCs) [16], provides an unprecedented opportunity to establish human in vitro models of cardiovascular disease as well as to develop platforms for the testing of novel cardiac therapeutics. Advances in stem cell biology enable us to reliably obtain human cardiomyocytes (CMs) from stem cells [15, 16] allowing for the study of human diseases and drug responses in vitro.

With human embryonic stem cells, a variety of methods can be employed to study cardiac diseases through genetic modifications. We can knockout genes or knockin missense mutations into genes or reporters in gene loci [8]. This can be done through homologous recombination [17] and electroporation [18] but each of these methods has a poor efficiency. Recently, the most effective way to modify human ES cells has been through the introduction of a double stranded break in genomic DNA for modifications to be done by zinc-finger proteins [19].

The percentage of successful modification of hES cells can be low and unless the gene position is known for knockout or kockin modifications, a specific disease state cannot be created. However, with human pluripotent stem cells the inherent disease is present and all that needs to be done is the differentiation of the cell into the desired somatic cell type [20] (although this can also be a difficult task). However, one of the main difficulties with iPSC disease modeling is that a patient with the disease needs to be found.

Once we have our desired end stage cardiomyocytes, whether they are “healthy” cardiomyocytes or induced disease state cardiomyocytes, the study of a particular cardiac disease or testing of how cardiac drugs interact with these cardiomyocytes can be performed, at least in theory. While this brief summary shows that we are able to readily differentiate hPSCs into cardiomyocytes and that we can also modify hPSCs to induce a diseased state for later use in
disease modeling or drug testing, a major issue of the maturation state of these cells, as determined through ultrastructure features and electrophysiology, has not been addressed.

2.2 hPSC-CM State of Maturity

The major problem with hPSC-CMs is that they are typically characterized as fetal-like cells and are not representative of the adult cardiomyocyte phenotype; whether the cell is atrial, ventricular or a pacemaker like cell.

hPSC-CMs are able to respond to both adrenergic and cholinergic responses due to functioning α and β-adrenergic receptors. The adrenergic agonists adrenaline, noradrenaline and the β-adrenergic specific agonist isoproterenol all increase beating rate in hESC-CMs in a dose dependent manner [21-23]. However, the β2-adrenergic agonist clenbuterol increases the beating rate of hESC-CMs only after the cells have been left in culture until days 61-72 [24], in comparison to the α1-adrenergic agonist phenylephrine which can increase the beating rate of day 15-20 hESC-CMs [25]. This points to the fact that hESC-CMs, once differentiated, seem to have functioning adrenergic and cholinergic responses yet it is not fully developed and time left in culture to allow the cells to further develop is required.

One way to determine the maturity of a hESC-CM is to perform patch clamping and examine the electrophysiological properties of the cell. The key parameters to examine are maximum diastolic potential, upstroke velocity, action potential duration, action potential amplitude, beating rate and diastolic depolarization rate [26]. These parameters are tabulated in an excellent review by Blazeski et al. and show that there is a large amount of heterogeneity found between cell lines [27], different differentiation protocols and whether or not the cells are patched in clumps or individual cells [26, 28]. While the specific values of the different regions of the hESC-CM action potential do not match that of their adult cardiomyocyte counterpart (atrial, ventricular or pacemaker) it has been shown that hESC-CMs are somewhat sensitive to drugs that cause distortions in the cell’s action potential like early after depolarization from E-4031 treatment [29] or decreased upstroke velocity through the use of TTX a sodium channel blocker.
Similar to the α and β-adrenergic receptor examination, the ion channels that comprise hESC-CMs (which shape the action potential of the cell) are present and are working to a limited extent but, are not functioning at the same currents as the ion channels are in the adult cardiomyocyte.

Finally, force generation and calcium handling within hESC-CMs has been shown to be immature. The force of contraction in hESC-CMs has been shown to increase in the presence of increased extracellular calcium concentrations [30, 31], addition of isoproterenol [30] and with increased IP3R activation [32]. However, a positive force-frequency relation was not found in hESC-CMs, which is typically exhibited in the adult myocardium due to a cardiomyocyte’s ability to store calcium in the sarcoplasmic reticulum and increase the calcium release when needed to generate a stronger force of contraction [33]. This immaturity of the sarcoplasmic reticulum was further shown through the inability of ryanodine and sarcoplasmic reticulum ATPase 2A blockers to disrupt hESC-CM contraction amplitude [33, 34]. Consequently, while hESC-CMs hold the promise to model the human heart, the current state of hESC-CMs does not generate a cell type that is representative of the adult cardiomyocyte but rather an immature fetal stage cardiomyocyte. This provides a limitation in utilizing these cells when trying to create a high fidelity platform to test sensitive drugs or model adult stage cardiac diseases.

2.3 The Sarcomere Unit

During human cardiac development, the heart first develops from the splanchnic mesenchyme during the third week of gestation followed by a fusing of two endocardial heart tubes to form a single heart tube. During weeks four to seven the heart tube loops and partitions into the four chamber heart [35]. Cardiomyocytes found in the ventricles or atria in the seventh week are immature and continue to develop through hypertrophy during gestation. In the early weeks of gestation (weeks 17-24) cardiomyocytes are typically small and rounded with sparse myofibrils and sarcoplasmic reticulum, clearly identifiable early intercalated disks, and not fully formed Z lines. During mid-fetal gestation (weeks 24-30), a greater concentration of aligned myofibrils
are apparent with intercalated discs taking on a more mature appearance with greater zigzags and the presence of desmosomes, fasciase adherents and gap junctions along the intercalated disks. Myofibrillar branching occurs at the Z centers with well distributed myofibrils throughout the sarcoplasm. By this time, the myofibrils also become more mature as the appearance of A and I bands becomes clearer. Finally, in the late stage of fetal gestation, (weeks 30-45) cardiomyocytes display densely packed myofibrils, adult like intercalated disks, easily observable sarcoplasmic reticulum along with the presence of T-tubules. One of the few features not present at this late stage of cardiomyocyte development are M bands, which mark the final end-state of myofibrillar maturation [35].

A schematic of a sarcomere unit can be seen in Figure 1 which details various structural proteins of the sarcomere unit. Each sarcomere unit is bound by two Z-disks. Each Z-disk has actin filaments that are cross-linked to the Z-disk by α-actinin. Actin serves as the ropes upon which the thick filaments move along to contract the sarcomere unit. The thick filaments run along the length of the sarcomere and are attached to the Z-disk through an elastic-like protein called titin.

Different electron dense regions in the sarcomere unit that correspond to specific protein overlapping regions have been given labels: I- and A-band, H-zone, and M-line. The I-band is termed the region from the Z-disk to the thick filaments. The A-band is the span of the thick filaments along the sarcomere unit. The H-zone is the less electron dense region right in the center of the sarcomere unit and the M-line is an electron dense line along the center of the H-zone (and center of the sarcomere unit). These structural features are used to describe states of sarcomere maturity and disease. Distortion of these regions ultimately means distortion of the structural proteins that constitute these patterns.
2.4 Pathological Vs. Physiological Hypertrophy

Cardiac hypertrophy is an increase in the size of the heart as a result of an increase in the size of its cells, cardiomyocytes, [36] in response to an increase in biomechanical stress [37]. This increased biomechanical stress can be either extrinsic, caused by increase blood pressure or valvular heart disease, or intrinsic, caused by inherited heart defects; cardiomyopathies. The purpose of cells undergoing hypertrophy is to normalize the increased wall tension in the myocardium due to the increased biomechanical stress being applied.
While hypertrophy is considered an adaptive response to increased cardiac stress, prolonged hypertrophy has been shown to be associated with increased risk for sudden death or progression to heart failure [37]. It is currently not understood why physiological hypertrophy, which occurs during postnatal development and in response to exercise, and pathological hypertrophy, which occurs during increased demands on the heart such as hypertension, result in different end points when they are both occurring due to an increase in biomechanical stress [37, 38].

During physiological hypertrophy cardiomyocytes increase in size, there is enhanced protein synthesis, and a higher organization of the sarcomere, which is the contractile unit of cardiomyocytes [36, 37]. These changes in cell phenotype are a result of cardiomyocytes undergoing a reversion to a fetal-like state and re-expressing fetal genes. Common fetal genes that are re-expressed are: atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and α-myosin heavy chain (αMHC) [39]. However, during pathological hypertrophy, a sustained fetal state will be induced resulting in the myocardium dilating and degenerating. Pathological hypertrophy has been thought to cause disturbances in the electrical properties of cardiomyocytes, which may result in the down regulation of connexion 43 (Cx43), which encodes for the gap junction protein for cell to cell signalling. Furthermore, the degeneration of the myocardium is a result of the sarcomere units becoming less organized and not contracting properly. The various components that the sarcomere consists of become distorted. Specifically, there is an increase in the β-form of myosin heavy chain, found in the thick filaments of each sarcomere unit [40], disorganization of the z-bands [41] which provide uniform structure to the contractile unit, and a breakdown of calcium uptake [39] which distorts the rate and strength at which the sarcomere can contract.

Due to the lack of understanding of how the heart undergoes hypertrophy, and the need to create ways in which to predict when someone has a heart undergoing pathological hypertrophy, more accurate \textit{in vitro} models need to be created to study how and why human cardiac tissue transforms from a healthy state to a hypertrophic state.
2.5 Previous Cardiac Hypertrophy Studies

Previous studies of pathological cardiac hypertrophy and cardiac tissue engineering have looked into elucidating cell cytoplasmic signaling pathways [39] in hypertrophic hearts. For instance, some hypertrophic responses can be partially reversed by using angiotensin-converting enzyme inhibitors, β-adrenergic receptor blockers, and calcium channel blockers [39]. The main problem with studies that have been looking into cytoplasmic signaling pathways are that they use animal models, specifically knock-out mice. These animal models are excellent at preliminary studies for elucidation of cell signaling pathways for cardiac hypertrophy, however, for a more accurate model of human cardiac hypertrophy, human cardiac tissue will need to be created and tested.

Other groups have been specifically looking into how neurohormones generate cardiac hypertrophy. One group looked into the effect angiotensin II has on rat CMs in 2D cultures [42]. This study showed that angiotensin II (AngII) causes the release of endothelin-1 Et-1 and TGF-β1, which cause cardiomyocyte hypertrophy [42]. This study provides a good starting point for research into cardiac hypertrophy, however, the main problems with this study are that it was conducted with rat instead of human cells and it was done in 2D culture instead of 3D. As stated before, using rat cells instead of human cells do not provide an accurate representation of what will happen within a human and, culturing cells in 2D monolayers is not the natural environment cells experience which could cause cell culture artifacts to arise.

2.6 Mechanical Stimulation

In the adult human heart, ventricular cardiomyocytes have a number of stretch activated ion channels that regulate cardiomyocyte functionality [43]. Furthermore, the natural function of the ventricles is to fill with blood and expand, then contract to expel this blood into the next heart chamber. Knowing the importance of physically stretching cardiomyocytes in vivo made it clear that the same stretching to an EHT in vitro, mimicking a portion of the native environment of the
cardiomyocyte, could help to mature neonatal cardiomyocytes from a neonatal stage to that of an adult stage.

**2.6.1 Mechanical Stimulation: Monolayers**

To begin the discussion of mechanically stretching cardiomyocytes, we look towards research performed on neonatal rat cardiomyocytes stretched on elastomers. The main way in which the heart grows after birth is through hypertrophy. As a result, one of the first questions to ask is how can we mature neonatal cells to undergo physiological hypertrophy, can the induction of hypertrophy be achieved purely through hormonal signalling and is there a difference between mechanically (stretch) induced hypertrophy and hormonal induced hypertrophy?

When rat neonatal cardiomyocytes were culture on an elastomer to be stretched physically up to 112% (statically stretched) and compared to neonatal rat cardiomyocytes that were cultured on an elastomer but not stretch and instead exposed to angiotensin II or phenylephrine (PE), differences between mechanical induced hypertrophy and neurohormonal induction of hypertrophy were found. Physical stretching of the cardiomyocytes and α-adrenergic stimulation with phenylephrine led to a comparable degree of hypertrophy and a similar induction of the fetal gene program [44]. However, during the genome wide analysis the research group had performed screening for 28,000 genes and found a subset of 24 genes that were stretch specifically regulated, suggesting a possible specific gene expression program for stretch induced hypertrophy [44]. Two genes of interest were the cardioprotective cytokine GDF15 and heme oxygenase 1 (Hmox1), which were found up-regulated in the stretch condition and not in the phenylephrine condition. Furthermore, the up-regulation of these genes was found to be blunted when the angiotensin 1 receptor was blocked by irbesartan, suggesting that the angiotensin 1 receptor plays a role in signaling the biomechanical induction of these genes [44].

Noting the importance of the angiotensin 1 receptor in mechanical stretch, we can look at studies that have examined cyclic stretch of neonatal rat cardiomyocytes on an elastomer and the role of autocrine/paracrine signaling that occurs. What was found was that during cyclic stretch,
expression of mRNA levels of Et-1, angiotensinogen, and angiotensin converting enzyme were elevated [45]. They also showed that the supernatant from cyclically strained constructs when used to culture statically strained constructs lead to a dose dependent increase in BNP levels [45]. The main findings from this study are that locally generated AngII and Et-1 are able to act in series in an autocrine/paracrine signalling capacity to mediate strain-dependent activation of the cardiac specific gene expression.

Moving from fetal gene regulation induction via cyclic stretch to morphological changes induced through cyclic stretch of neonatal rat cardiomyocytes, we see that cardiomyocytes orientation and polarization of gap junctions is also affected. It was found that cyclic stretch modulates the expression of Cx43 and its location of expression. During cyclic stretch, Cx43 mRNA and protein expression is up-regulated and the expression of the Cx43 becomes localized at the poles of the cardiomyocyte [46]. Furthermore, it has been shown that N-cadherin also polarizes in a stretch dependent manner and co-localizes with Cx43 at the cardiomyocyte poles and that this was regulated through the ERK1/2 pathways [46, 47]. Two points to note, however are that there was no up-regulation of Na⁺/K⁺ –ATPase and L-type Ca²⁺ channel expression [46, 48-50] and that while the cardiomyocytes elongated due to stretch they did so in a transverse manner to the stretch direction, not parallel [46, 51, 52]. These two points show 1) that stretching induces cardiac hypertrophy but possibly no improvements to calcium handling, and that 2) culturing cells in 2 dimensions seems to create bizarre artifacts, where in this case cells do no elongate in the direction of stretch and rather elongate perpendicular to the stretch direction.

### 2.6.2 Mechanical Stimulation: Engineered Heart Tissues

To better mimic the *in vivo* microenvironment of the heart, engineered heart tissue (EHTs) became a new way to study cardiac tissue *in vitro*. This was primarily done through using a cell source of choice, usually still neonatal rat cardiomyocytes, and seeding these cells onto a biomaterial of choice, such as a collagen sponge or suspended within a collagen or fibrin hydrogel [53]. EHTs allow for the study of how cardiomyocytes would behave in a three dimensional environment under different conditions. In a paper by Wolfram Zimmermann *et al.*,
he shows how in their EHT set-up there is an optimal cell number for a maximal twitch tension, how the force of contraction increases over an 18 day period and remains constant from day 18 to 26, and that the EHTs, consisting of neonatal rat cardiomyocytes, are able to display the Frank Starling behaviour [54].

This work was further improved through the integration of mechanical cyclic stretching of their EHTs at 10% and 2Hz. Interestingly, Zimmermann et al. were able to mature neonatal rat cardiomyocytes to an adult stage. This was seen most convincingly in their transmission electron microscopy (TEM) sections as the presence of Z, I, A, and H bands were clearly seen, sarcoplasmic reticulum could be easily identified but most importantly T-tubules, which are not found in rat neonatal cardiomyocytes but rather adult cardiomyocytes, were clearly found multiple times [55]. On a side note, the cardiomyocytes were aligned parallel to the stretching force unlike what was found in the 2D model. The notion that mechanical stretching matured neonatal rat cardiomyocytes in EHTs was further proven through a study of the hypertrophic response these EHTs were undergoing and the fetal gene response that was occurring [56, 57].

### 2.6.3 Mechanical Stimulation: hPSC-CM based Engineered Heart Tissues

It has been shown that mechanically stretching EHTs brings about their maturation from a neonatal state to that of an adult state, but what if the starting cell population was fetal and not neonatal? When creating platforms for human disease modeling and drug testing, the input cells are hPSC-CMs that are a fetal state. In order to create adult human cardiac tissue in vitro, ideally, applying a similar stretching regiment as previously discussed would mature hPSC-CMs from a fetal through neonatal to an adult state.

Tulloch et al. showed that through a cyclic stretch regimen, a hypertrophic response in the hPSC-CMs could be induced along with enhanced cell architecture. Specifically, cells aligned parallel to the mechanical loading force, with cyclically stretched constructs displaying increased DNA synthesis, cardiomyocyte area, and increased RNA levels of β-myosin heavy chain (MYH7), cardiac Troponin T (TNNT2), L-type calcium chanel (CACNA1C), ryanodine receptor
(RYR2), and sarco/endoplasmic reticulum calcium ATPase 2A (ATP2A2) over control no stress constructs [58].

Another paper was published on cyclically stretched EHTs by Schaaf et al. which showed that their EHTs displayed a chronoptropic response to calcium and the β-adrenergic agonist isoprenaline. Furthermore, they tested pro-arrhythmic compounds E-4031, quinidine, procainamide, cisapride, and sertindole and found that their EHTs relaxation velocity and irregular beating would occur in a dose dependent manner and that these effects were reversible [30].

Tulloch et al., Schaaf et al., and a 2D analysis of hypertrophy on hPSC-CMs [59] have shown that hPSC-CMs are able to respond to cyclic stretch by activating a fetal gene response for hypertrophy. Furthermore, it has been shown that this hypertrophic response can also be induced through neurohormones such as AngII, PE, and Et-1, and that these hPSC-CMs/EHTs can respond to chemical agents to be used as a preliminary functional screening platform for cardiac toxicity and proarythmic drugs. However, it was noted that cyclic stretching resulted in abnormally long action potential durations, up to 1200ms, and that the resting membrane potential of EHT CMs was -49.1mV, less negative than the comparable 7-8 week old EBs which had -60.7mV resting potential. Consequently, we see that mechanical stimulation does not greatly mature the calcium handling capabilities of hPSC-CMs. Furthermore, even though mechanical stretch does induce a hypertrophic response, major ultrastructural makers such as H-zones, aligned Z-disks, T-tubules, and others are missing from this work. To address the first concern of calcium handling we will now take a look at how electrical stimulation affects in vitro cultures of cardiomyocytes.
2.7 Electrical Stimulation

2.7.1 Electrical Stimulation: Monolayer

Early on in electrical stimulation of cardiomyocytes, the cell type of choice was adult feline or rat ventricular cardiomyocytes. They were placed as isolated cells into an electrical field to determine how these adult cardiomyocytes change if they were field stimulated during culture. What was found was that after 24hrs of 1Hz stimulation the fractional shortening percentage, shortening velocity and relaxation velocity were all higher in comparison to the unstimulated controls [60]. Furthermore, in a separate study, the rate of contraction (controlled through electrical stimulation at 1Hz) was linked to an accelerated rate of MHC synthesis via an increase in translational efficiency [61].

2.7.2 Electrical Stimulation: Engineered Heart Tissues

To examine whether or not neonatal rat cardiomyocytes mature under electrical stimulation we look towards the work of Radisic et al. Over an 8 day period, 3 days of preculture and 5 days of 1Hz field stimulation, it was found that electrically stimulated samples were able to form well aligned resisters of sarcomeres as well as having defined M and Z lines and H, I, and A bands [62].

2.7.3 Electrical Stimulation: hPSC-CM

Recently, Lieu et al. published on the effects of hPSC-CM maturation through the use of 1-3Hz field stimulation. They showed that their hPSC-CM atrial or ventricular cells when exposed to field stimulation showed upregulation of structural proteins such as α and βMHC and the atrial and ventricular MLCs in comparison to their non-spontaneous beating counterpart control cells. Furthermore, they showed that on the mRNA level calcium handling gene expression of calsequestrin, triadin and junctin were upregulated in comparison to non-stimulated controls. Finally, when looking at the ultrastructure of the cells using transmission electron microscopy
electrical stimulation was shown to have induced improved ultrastructure with enhanced myofilament structure and organization [63]. These results point to the fact that electrical field stimulation can induce a maturation effect on hPSC-CMs in 2D monolayer set-ups but, still leave room for improvement as the mature adult CM phenotype was not produced.
3 Objectives and Hypothesis

3.1 Cardiac Maturation: Electrical Stimulation Utilizing the Biowire Platform

3.1.1 Objective

The objective of this project was to examine how the biowire platform in conjunction with a field stimulation ramp-up regiment, either to a maximum rate of 3Hz or 6Hz, effected the maturation of hPSC-CMs in vitro.

3.1.2 Hypothesis

It was hypothesised that the gel compaction of the suspended hESC-CMs around a relatively rigid suture would direct the alignment and elongation of hESC-CM. hESC-CM ultrastructure would be further enhanced when exposed to an electrical field stimulation with increasing ultrastructural features seen as the rate of stimulation increased. Ultrastructural improvement would be seen in greater alignment of Z-disks and the presence of more mature sarcomere features such as H-Zones and I-Bands.
3.2 Cardiac Disease Modeling: Pathological Hypertrophy Stimulation Utilizing the Biowire Platform

3.2.1 Objective

The objective of this project was to optimize a hESC-CM based EHT *in vitro* to measure induced pathological hypertrophy. Pathological cardiac hypertrophy was induced *in vitro* by pharmacological agents and optimized based on fetal gene upregulation.

3.2.2 Hypothesis

It was hypothesized that using Endothelin-1 (Et-1), Angiotensin II (AngII) or isoproterenol (Iso) would elicit a hypertrophic response within the EHT [42]. Et-1 and AngII are hormones and peptides involved with vasoconstriction [42] while Iso is a synthetic cardiac drug [64]. This hypertrophic response would induce the up-regulation of fetal cardiomyocyte genes such as atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and α-myosin heavy chain (αMHC) [65].
3.3 Cardiac Maturation: Mechano-Electrical Stimulation Utilizing the Post Deflection Platform

3.3.1 Objectives

The objectives of this project were threefold.

1. Designed and created a platform that allowed for functional screening of EHTs by measuring EHT force of contraction.

2. Augmented the platform to facilitate mechanical and electrical stimulation to be simultaneously applied to EHTs.

3. Optimized a regiment of mechanical and electrical stimulation to obtain the greatest amount of maturation in the EHTs.

3.3.2 Hypothesis

It was hypothesised that a regiment of 2Hz field stimulation and 5-10% static stretch would lead to EHTs producing a greater contractile force than electrically or mechanically stimulated EHTs and thereby produced the most mature EHTs. The cardiomyocytes that these mature EHTs were composed of will display a more developed sarcomere ultrastructure, seen through the alignment of Z-band and presence of I and H zones, and improved calcium handing, seen in the faster upstroke and downstroke of calcium released from the sarcoplasmic reticulum, when compared to cardiomyocytes of non-stimulated or just electrically or mechanically stimulated EHTs.
4 Materials and Methods

4.1 Biowire Projects

4.1.1 Human pluripotent stem cell maintenance and differentiation

We have used cardiomyocytes derived from one hESC lines (Hes2). The Hes2 line was maintained as described [15, 66]. Embryoid bodies (EBs) were differentiated to the cardiovascular lineage as previously described [15, 66]. In brief, EBs were generated by culture in StemPro-34 (Invitrogen) media containing BMP4 (1ng/ml). On day 1, EBs were harvested and re-suspended in induction medium (StemPro-34), basic fibroblast growth factor (bFGF; 2.5ng/ml), activin A (6ng/ml) and BMP4 (10ng/ml)). On day 4, the EBs were harvested from the induction medium and re-cultured in StemPro-34 supplemented with vascular endothelial growth factor (VEGF; 10ng/ml) and DKK1 (150ng/ml). On day 8, the medium was changed again and the EBs were cultured in StemPro-34 containing VEGF (20ng/ml) and bFGF (10ng/ml) for the duration of the experiment. Cultures were maintained in hypoxic environment (5% CO₂, 5% O₂) for the first 12 days and then transferred into a 5% CO₂ for the remainder of the culture period. EBs were dissociated for seeding in biowires at day 20 (EBd20).

4.1.2 Biowire device design and manufacture

The device was fabricated using soft lithography technique. A two-layer SU-8 (Microchem Corp., Newton, MA) master was used to mold poly(dimethylsiloxane) (PDMS). Briefly, device features were printed on two film masks (CADART) corresponding to the two-layer design. SU-8 2050 was spun onto 4-inch silicon wafer, baked, and exposed to UV light under the first-layer mask to create the first layer including the suture channel and the chamber with thickness of 185 μm. The second layer including only the chamber with thickness of 115 μm was spun on top. After additional baking, the second-layer mask was aligned to the features on the first layer and then UV exposed. Finally, the wafer was developed using propylene glycol monomethyl ether
acetate (Doe & Ingalls, Inc., Durham, NC). PDMS was then cast onto the SU-8 master and baked for 2hr at 70°C. PDMS templates were used to hold a piece of surgical suture centrally in the channel, Figure 2, to which the cardiac cell suspension gel was added.

4.1.3 Biowire generation

Day 20 EBs generated as described above were incubated in collagenase type I (1mg/ml; Worthington) and DNAs (1mg/ml, CalBiochem) in Hank’s Balanced Salt Solution (NaCl, 136 Mm; NaHCO₃, 4.16mM; Na₃PO₄, 0.34mM; KCl, 5.36mM; KH₂PO₄, 0.44mM; dextrose, 5.55mM; HEPES, 5mM) for 2hr at 37°C. EBs were centrifuged (800 r.p.m., 5 min), incubated with trypsin (0.25%, Gibco) for 5 min at 37°C and pipetted gently to dissociate the cells. After dissociation, cells were centrifuged (1,000 r.p.m., 5 min), counted and seeded at 0.5x10⁶ cells/wire of 0.5 cm in length. This ratio was maintained for the generation of longer biowires. Cells were seeded in Collagen Type I gels (4µl/0.5cm wire length; 2.1 mg/ml of rat tail collagen type I (BD Biosciences) in 24.9mM Glucose, 23.81mM NaHCO₃, 14.34mM NaOH, 10mM HEPES, in 1X M199 media + 10% of growth factor reduced Matrigel (BD Biosciences)) by pipetting the cell suspension into the main channel of the PDMS template (Figure 2). After seeding, cells were kept in culture for 7 days to allow collagen matrix remodeling and assembly around the suture (Figure 2).

4.1.4 Pathological hypertrophy stimulation setup and conditions

After preculture for 7 days, biowires were transferred into a 6-well dish where drug treatment was applied to the biowires for another 7 days. The media was changed every other day with fresh drugs added to the media during the drug treatment period. A concentration of 200nM Angiotensin II (Sigma), 150nM Endothelin 1 (Sigma) and 100nM Isoproterenol (gift from the Gramolini lab) was used. On day 14, experiments were stopped and wires were harvested for analysis, Figure 3.
4.1.5 Electrical stimulation setup and conditions

After preculture for 7 days, biowires were transferred to stimulation chambers fitted with two 1/4-inch-diameter (0.635cm) carbon rods (Ladd Research Industries, Burlington, VT) placed 2cm apart and connected to a cardiac stimulator (Grass s88x) with platinum wires (Ladd Research Industries, Burlington, VT). Biowires were placed perpendicular to the electrodes and were either submitted to electrical stimulation (rectangular, biphasic, 1ms, 3-4V/cm) or cultured without electrical stimulation (non-stimulated controls or CTRL) for 7 days. We utilized two electrical field stimulation protocols: (I) where stimulation started at 1Hz and increased gradually and daily to 3Hz (1, 1.83, 2.66 and 3Hz) where it was maintained for the remainder of the week or (II) where stimulation started at 1Hz and increased gradually to 6Hz throughout the week (1, 1.83, 2.66, 3.49, 4.82, 5.15 and 6Hz, daily frequencies), Figure 4. Since increased time in culture is shown to affect maturation [67, 68], we have utilized age matched EBs (EB d34) as an additional control. To verify that biowire-stimulated cardiomyocytes truly exhibited maturation on a single-cell basis we performed assays in which single cells were used. With this goal, biowires were digested with collagenase type I (1mg/ml; Worthington) and DNAse (1mg/ml, CalBiochem) in Hank’s Balanced Salt Solution (NaCl, 136mM; NaHCO3, 4.16mM; Na3PO4, 0.34mM; KCl, 5.36mM; KH2PO4, 0.44mM; dextrose, 5.55 mM; HEPES, 5mM) for 4hr at 37°C, centrifuged (800 r.p.m., 5 min), incubated with trypsin (0.25%, Gibco) for 5 min at 37°C and pipetted gently to dissociate the cells. Isolated single cells were seeded on Matrigel covered 12-well plates for area measurements.

4.1.6 Assessment of Electrically Stimulated Biowires

The progression of tissue assembly was assessed at various levels after 2 weeks in culture (i.e. 7 days of gel compaction followed by 7 days of stimulation): functional (excitation threshold (ET), and maximum capture rate (MCR)); ultrastructural (sarcomere development, frequency of desmosomes), cellular (cell size and shape, proliferation, distribution of cardiac proteins: actin, troponin T and α-actinin), and molecular (expression levels of cardiac genes and proteins).
4.1.7 Immunostaining and Fluorescence Microscopy

Immunostaining was performed using the following antibodies: mouse anti-cTNT (Abcam; 1:100), mouse anti-α-actinin (Abcam, 1:200), goat anti-mouse-Alexa Fluor 488 (Jackson ImmunoResearch; 1:400), anti-Ki67 (Millipore, 1:250), donkey anti-rabbit-TRITC (Invitrogen; 1:400). DAPI was used to counterstain nuclei. Phalloidin (Invitrogen 1:1000) was used to detect actin fibers. The stained cells were visualized using a fluorescence microscope (Leica CTR6000) and images captured using the Leica Application Suite software. For confocal microscopy cells were visualized using a fluorescence confocal microscope (Zeiss LSM-510).

4.1.8 Transmission Electron Microscopy

Tissue was fixed with 4% Paraformaldehyde, 1 % Glutaraldehyde in 0.1M PBS for at least 1hr and washed 3 times with PBS pH 7.2. Post-fixation was done with 1% Osmium Tetraoxide in 0.1M PBS, pH 7.2 for 1hr and dehydrated using ethanol series from 25 to 100%. Tissue was infiltrated using Epon resin and polymerized in plastic dishes at 40°C for 48hr. Tissue was stained with Uranyl Acetate and Lead Citrate after sectioning. Imaging was performed at Hitachi H-7000 transmission electron microscope.

4.2 Post Deflection Platform

4.2.1 Electromechanical platform design and manufacture

A 3D computer generated drawing of the mould for the electromechanical platform was created using AutoCad, Figure 5. This AutoCad drawing was inputted into a milling machine so that the designed mould could be created out of aluminum Figure 5. To create the final device, the aluminum negative mould was filled with PDMS and allowed to cure at 80°C for 2hr at which point the PDMS platform was removed from the aluminum mould Figure 6.

The PDMS platform would then be inserted into the pneumatically driven stretch device as seen in Figure 6. The ends of the PDMS platform were sandwiched between two metal plates that
served to anchor the PDMS platform to the stretch device. Compressed air was pumped into the pneumatic pistons to move the two plates of the stretch device apart stretching the entire PDMS platform. Finally, carbon rods (Ladd) were inserted into the mould to provide the electrical field stimulation.

4.2.2 Post deflection set-up

Engineered heart tissues were created from day 20 embryoid bodies (EBD20) that were differentiated to the cardiovascular lineage using human embryonic stem cells and a growth factor based differentiation protocol [15]. EBD20s were then dissociated into a single cell suspension using a collagenase digestion followed by trypsin-EDTA treatment. These cells were then suspended in a collagen gel and pipetted into the post deflection set-up and placed into the incubator at 37°C for 30 minutes to allow the collagen hydrogel to gel. Constructs were then cultured for two weeks in the mould with media changes every other day. After two weeks of culture, a third week of electrical stimulation at 2Hz was performed for the electrically stimulated samples or for control samples no treatment was performed and they were left to continue culturing for the final week.

4.2.3 Confocal microscopy

At the final time point of three weeks, EHTs are fixed in 4% paraformaldehyde. Immunostaining was performed using the following antibodies: mouse anti-cTNT (Abcam; 1:100), mouse anti-α-actinin (Abcam, 1:200), rabbit anti CX43 (Abcam, 1:500), goat anti-mouse-Alexa Fluor 488 (Jackson ImmunoResearch; 1:400) and donkey anti-rabbit-TRITC (Invitrogen; 1:400).

4.2.4 Beam deflection measurements

To determine the force of contraction of each EHT, a cantilever beam-partial uniform load equation was used. After three weeks, the post deflection device was transferred into a temperature controlled, at 37°C, microscope. To determine the force of contraction of each EHT,
the top surface of a post for one EHT (at a time) was video recorded using the microscope Olympus software. The video was then exported as a series of Tiff images and opened in ImageJ. Each image was 0.05 seconds in length. By moving sequentially through the images and using the edge of the post as a marker, the displacement of the post could be measured. Multiple measurements were recorded and then averaged to determine the maximum post deflection, $\delta_{max}$. The elastic modulus of the PDMS was determined prior and an average obtained. The height of the post and the height of the tissue on the post were individually measured for each construct so that a precise calculation for each EHT could be determined. The formula governing the cantilever beam-partial uniform deflection is seen in Equation 1, schematic found in Figure 7, and variables for Equation 1 defined in Table 1.

If the tissue was not situated at the bottom of the post, Equation 1 became invalid and instead the method of superposition was implemented to determine the distributed load on the cantilever beam. In this method, as seen in Figure 8, to determine a partial uniform load that was at a specific distance along the beam’s length one can subtract two different partial uniform distributed loads that all originate at the fixed end to determine the “floating” distributed load. This was achieved using Equation 2 with the variables explained in
To determine the force per cross sectional area of each EHT, the force per length $q$, was converted into a point load and divided by the cross sectional area of the EHT. Each cross sectional area of the EHT was individually measured. Four points along the length of the EHT were measured and averaged to obtain the diameter of the EHT, assuming circular cross section, to determine each specific EHT’s area. Equation 3 shows the equation that was used and Table 1 outlines the variables.

4.2.5 Post deflection time measurements

To determine the length of time the tissue was undergoing contraction, relaxation, and resting the videos of post deflection were analyzed. The exported video in Tiff series format was viewed in ImageJ. Each image represented 0.05 seconds. Knowing this, the number of frames it took for post deflection, relaxation and resting to occur were counted and then multiplied by 0.05 seconds to determine the total time for each process.

4.3 Statistical Analysis

Statistical analysis was performed using SigmaPlot 12.0. Differences between experimental groups were analyzed by Student’s t test or two-way ANOVA. Normality test (Shapiro-Wilk) and pairwise multiple comparison procedures (Holm-Sidak method) were used for two-way ANOVA tests. In groups were normality failed, statistics was done using one-way ANOVA on Ranks (pairwise multiple comparison, Dunn’s method). $P < 0.05$ was considered significant for all statistical tests.
4.4 Materials and Methods Figures

Figure 2: Biowire set-up and morphology.
Biowire PDMS template. I, Surgical suture (black) is placed in the center of the channel. II, Cardiomyocyte suspension in collagen type I gel is seeded into the main channel around the suture. III, Pre-culture of hESC-cardiomyocyte in biowire template for 7 days allows cells to remodel the gel and contract around the suture. IV, Biowires maintain structure after removal from PDMS templates.

Figure 3: Pathological hypertrophy regimens applied.
Pre-culture biowires were submitted to drug treatments of either Endothelin-1, Angiotensin II or Isoproterenol for one week. Fresh drugs were added every other day during media changes.
Figure 4: Electrical stimulation regimens applied.

Pre-cultured biowires were submitted to electrical stimulation at 3-4V/cm for 1 week. (Low Frequency) Electrical stimulation started at 1Hz and was progressively increased to 3Hz where it was kept for the remainder of the week (low frequency ramp-up stimulation regimen or 3Hz). (High Frequency) Stimulation rate was progressively increased from 1 to 6Hz (High frequency ramp-up stimulation regimen or 6Hz).
Figure 5: 3D rendering and final aluminium mould for the PMDS platform.

The top figure shows the Auto-CAD drawing of the mould for the PDMS platform. The bottom figure shows the final mould made of C&C machined aluminium.
Figure 6: Electromechanical PDMS platform and stretch device.
The top figure shows the PDMS platform that consists of eight tissue wells each with two posts per well. The PDMS platform can also house two sets of carbon electrodes for electrical field stimulation. The bottom figure shows the stretch device loaded with a PDMS platform filled with culture medium.
Equation 1: Cantilever beam-partial uniform load solved for force per length

\[ q = \frac{24 \delta E I}{a^3 (4L - a)} \]

Equation 2: Superposition Method

\[ \delta_B = -v(L) = \frac{q a^3}{24 E I} (4L - a) \]
\[ - \frac{q a'^3}{24 E I} (4L - a') \]

In this case \( a = 2L/3 \) and \( a' = L/3 \)

Equation 3: Force per area of engineered heart tissue

\[ FPA = \frac{qa}{\pi r^2} \]
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Units</th>
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<tbody>
<tr>
<td>q</td>
<td>Force per length</td>
<td>$\frac{N}{m}$</td>
</tr>
<tr>
<td>a</td>
<td>Distance of uniform load</td>
<td>m</td>
</tr>
<tr>
<td>E</td>
<td>Elastic Modulus</td>
<td>$\frac{N}{m^2}$</td>
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<tr>
<td>I</td>
<td>Area of moments of inertia (circle = $\frac{\pi r^4}{4}$)</td>
<td>m$^4$</td>
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<tr>
<td>L</td>
<td>Length of post</td>
<td>m</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Maximum deflection at free end of beam</td>
<td>m</td>
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<tr>
<td>FPA</td>
<td>Force per area</td>
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<td>r</td>
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5 Cardiac Maturation: Electrical Stimulation Utilizing the Biowire Platform

Information for the Results and Discussion section in this chapter were taken from the cited paper below. The works taken from the paper are of experiments that I directly performed and analyzed.


5.1 Introduction

As discussed in section 2.2, hPSC-CMs are immature at their end stage of differentiation (around day 20). Consequently, it becomes difficult to compare gene or protein expression profiles generated from these immature cells to the mature adult cardiac phenotype. While Chapter 6 makes a good first attempt at harnessing the potential of hESC-CMs to model human cardiac disease, a step-back needs to be taken to examine hESC-CM maturation. The focus now needs to shift to creating high fidelity adult like hESC-CMs utilizing bioengineering principles so that our in vitro models are truly representative of the adult cardiomyocyte phenotype.

In this chapter I propose the idea of recapitulating part of the cardiomyocyte microenvironment through not only the physical cues that the biowire platform provides but, in conjunction with electrical field stimulation. As described in section 2.7 electrical stimulation has been shown to push neonatal cardiomyocytes to the adult phenotype [62] and more recently, electrical stimulation on hPSC-CMs has been shown to improve aspects of the cells electrophysiology [63]. As a result, here we will compare control biowires to biowires that have been stimulated at
a 3Hz or 6Hz electrical field stimulation ramp up regiment. After the electrical stimulation
regiment the biowires will be examined for cardiomyocyte ultrastructure, using confocal and
transmission electron microscopy, and cell gene regulation profiles using real time quantitative
polymerase chain reaction. The goal is to explore the effects that electrical stimulation has on
maturing hESC-CMs in conjunction with the biowire platform.
5.2 Results

5.2.1 Engineering of human cardiac biowires.

hESC-cardiomyocytes and supporting cells obtained from directed differentiation protocols were used to generate 3D, self-assembled cardiac biowires by cell seeding into a template polydimethylsiloxane (PDMS) channel, around a sterile surgical suture in type I collagen gels as seen in Figure 2. Seeded cells remodeled and contracted the collagen gel matrix during the first week with ~40% gel compaction around the suture (Figure 9A and B, final width ~600µm). This allowed the suture to be removed from PDMS template without damaging the biowire structure.

Staining of cell seeded biowires with hematoxylin and eosin (H&E) and Masson's trichrome (MT) revealed cell alignment along the axis of the suture (Figure 9C). Human cardiac biowires started to beat synchronously and spontaneously between 2 and 3 days post-seeding and kept beating during gel compaction, demonstrating that the biowire setup allows for electromechanical coupling of the cells within the collagen type I matrix. Biowires could be electrically paced and responded to physiological agonists such as epinephrine (β-adrenergic stimulation) by increasing spontaneous beating frequency.

After pre-culture for 1 week, biowires were either submitted to electrical field stimulation or cultured without electrical stimulation (non-stimulated controls) for 7 days. We utilized two different protocols where stimulation rate was progressively and daily increased from 1 to 3 Hz (Figure 4, Low frequency ramp-up regimen, referred to as low frequency or 3Hz from here on) or from 1 to 6Hz (Figure 4, High frequency ramp-up regimen, referred to as high frequency or 6Hz from here on) to assess whether electrical stimulation alone is important, or if effects are dependent on stimulation rate.
5.2.2 Cardiomyocyte physiological hypertrophy in stimulated biowires.

After 2 weeks in culture, immunostaining showed that cells throughout the biowires strongly expressed cardiac contractile proteins sarcomeric α-actinin, actin and cardiac Troponin T (Figure 10a; Hes2). Sarcomeric banding of the contractile apparatus (Figure 10a) and myofibrillar alignment along the suture axis was qualitatively similar to the structure of adult heart [69]. Biowires kept in culture for 3 and 4 weeks maintained cell alignment and their contractile apparatus structure as evidenced by confocal microscopy upon immunostaining (sarcomeric α-actinin, α-actin and cardiac troponin T) and transmission electron microscopy.

Early in cardiac development, cardiomyocytes are round shaped cells differentiating into rod-shaped phenotype [70] after birth. Adult human cardiomyocytes display a more structurally rigid architecture when dissociated, retaining a rod-like shape [71] immediately after dissociation while hESC-cardiomyocytes tend to remain round. We dissociated age matched embryoid bodies (EBd34) and biowires and seeded the cells into Matrigel-coated. While ~80% of cardiomyocytes from EBd34 displayed a round phenotype, this number was significantly lower (~50% less) in electrically stimulated samples (Figure 10b). The percentage of rod-like cardiomyocytes was significantly higher (~4 fold) in electrically stimulated biowires (Figure 10b) as compared to EBd34.

During development, cardiomyocytes undergo physiological hypertrophy which is characterized by an increase in cell size accompanied by a change in sarcomere structure and followed by a downregulation of fetal genes after the hypertrophic response [72, 73]. There was a significant increase in cardiomyocyte size (area of plated cells) in biowire conditions compared to cardiomyocytes from age matched EBs (EBd34) (Table 2, EBd34 vs. CTRL p=0.034; EBd34 vs. 3Hz p=0.003; EBd34 vs. 6Hz regimen p=0.01). Atrial natriuretic peptide (ANF), brain natriuretic peptide (BNP) and α-myosin heavy chain (MYH6) are molecules highly expressed in fetal cardiomyocytes and upregulated during pathological hypertrophy in diseased adult ventricular cardiomyocytes. Downregulation of the fetal cardiac gene program (ANF, BNP,
MYH6) in hESC-derived cardiomyocyte biowires (Figure 11), compared to age matched EBs, in concert with increase in cell size, suggests physiological cardiomyocyte hypertrophy and a more mature phenotype. Potassium inwardly-rectifying channel gene (KCNJ2), that plays important roles in cell excitability and K⁺ homeostasis [74], was upregulated compared to EBd34 (Figure 11).

hESC-cardiomyocytes cultured in biowires also displayed lower proliferation rates than those cultured in EBs (Figure 12, EBd20 vs. EBd34, p=0.002; EBd34 vs. CTRL, p=0.019; EBd34 vs. 3Hz, p=0.016; EBd34 vs. 6Hz, p=0.015) and the percentage of cardiomyocytes in each condition remained unchanged after culture for 2 weeks (48.2 ± 10.7%, Figure 13). Initial percentages of CD31 (2.4 ± 1.5%, endothelial cells [75]), CD90 (34.4 ± 23%, fibroblasts [75]), calponin (35 ± 22%, smooth muscle cells) or vimentin (80 ± 22%, non-myocytes) positive cells in EBd20 population, were largely maintained after biowire culture, suggesting that the improvements observed were not related to the induction of a particular cell type in culture.

### 5.2.3 Maturation of contractile apparatus in electrically stimulated biowires.

Cells within the 3D biowires that did not undergo electrical stimulation (non-stimulated controls) displayed well-defined Z discs and myofibrils (Figure 10c, black arrows;) but no signs of Z disc alignment. In contrast, biowires stimulated under the high frequency regimen showed signs of maturation, such as organized sarcomeric banding with frequent myofibrils that converged and displayed aligned Z discs (Figure 10c, 6Hz,), numerous mitochondria (Figure 10c, 6Hz, m;) and desmosomes (Figure 10c, arrows). In the 6Hz ramp-up condition, mitochondria were positioned closer to the contractile apparatus than in non-stimulated controls or 3Hz condition.

Electrically stimulated samples displayed a sarcomeric organization more compatible with mature cells than non-stimulated controls as shown by a significantly higher presence of H-zones per sarcomere (Figure 10d, sarcomere structure, white arrows; CTRL vs. 6Hz, p=0.005) and I-bands per Z disc (Figure 10d, CTRL vs. 3Hz, p=0.01; CTRL vs. 6Hz, p=0.003). No M-lines or
T-tubules were detected in any conditions. Biowires that underwent electrical stimulation in the 6Hz regimen also displayed a significantly higher number of desmosomes per membrane length than both non-stimulated controls and 3Hz-stimulated biowires (Figure 10d, p=0.0003).
5.3 Discussion

This is the first study to examine the effects of electrical stimulation and different pacing regimens on hESC-derived cardiomyocyte maturation and phenotype. Here, we showed for the first time that the combination of geometric control of 3D tissue assembly and electrical stimulation of hESC-derived cardiomyocytes and supporting cells improved electrical and ultrastructural properties of human cardiac tissue, resulting in physiological hypertrophy and maturation. Although electrical field stimulation was used previously with cells from primary sources and animal tissues [69, 76], we demonstrate that a stimulation regimen of progressive frequency ramp-up results in improvements in hESC-cardiomyocytes.

We have chosen a natural matrix of collagen type I, one of the major components of human myocardial matrix, since it enables the control of tissue architecture and does not elicit foreign body response when implanted. The biowire suture remained anchored to the device platform during matrix remodeling, generating tension in the matrix that resulted in cell alignment along the suture axis. Electrical stimulation induced the development of long, well aligned registers of sarcomeres, containing compact and clearly visible Z discs, H zones and I bands that resembled those of native myocardium [69]. However, no M lines and T-tubules were detected, which is consistent with previous reports [77, 78]. Appearance of these features takes place at later stages of development [79], indicating that despite the more mature phenotype present in 6Hz-stimulated conditions these cardiomyocytes do not correspond to terminally differentiated, adult, working cardiomyocytes.

The normal human fetal heart rate varies significantly during development, being maintained at ~3Hz for most of the time [80] while the adult resting heart rate is ~1Hz [80]. The rate change is associated with changes in contractile protein expression (higher expression of \( \alpha \)- than \( \beta \)-myosin heavy chain in fetal vs. higher \( \beta \) than \( \alpha \)-myosin heavy chain in adult human cardiomyocytes) and suggests a possible dependence of cardiac maturation on stimulation rate. The fact that the progressive increase from 1 to 6Hz was the best condition tested, and not 1 to 3Hz, was
surprising since 3Hz is the average fetal heart rate [80]. This could be a compensatory mechanism for the lack of other important cells types such as endocardial cells [81] and cell-cell developmental guidance in the in vitro setting. Despite the MCR upon point stimulation being ~4Hz, the MCR measured upon field stimulation was appreciably higher, 5.2Hz for the 6Hz ramp-up group. Since we applied field stimulation and a ramp-up protocol during culture where stimulation frequency was gradually increased over 7 days, the 6Hz group might only lose capture (exceed the rate of 5.2 Hz) at the very last day of stimulation. Collectively our results suggest that it may be the stimulation at the highest possible rate, and not the rate per se, that is the governing cue for cardiomyocyte maturation in vitro. Additionally, since the differentiated progeny of hESCs are described to be reflective of very early human development (< 6 weeks) [82], it is likely that electrical stimulation rates required for maturation in vitro may differ from in vivo embryo development. Regardless, the remarkable maturation of the in vitro cardiac biowires obtained with progressively higher electrical stimulation rates provides an important tool to generate more mature contractile cardiac tissues in vitro.

Notably, improved cell structure in stimulated conditions did not correlate with upregulation of structural proteins expression. Instead, it correlated with better electrical properties of stimulated biowires such as lower ET, higher MCR, and upregulation of potassium inwardly-rectifying channel gene (KCNJ2). Although there was a downregulation of structural proteins mRNA in biowires compared to EBs, no changes were observed in protein expression which is consistent with the lack of cardiac load mimic. However, there were significant improvements in myofilament structural arrangement. Mechanical stimulation was reported to lead to a robust induction of structural proteins such as myosin heavy chain and induce proliferation of hPSC-derived cardiomyocytes [30, 83]. The fact that we observe a decrease in gene expression of myosin heavy chain and show very low percentage of proliferating cardiomyocytes is therefore in contradiction to the idea that 6Hz electrical stimulation is simply a better mechanical stimulation regime. Instead, the observed effects are likely due to the combined excitation-contraction coupling process.
Although application of mechanical stretch led to improved sarcomeric architecture in previous studies, it was not sufficient to electrophysiologically mature hESC-derived cardiomyocytes, even in the presence of a 3D matrix and gel compaction [30]. Studies in different animal models [84, 85] and with hESC-derived cardiomyocytes [83, 86] indicate that mechanical loading or cultivation on stiff matrices for prolonged time (100 days) [68] leads to myofibrilogenesis. Thus, the use of electrical stimulation in conjunction with stretch as a mimic of cardiac load [83], concurrent or sequentially, might be required to induce terminal differentiation in hPSC-derived cardiomyocytes, upregulate the expression of myofilament proteins and generate cardiac tissues that function like adult working myocardium. Other strategies might include cultivation in the presence of T3 thyroid hormone, shown to improve fetal sheep cardiomyocyte maturation [87] or insulin like growth factor-1, shown to induce physiological CM hypertrophy in mice [88]. Alternatively, point instead of field stimulation and addition of other cell types such as endocardial cells [81] might also be used. Addition of laminin to the gel matrix might lead to phenotype improvements [71]. Incorporation of native decellularized heart ECM into the hydrogel mixture for biowire seeding might also be used to further improve maturation [89]. Recent work showed that both rat neonatal cardiomyocytes [90] and early stage hESC cardiomyocytes [91] increased contractility on stiffer substrates which could also be used as a strategy to promote cardiomyocyte maturation.

The described biowire technology can serve as a model to study distinctive characteristics of human cardiac development, determine parameters that affect cardiac maturation and function as a drug-screening platform. Correlating the properties of hESC-cardiomyocytes in biowires with mouse or human cardiomyocytes development could be helpful to gauge a maturation stage. However, since rodent cardiomyocytes differ significantly from human (i.e. different expression of contractile proteins such as α- and β-myosin heavy chains in embryo vs. adult, different beating rates and heart size etc), any comparison to rodent cardiomyocytes would be misleading. Age-defined human samples that could surgically be obtained from diseased adult or infant patients usually display abnormal physiological and morphological properties. Limited access to samples from abortion clinics prevents defined developmental stage comparisons, as only early
gestation specimens are available. Invariably, these comparisons are complicated by the presence of non-cardiomyocytes in both hESC-derived cardiomyocyte preparations and in cardiac biopsies [92]. Additionally, in vitro maturation might not be compatible with embryo development since we are clearly enhancing the electrical properties but not inducing myofibrilogenesis. Taken together, these limitations make it difficult to accurately gauge the developmental stage of hPSC-derived cardiomyocytes against healthy human cardiomyocytes from primary sources.

The presence of the suture prevents both direct measurements of active force developed by the cardiomyocytes and mechanical stimulation since the suture is stiffer than the surrounding cardiac tissue. This is a limitation of the technology in the present form and can be changed in the future by using biodegradable sutures that may allow force measurements to be performed in vitro upon suture degradation.

In addition to the drug screening potential, hPSC-derived cardiomyocytes are promising for use in cell replacement therapies. For effective therapeutic use, cells must fully integrate with the host myocardium to contribute to contractile function and to respond to various physiological stimuli. Yet, there is still controversial opinions as to what stage of development would be most effective for cell transplantation [93]. We have now generated a unique platform that enables generation of human cardiac tissues of graded levels of maturation that can be used to determine, in future in vivo studies, the optimal maturation level that will result in the highest ability of cells to survive and integrate in adult hearts with the lowest side effects (e.g. cardiac arrhythmias).

The small size (radius of ~300μm) of biowire upon gel compaction was selected to be close to the diffusion limitations for oxygen supply [94] to ensure that the biowires can be maintained in culture without perfusion and without a fully functional vasculature. Addition of other cell types to purified cardiomyocytes will be imperative for improving survival and integration with the host tissue in future in vivo studies, as described recently by Tulloch et al [83].
5.4 Conclusion

The objective of this project was to examine how the biowire platform in conjunction with a field stimulation ramp-up regiment, either to a maximum rate of 3Hz or 6Hz, effected the maturation of hPSC-CMs in vitro.

In conclusion, our findings demonstrate that the combination of tissue engineering techniques and electrical stimulation can 1) improve hESC-cardiomyocyte architecture and induce physiological hypertrophy and 2) induce sarcomere maturation of engineered cardiac tissues \textit{in vitro} in a stimulation frequency dependent manner representing an important first step towards obtaining adult-like human cardiomyocytes.
5.5 Cardiac Maturation Figures

(a) Pre-culture of hESC-cardiomyocyte in biowire template for 7 days allows cells to remodel the gel and contract around the suture.  
(b) Quantification of gel contraction shows compaction of ~40% (average ± SD, n=3-4 wires).  
(c) Hematoxylin and Eosin (H&E) and Masson’s Trichrome (MT) staining of biowire sections show cell alignment along the suture axis (arrows represent suture axis).

Figure 9: Generation of human cardiac biowires.
a  

Control  

α-actinin/actin  cardiac troponin T  

3Hz  

6Hz  

b  

Cell shape (%)  

CTRL  3Hz  6Hz  EBD34  

p=0.01  p=0.03  

*
Figure 10: Culture in biowire in combination with electrical stimulation promotes physiological cell hypertrophy and improves cardiomyocyte phenotype.

(a) Representative confocal images of non-stimulated (control) and electrically stimulated biowires (3 and 6Hz ramp up) showing cardiomyocyte alignment and frequent z-bands (arrows represent suture axis). (b) Analysis of cardiomyocyte cell shape in different conditions reveals that biowires cultivated under electrical stimulation displayed significantly less round cells and more rod-like cells (average ± SD, EBd34 vs. 3Hz p=0.01 for both rod and round like; EBd34 vs. 6Hz p=0.03 for both round and rod-like). (c) Ultrastructural analysis shows that electrical stimulation at 6Hz induces cardiomyocyte self-organization. Representative images of non-stimulated (control) and electrically stimulated biowires showing sarcomere structure (Sarcomere panel, white bar; Z bands, black arrow; H zones, white arrows; m, mitochondria) and presence of desmosomes (Desmosomes panel, white arrows). Scale bar 1µm. (d) Morphometric analysis (average ± SD) showing ratio of H zones to sarcomeres (CTRL vs. 6Hz, p=0.005) ratio of I bands to Z-disks (CTRL vs. 3Hz, p=0.01; CTRL vs. 6Hz, p=0.003) and number of desmosomes per membrane length (CTRL vs. 6Hz, p=0.0003). *denotes statistically significant difference between group and control. In normal adult cells the ratio of H zones to sarcomeres is 1 and ratio of I bands to Z-disks is 2. n=3-4 per condition.(a-d)
Table 2: Culture in biowire and electrical stimulation induce cardiomyocyte hypertrophy.
EBd34 vs. CTRL p=0.034; EBd34 vs. 3Hz p=0.003; EBd34 vs. 6Hz p=0.010. Measurements performed on single Hes2 hESC derived cardiomyocytes dissociated from biowires at the end of cultivation. * denotes statistical significance between group and EBd34. Cell area ± SD (µm²).

<table>
<thead>
<tr>
<th></th>
<th>EBd34</th>
<th>Non-stimulated control</th>
<th>3Hz</th>
<th>6Hz</th>
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<tbody>
<tr>
<td>Round</td>
<td>195 ± 31</td>
<td>248 ± 94</td>
<td>279 ± 93</td>
<td>208 ± 115</td>
</tr>
<tr>
<td>Rod-like</td>
<td>562 ± 79</td>
<td>857 ± 187*</td>
<td>977 ± 246*</td>
<td>917 ± 171*</td>
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Figure 11: Gene expression analysis shows downregulation of cardiac fetal gene program and upregulation of potassium channel gene.

Cardiac Troponin T (cTNT), Connexin43 (Cx43), Brain natriuretic peptide (BNP), Atrial natriuretic peptide (ANF), α-myosin heavy chain (MYH6), β-myosin heavy chain (MYH7), ryanodine receptor 2 (Ryr2), potassium inwardly-rectifying channel
gene (KCNJ2). Cardiac fetal genes ANF, BNP and MYH6 were significantly downregulated in hESC-derived cardiomyocyte biowires while KCNJ2 was upregulated compared to age matched EBs (EBd34; average ±SEM, n=3-6).
Figure 12: Cardiomyocyte proliferation in biowires is lower than in EBs.

Proliferation was assessed by double staining for sarcomeric α-actinin and Ki67 (n= 3-4 per condition). *, **, # and & represent statistically significant difference compared to EBd34. Measurements were performed on single Hes2 hESC derived cardiomyocytes after dissociation from biowires.
Figure 13: Cell population in the biowires does not vary significantly after cultivation. Percentage of cells positive for the different markers normalized to EBd20 (starting population). Cells from EB day 20, EB day 34 and biowires (CTRL and 6Hz) were stained for CD31 (endothelial cells), CD90 (fibroblasts), calponin (smooth muscle cells), vimentin (all non-myoocytes) and cardiac Troponin T (cardiomyocytes), and analyzed by flow cytometry (n=3-6 per condition). Results with hESC-derived cardiomyocytes Hes2 cell line after dissociation from biowires are shown.
6 hPSC-CM Disease Modeling: Pathological Hypertrophy Stimulation Utilizing the Biowire Platform

6.1 Introduction

Cardiovascular disease is the leading cause of death globally and is predicted to continue being the leading cause of death until at least 2030 [1]. While there have been many improvements to help relieve acute complications of cardiovascular disease there is no permanent solution to a failing heart other than a heart transplant. Consequently, the majority of patients suffering from chronic cardiovascular disease, such as heart failure, is increasing to epidemic levels and is becoming a serious burden on the medical system [2]. In Canada, heart failure affects more than 1% of the population and results in 9% of all deaths. Furthermore, heart failure is the most common cause of hospitalization of people over 65 years of age [3]. In order to ease this burden on the medical system, a better understanding of various cardiovascular diseases needs to be improved and better early detection methods need to be created [4]. One such area of cardiovascular disease that needs improved understanding and early detection methods is cardiac hypertrophy.

Due to the lack of understanding of how the heart undergoes hypertrophy, and the need to create ways in which to predict when someone has a heart undergoing pathological hypertrophy, more accurate in vitro models need to be created to study how and why human cardiac tissue transforms from a healthy state to a hypertrophic state.

In this project I propose the generation of an in vitro model of human cardiac pathological hypertrophy utilizing human embryonic stem cell derived cardiomyocytes and the biowire platform. The biowire platform will allow the generation of 3D engineered heart tissues that will better mimic the microenvironment of the heart while providing structural cues to the hESC-CMs to improve cell alignment and structure. After a pre-culture period of 7 days, a 7 day drug induced pathological hypertrophy will be implemented. The extent of the pathological
hypertrophic response will be analyzed through the study of cardiomyocyte ultrastructure using confocal and transmission electron microscopy, and through the cells gene regulation profile using real time quantitative polymerase chain reaction. The goal of this project was to create a high fidelity *in vitro* model of pathological cardiac hypertrophy to better understand this disease phenotype with the intention to better treat this disease.
6.2 Results

6.2.1 Sarcomere structural proteins breakdown in the presence of hypertrophic agonists

One of the hallmarks of pathological cardiac hypertrophy and pressure-overloaded ventricles is a disorganization of the muscle fibers. Specifically, the muscle fibers show a “whirling” pattern instead of the regularly aligned cardiac fibers [95, 96]. In each of the three conditions of our in vitro model of pathological hypertrophy: Endothelin-1, AngiotensinII and Isoproterenol, we examined the ultrastructure of the cardiomyocytes to see if we were able to disrupt the sarcomere unit thereby inducing a similar pathological hypertrophic phenotype.

Figure 14 shows confocal microscopy images at the end time point of the three pathological hypertrophy inducing conditions. After one week of Et-1, AngII or Iso stimulation we found that α-actinin actin colocalization began to breakdown and instead of the regularly striated pattern of α-actinin, marking the Z-bands of the cardiomyocyte sarcomere unit, with actin running in between as seen in the control, protein degradation was found. The greatest effect was seen in the Et-1 condition as the α-actinin looks as if it had been smeared across the sarcomere unit. The AngII condition also presented a distorted α-actinin actin structure (Iso condition not examined for α-actinin and actin). The same effect was found with cardiac troponin-T, Figure 14 demonstrates that the regularly striated pattern of cTnT found in the control was disrupted in all three conditions.

6.2.2 Hypertrophic agonists cause an up-regulation of cardiomyocyte fetal genes (BNP, ANF) and an increase in the β/α MHC ratio

One of the main indicators of the cardiac hypertrophic diseased state is the up-regulation or reversion to a fetal gene profile [37, 65, 97]. Two key fetal genes used to asses a hypertrophic state are: atrial natriuretic factor and brain natriuretic peptide [97]. All RT-qPCR results found in Figure 15 are normalized to the housekeeping gene Glyceraldehyde 3-phosphate
dehydrogenase (GAPDH) and then normalized again to EBD20, the starting population gene expression for the biowire set-up. EBD34 represents the end point cells that have been left in EB format to serve as an age matched untreated (no biowire or pathological hypertrophic agonist) control.

In general, as the hESC-CMs stayed in culture longer and were allowed to “mature” over time, the fetal gene program should have been down-regulated. This was seen when comparing EBD20 to EBD34 conditions for BNP and ANF, Figure 15. Next, looking at the biowire set-up alone, control samples showed that they have the ability to down-regulate the fetal gene program at a faster rate than time in culture alone. When examining the treated groups, BNP upregulation was seen in Et-1 and Iso (P<0.05 for Iso Vs. CTRL ANOVA on Ranks) conditions and ANF upregulation was seen with all three hypertrophic agonists, however, no statistical significance was found. An increase in the β/αMHC ratio also indicated a hypertrophic state as αMHC is down-regulated during cardiac hypertrophy [98]; this was most evident in the Et-1 condition. Finally, smooth muscle actin (SMA) was shown as it should stay constant throughout the various conditions. There was no statistical significance found between control and the three conditions for SMA.

This PCR data suggested that the Iso condition, at its concentration of 100nM, was able to stress the hESC-CMs the most in comparison to Et-1 and AngII. Furthermore, out of the two neurohormonal stimulants, Et-1 and AngII, AngII showed the weakest pathological hypertrophic effect. One possible reason for this is that Et-1 is one of the downstream signalling molecules of AngII to induce hypertrophy in cardiomyocytes. Since AngII induces the release of Et-1 and TGF-β from cardiac fibroblasts [42], it stands to reason that the current dose of AngII may not be high enough to induce a large enough endogenous release of Et-1 for paracrine signalling between the non-myocyte population and the cardiomyocyte population. While it is true that cardiomyocytes express angiotensin receptor 1 (AT1), it has been previously shown that in the absence of a non-myocyte population the effects of AngII on cardiomyocytes is diminished [42]. For instance, AngII does not modulate cardiomyocyte contraction amplitude or velocity when no
non-myocytes are present [99]. Furthermore, in pathological settings it is the fibroblast population that responds to AngII by increasing the number of AT1 receptors rather than the myocyte population suggesting that AngII primarily affects fibroblasts which in turn affects the myocyte population through paracrine signalling [100, 101]. As a result, the PCR findings suggest that the direct stimulation with Et-1 sidestepped the need for side population paracrine effects and directly stimulated the cardiomyocytes resulting in a more noticeable fetal gene upregulation.

It should be noted that all agonist were used with an initial concentration of 100nM. This concentration was further optimized based on BNP and ANF expression leading to final concentrations of 100nM, 150nM and 200nM for Iso, Et-1 and AngII. We also were continuously examining the constructs with confocal at each of these concentrations to ensure cardiomyocytes were undergoing pathological hypertrophy, as seen through sarcomere disorder, and not apoptosis. With these concentrations, we were satisfied that we were on the right track in terms of finding an ideal dosage of each stimulant to induce a pathological hypertrophic response in the biowire model while not inducing a large amount of cell death.

### 6.2.3 Pathological Hypertrophic Regiment Increases Cell Size

Another key feature of both pathological and physiological hypertrophy found in cardiomyocytes is an increase in cell size due to increased protein expression resulting in the production of new sarcomere units [37]. Examining the cell area is another means to probe the efficacy of the hypertrophic stimulus being used.

Figure 16 shows cell area in μm² as determined by digesting the biowires at the end time point, plating the cells onto matrigel for 24 hours and then fixing and staining with cTnT to see the cardiomyocyte boarder. What was found is that the Et-1 condition had the largest area at 532±46μm² with a statistical significance between CTRL and Et-1 using student T-test (P=0.009). The areas of AngII and Iso were 360±128μm² and 352±68μm² with no statistical significance with the control.
Similar to the PCR results, the neurohormones Et-1 and AngII display the similar trend of Et-1 being more effective at inducing a hypertrophic response in comparison to AngII. Again, the reason for this is probably due to the AngII concentration not being high enough to elicit a strong enough paracrine signalling effect of Et-1 from the fibroblast population in the biowire. As for the Iso condition, it was at first surprising to see a minimal increase in cell area when the Iso condition showed a strong fetal gene upregulation. However, the way in which Iso interacts with cardiomyocytes is different than the neurohormones. While endothelin-1 receptors and AT1 have their hypertrophic response mediated by the Gαq protein, this is not the case for isoproterenol as it signals cardiomyocytes through the β-adrenergic receptor which is mediated by Gαs and Gαi proteins [39]. This result that βAR agonist isoproterenol does not increase cell size in a dose-dependent manner was shown in the work by Simpson et al. [102, 103].

Since the isoproterenol group showed the strongest upregulation of the stress induced fetal gene (ANF and BNP) program along with distorted ultrastructure and an expected cell size, we elected to move ahead with the isoproterenol group and not the neurohormonal groups for the subsequent studies.

### 6.2.4 Hypertrophic agonist, isoproterenol, results in a decrease in the number of Z-disks per sarcomere in comparison to controls

To better quantify the pathological hypertrophic response and its breakdown of the sarcomere ultrastructure, transmission electron microscopy (TEM) was performed. Figure 17, shows representative images of control and isoproterenol sarcomeres at the end time point. As seen in the TEM images, the isoproterenol group had less defined Z-disks in comparison to the control (black arrows). The number of intact Z-disks per sarcomere was then quantified showing that the Iso group had a drastic reduction in Z-disk structure in comparison to control (P=0.0015 student-T test). This finding correlates well with our previous findings in Figure 14 showing the ultrastructure proteins of cTnT and αActinin breakdown and with a rat animal model using low
doses of isoproterenol shown to induce cardiac lesions and cardiomyocyte ultrastructure breakdown [64].
6.3 Discussion

Currently, the majority of studies performed to study pathological hypertrophy are done using the mouse or rat as the model system. For instance, using knock-in mice to study the cytoplasmic signaling pathways [39] in hypertrophic hearts or using 2D culture systems of rat CMs to study the hypertrophic response to various neurohormones or mechanical stimulation [42, 59]. However, each of these animal models has different electrophysiological properties of CMs between themselves and compared to humans. They also have different protein expression under stressed conditions, for instance in the rat α and β-MHC expression is reversed compared to humans [30]. With each mounting difference between the various animal models and humans, it becomes more difficult to tease out mechanisms, protein isoform changes and other symptoms of pathological hypertrophy that are specific to humans. While, animal models are excellent at preliminary studies for elucidation of possible similar cell signaling pathways of cardiac hypertrophy, for a more accurate model of human cardiac hypertrophy, human cardiac tissue needs to be created and tested.

Our *in vitro* model of pathological hypertrophy in the biowire platform, optimized using isoproterenol as the hypertrophic agonist, resembled various hallmarks of pathological hypertrophy found within humans: re-expression of certain aspects of the fetal gene program (ANF and BNP) [37] and a breakdown of ultrastructural proteins [104, 105] including Z-band smearing [106]. While these findings are not novel, the achievement in this project was the creation of a human *in vitro* model of pathological hypertrophy.

Another boon to the biowire platform, other than the use of human CMs, is that a heterogeneous population of cells is present. Since the heart is comprised of a heterogeneous population of cells, an *in vitro* model of the human heart should also mimic this population to ensure accurate crosstalk between cell populations is being modeled. In the mouse ventricle, 56% of cells are myocytes, 27% fibroblasts, 7% endothelial cells and 10% vascular smooth muscle cells [107]. The biowire consists of all these populations at similar amounts (CD31 (2.4 ± 1.5%, endothelial
cells [75]), CD90 (34.4 ± 23%, fibroblasts [75]), calponin (35 ± 22%, smooth muscle cells) and vimentin (80 ± 22%, non-myocytes)) and we believe that this heterogenous cell composition helps to generate a higher fidelity in vitro model of cardiac hypertrophy specifically, since we know that cross talk between fibroblasts and myocytes occurs and is necessary to recapitulate the pathological hypertrophic response [42].

However, the model is not without its limitations especially in the face of iPSC-CM disease modeling. While the results shown here indicate some features of pathological hypertrophy can be recreated, we do not know what type of hypertrophy we are stimulating. Are we generating right or left ventricular hypertrophy? Is this cardiac hypertrophy model more similar to an afterload or preload hypertrophic model? Since we have atrial like cells present are we also getting an atrial hypertrophic response mixed in as well? Using iPSC-CM diseasing modeling [20] allows for a much more controlled and specific examination of cardiac hypertrophy. One excellent example of this is the work by Lan et al. where a familial hypertrophic cardiomyopathy was examined in a family that had a missense mutation in the MYH7 gene [108]. Using iPSC-CM with an inherit defect allowed for the study of this MYH7 based hypertrophic model leading to a better understanding of how a dysregulation of calcium handling is a central factor behind the pathological hypertrophic response. The main short coming of this technique in comparison to our model of cardiac hypertrophy is that using iPSC-CMs to study a genetic disorder based pathological hypertrophy is very specific and may only be beneficial to a small subset of the population. Contrary to this, inducing pathological hypertrophy via neurohormones or cardiac therapeutics allows us to model pathological hypertrophy that occurs in the majority of the aging population that suffers from high blood pressure, due to poor diet and older age, along with patients currently trying to treat heart failure with pharmaceuticals.

Of course, the main drawback of using any type of hPSC-CM is the maturation state of the cell. In both methods, either inducing pathological hypertrophy using drugs or a disease iPSC-CM line, the cardiomyocytes that are generated are not representative of the adult phenotype as shown by [109]. Consequently, it is probably necessary to take a step back and look at how we...
can mature these PSC-CMs to a more adult like phenotype before we move forward in using these cells to model human diseases. As I have previously discussed in Chapter 5, electrical stimulation is one way to help mature hPSC-CMs, however, it does not produce the adult cardiomyocyte phenotype. The subsequent chapter will continue to explore physiological hypertrophy and hPSC-CM maturation through the combined us of mechano-electrical stimulation.
6.4 Conclusion

The objective of this project was to optimize a hESC-derived CM based EHT in vitro to measure induced hypertrophy. Pathological cardiac hypertrophy was induced in vitro by pharmacological agents and optimized based on fetal gene upregulation.

An in vitro model that recreates certain aspects of pathological hypertrophy was created using three different agonist: Angiotensin II (200nM), Endothelin-1 (150nM) and Isoproterenol (100nM). It was found that Isoproterenol elicited the strongest pathological hypertrophic response as determined through the induction of stress genes ANF and BNP and cardiomyocyte ultrastructure breakdown. Further analysis of the isoproterenol group using TEM revealed that a breakdown in sarcomere ultrastructure, specifically Z-band disruption, was evident and significantly different in comparison to controls. In conclusion it was possible to re-capitulate certain aspects of the adult pathological cardiac hypertrophy disease using the biowire platform along with hESC-CM to generate this in vitro model.
6.5 Future Work

While this first attempt showed some positive results, much of the PCR is not statistically significant along with weak and inconclusive electrophysiological data for the isoproterenol group (not shown). Moving forward, a combined Et-1 and Iso treatment should be optimized to elicit a greater hypertrophic response. Once this has been optimized, calcium handling of individual cells should be examined using confocal microscopy for calcium transients. Furthermore, immune-precipitation experiments for cardiac Troponin-T should be performed on the supernatant and compared to clinical values of patients undergoing heart failure due to cardiac hypertrophy.

Other concerns that need to be addressed are the cell population and maturity state of these cells. With the current model using a heterogeneous population of both myocytes and non-myocytes, it is difficult to determine what type of hypertrophy model is being generated, ventricular or atrial. Since there are differences between ventricle and atrial hypertrophies [110], once improved differentiation protocols have been established a more controlled cell population should be utilized. Furthermore, before a hESC-CM in vitro model can be truly used to model adult cardiac diseases, the problem of maturity of the hESC-CMs needs to be addressed.
6.6 Pathological Hypertrophy Figures

Control

Endothelin 1 (150nM)

Angiotensin II (200nM)
Isoproterenol (100nM)

Figure 14: Sarcomere structural proteins breakdown in the presence of hypertrophic agonists.
The left hand column shows sarcomeric actin (red) with α-actinin (green). The regularly repeated striation pattern of α-actinin can be clearly seen in the control while the breakdown of α-actinin can be seen in the Et-1 and AngII groups. The right hand column shows cardiac troponin-T (green) and connexion 43 (red). Again, a regularly repeated striated pattern is seen in the control while the pathological hypertrophy groups show a breakdown of the cardiac Troponin T protein.
Figure 15: Pathological hypertrophic agonists induce re-expression of the fetal gene program.
Real time polymerase chain reaction was performed at the 2 week end time period on control and drug treated biowires. During pathological hypertrophy the fetal gene program is re-expressed as seen in the following groups: atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and the β to α-myosin heavy chain ratio. Smooth muscle actin is shown as this gene should not be altered from the drug treatment. Statistical significance was seen in the BNP group between CTRL and ISO biowire conditions. *=P<0.05 ANOVA on ranks.
Figure 16: Endothelin-1 induces the greatest amount of cell hypertrophy as determined by cell area.

At the end of the drug treatment week biowires were digested and plated as single cells on matrigel. After 24 hours the cells were fixed and stained with cardiac troponin T to determine cell area. Cell area increased with all pathological hypertrophic agonists with a statistical significance found between the control and Et-1 groups (*: \( P=0.009 \) for control versus Et-1 using Student-T test).
Figure 17: Isoproterenol causes Z-disk protein breakdown and sarcomere disorder.

TEM images of control and isoproterenol treated biowires at the end of the two week period are shown. Control biowires show clear z-disks (black arrows) and a clear sarcomere unit shown with the white bar. Isoproterenol treated biowires show a breakdown of the Z-disk structure (black arrows) and disorganized sarcomere units. The graph shows that the number of z-disks per sarcomere in control versus isoproterenol treated biowires is statistically different (P=0.0015 using Student-T test)
7 Mechano-Electrical Physiological Hypertrophy Utilizing the Post Deflection Platform

7.1 Introduction

I propose that a combined electrical and mechanical stimulation of engineered heart tissue will result in a greater maturation of the tissue in comparison to each of the stimulating regiments alone or no stimulation. Based on the literature, we see that mechanical and electrical stimulation of hPSC-CMs each bring about a certain degree of maturation but each technique has its own shortcomings. Mechanical stimulation leads to physiological hypertrophy but induces elongation of action potential durations and does not seem to mature the calcium handling of the cardiomyocytes. Interestingly, electrical stimulation does improve cardiomyocyte calcium handling and also elicits a physiological hypertrophic response, albeit incomplete.

The purpose of pursuing this project using hPSC-CMs and not using neonatal rat cardiomyocytes, as an example, is twofold. The first is that ample work has already been performed on neonatal rat cardiomyocytes showing that mechanical and electrical stimulation can bring about the adult like phenotype. This is explicitly seen as t-tubules are brought about during mechanical stimulation or electrical stimulation. It seems that neonatal cells have developed far enough that applying either mechanical or electrical stimulation can bring about the adult phenotype. However, this is not the case with hPSC-CMs due to their fetal like state at the end of the differentiation protocol. This makes it an exciting challenge to try and push these cells through to the neonatal state and if possible towards the adult state. The second reason for using hPSC-CMs is that we want to have a human based platform instead of rodent (or another species). This maturation platform can also be used as a disease modeling and/or toxicology platform for human cardiac tissue. As a result, a more representative in vitro cardiac tissue to
model the human myocardium can be achieved using hPSC-CMs in order to elucidate a better understanding of human cardiac disease and to test novel cardiac pharmaceuticals.

Due to the challenges in determining what stage of maturation hPSC-CM based EHTs are at, one reliable method to indirectly determine higher degrees of maturation is through functional screens. Currently, there are a variety of costly and timely assays that can be employed to determine the approximate maturation level of the hPSC-CMs. These include, confocal microscopy, TEM, RT-qPCR, immunohistochemistry, calcium transients, optical mapping and patch clamping. In order to obtain a complete picture of the hPSC-CMs, all of these assays are required. However, as previously stated these are time consuming and some assays, such as the immunohistochemistry and calcium transients, require the tissue to be digested at the end time point and plating of cells overnight which, can possibly introducing error. As a result, a quick method that uses the bulk tissue to determine maturation was needed. To address this, the platform that will be designed will have posts that can deflect under the contractile force of the tissue. Using two dimensional analytical solutions for a bending cantilever, the force of contraction of the tissue can be calculated. While this does not directly give the maturation state of the tissue, it is an effective way of screening different electromechanical regimens. Moreover, for the EHT to beat as a whole tissue and to beat faster in response to faster pacing the tissue must have functioning excitation-contraction coupling. Consequently, if one assumes that as contractile force increases so does the maturation state of the EHT then, screening for the most effective regiment will also mean screening for the conditions that bring about the highest degree of maturation in the tissue.

In order to mature hPSC-CMs a recapitulation of their native environment will be approximated through the creation of a 3D tissue construct in a collagen gel and by applying both mechanical and electrical stimulation. It has been previously shown that hESC-CMs do not have a fully functional sarcoplasmic reticulum or contractile apparatus [26]. We have shown in the previous chapter that through electrical stimulation a more functional sarcoplasmic reticulum can be induced within hPSC-CMs. Mechanical stretch has been shown to induce a positive force
frequency relation [58], via the Frank-Starling curve that was not present in non-mechanically stimulated hPSC-CMs [33]. These two findings motivate the creation of a combined electromechanical platform to mature cardiomyocytes in both their calcium handling and contractile apparatuses.

My research plan is to create a combined electromechanical stimulating platform out of polydimethylsiloxane (PDMS) in which the force of EHT contraction can be measured through the EHTs ability to deflect a post. The EHTs will consist of hESC-derived cardiomyocytes that will be suspended in a collagen gel and will be cultured in media for two weeks to allow for construct remodeling around two anchoring but deflectable posts. After two weeks, a regiment of mechanical and/or electrical stimulation will be applied for one week to mature the EHTs. Regiments that yield constructs displaying the highest force of contraction, calculated through post deflection, will be examined for maturation markers through a variety of techniques. Ultrastructure will be examined through confocal microscopy and transmission electron microscopy. Calcium handling will be examined through optical mapping and calcium transients. Finally, gene and protein regulation will be examined through real time polymerase chain reaction (RT-QPCR) and western blots.
7.2 Results

7.2.1 Validation of Stretch Platform

Validation of the force required to move the PDMS post was done using the Refined Myograph System (Kent Scientific). The PDMS post and part of the base was excised from the PDMS platform and placed upon a glass slide. Since the Myograph has a caliper attached to the force transducer, this allowed for specific displacements to be measured while recording a change in voltage from the transducer as the transducer applied force to the top tip of a single PDMS post. A displacement versus force graph was then generated as seen in Figure 18. Percentage error based upon the difference between the Myograph testing and the 2D analytical solution was then calculated as seen in Table 3 with an average percent error of 7.44±4.11%. During the final 3rd week of EHT culture, a 5-10% stretch would be implemented to provide static stretch. As seen in Table 4, the actual displacement of the posts was shown as a percent change in distance. For the 5% and 10% change in displacement the percent errors were 9.74% and 4.72%. As the desired displacement increases to 15% and 20% stretch the actual displacement of the posts begins to falter as seen in the percent error rising to 12.48% and 18.28%. Finally, a sensitivity analysis was performed to determine the relationship between tissue height and deflection of the post at the free end and to also examine the relationship between a tissue’s force of contraction and the deflection of the post at the free end.

Figure 19 shows how the deflection of the post at the free end changes based on how the height of the tissue varies from the bottom of the post to the top of the post. In this scenario it is assumed that the tissue thickness was 0.4mm and the percentage change in height was the difference between the tissue’s new height along the post and the ideal position of the tissue, which is when the tissue is sitting at the bottom of the post. Figure 19 shows that the system was very sensitive to the tissue moving up along the post. In fact an $x^2$ relationship is seen. Consequently, it will be important to either always guarantee that the tissue is at the bottom of the post during post deflection measurements or to determine the tissue’s height each time post
deflection is performed by cutting out the post and tissue construct from the PDMS platform to measure the tissue height from the bottom of the post.

Figure 20 shows how the strength of the EHT’s force of contraction, or the distributed load along the post, affects the maximum deflection at the free end. In this scenario, the original distributed load was set to be 0.5N/m, which is a point-load of 0.2mN. At 0.2mN the free end will deflect about 1μm. This value is probably the lower limit of the sensitivity that can be achieved on the microscope we are using at a magnification of 20X, the required magnification to see the post deflect. At higher magnifications the thickness of the PDMS is too great for the microscope to be able to focus on the top plane of the post while a lower magnification reduces the accuracy of measuring post deflection. A linear relationship governs the increase in distributed load to the increase in free end maximum deflection.
7.2.2 Post deflection EHT set-up

During the initial trials of the electromechanical platform, four key parameters were required to be optimized before a reproducible EHT set-up was achieved. Collagen concentration had to be optimized to ensure the constructs would not break apart during the gel compaction process, via the fibroblast like cells, and construct contraction, via the cardiomyocytes. If the collagen concentration was too low, the EHT would break apart after approximately a week.

Next, the number of cells used had to be optimized. While a low number of cells was desired due to the cost and difficulty of differentiating hESC-CMs, due to the relatively large size of the device a greater number of cells had to be used. If the cell density was too low, the contraction and beating of the construct would take longer than 2 weeks to occur and the force of contraction would be rather weak making it difficult to assess tissue contractile strength as the posts would not deflect a large degree.

The third parameter that needed to be tested was surface treatment. During preliminary studies with EHTs that contained the optimal collagen concentration and cell density, there were problems with the tissue staying attached to the walls of the PDMS channels. While the majority of the tissue would compact away from the side walls, the main issue lay in the EHT attaching to the bottom surface of the PDMS channels. This was a problem as when the tissue contracted, some of the force would have been dissipated in the regions attached to the side walls and bottom surface. Furthermore, the reproducibility of the EHTs, in terms of how the gel compacted around the post and the amount of gel attached to the bottom surface, from well to well varied greatly making it difficult to compare post deflection values. In order to overcome this challenge pluronic acid was used to coat the surface of the PDMS providing a hydrophobic surface treatment facilitating the detachment of the EHTs from the side and bottom walls during the two week gel compaction period.

The final parameter was the time required to culture the EHTs. The total time the EHTs were cultured for was three weeks where, the electrical stimulation treatment was applied during the
third week of culture. While three weeks was a long time point, it was a necessary one. During the first week of culture EHTs compact the collagen gel and twitching of the tissue was seen. During the second week of culture the gel continued to contract and by mid-week post deflection was seen; however, the tissue did not beat synchronously and gel compaction was not complete. It was not until the beginning of the third week that synchronous beating of the tissue was seen, most of the time, and that the majority of the gel compaction was completed. As a result, the beginning of the third week was an optimal time point to start electrical and/or mechanical stimulation. Figure 21 and Figure 22 show the three week time course of gel compaction along with the PDMS platform with no tissue and with tissue at the end of the three week period.

### 7.2.3 Force of contraction

In order to screen different testing conditions in a reliable and quick fashion, a post deflection method of measuring the force of contraction of each EHT was designed. The reason for doing so was to eliminate the need of running RT-qPCR, optical mapping, calcium transients and other methods currently used to determine the maturity level of the tissue. These methods are necessary; however, for initial screening of different electrical and/or mechanical regiments one can correlate the maturity level of the tissue with the force of contraction. This has been previously shown in the sheep’s heart as cardiac tissue increasing in contractile strength during gestation through birth and into adulthood Figure 23A [111]. Consequently, which ever regimen of electrical and/or mechanical stimulation provides the greatest force of contraction will ideally be inducing the greatest amount of maturation in the hESC-CMs of the EHT.

Referring to Figure 23B, the force of contraction for control and 2Hz stimulated three week old EHTs is shown. The force of contraction was measured under 1Hz and 2Hz pacing. All of the conditions were around the same value of 1.5mN/mm² except for the 2Hz stimulated EHT paced at 2Hz, which had an average force of contraction of about 2.3mN/mm². These results point to the electrically stimulated samples being “more mature” than the control samples due to the greater force of contraction seen in the electrically stimulated EHTs. Another way to interpret this finding was that the control EHT when paced at a higher frequency did not display a positive
force frequency relationship while the 2Hz stimulated group showed this relationship [112]. These findings indicated that the most likely explanation was that the 2Hz paced EHT had a more developed calcium handling system allowing for the positive force frequency relationship. This resulted in the 2Hz EHT being more mature, at least, in terms of calcium handling in comparison to control EHTs.

The native human adult myocardium has a peak twitch force of 34-54mN/mm$^2$ [113], which is significantly greater than the force of contraction produced by the EHTs. The most obvious reason as to why there is such a large discrepancy is that the EHTs are created from fetal like hESC-CMs that do not consist of the same ordered mechanical apparatus and calcium handling capabilities as adult cardiomyocytes. Furthermore, the cell density of cardiomyocytes within the native myocardium is 1-10*10$^8$ cells/cm$^3$, while the EHTs consist of one to two orders of magnitude less cell density in comparison. While these preliminary results may seem discouraging, if we examine the contractile forces produced by rat neonatal EHTs (2-4mN/mm$^2$ [114]) and other groups that have looked at mechanical stretch to stimulated hPSC-CM EHTs (0.1-0.4mN/mm$^2$ [58]), the results generated here become a bit more encouraging. Currently, this electromechanical deflection platform is able to produce EHTs that generate force that is greater than other hPSC-CM based EHTs and has produced EHTs with comparable force of contraction as neonatal rat EHTs.

### 7.2.4 Excitation threshold and Maximum capture rate

Excitation threshold (ET) and maximum capture rate (MCR) were performed on EHTs at the three week time point. ET was performed at a constant 2Hz frequency and MCR was performed at a constant 4V/cm. The 2Hz electrically stimulated group had a lower ET and higher MCR compared to the control group, Figure 24, indicating that electrical stimulation leads to improved functional properties of the EHT.
7.2.5 Post relaxation, contraction, and rest time

Upon examining the tissue, specifically after electrical stimulation had begun, it became apparent that there was a significant difference in the time it took the tissue to contract and relax when comparing between the control and stimulated groups. Consequently, an examination of the duration of contraction and relaxation transient was performed. This was then normalized to either 1 second or 0.5 seconds depending on the pacing rate to determine the percentage of time the tissue was undergoing contraction and relaxation (the rest of the time the tissue was resting) Figure 25A. What was found is that the electrically stimulated tissue was faster at going through the contraction and relaxation period in comparison to the control group at both the 1Hz and 2Hz pacing. Both control and electrically stimulated EHTs when paced at the higher frequency, 2Hz, had less resting time. This was clearly evident in the control tissue as 97% of the time the tissue was contracting/relaxing.

To quantify the duration of the transient more rigorously, the time it took the post to contract, relax, and resting time was determined, Figure 25B. What was found is that the electrically stimulated group had approximately equal times for both contraction and relaxation while the control group did not have an even distribution. Moreover, the electrically stimulated group at 1Hz showed a 300ms contraction and relaxation time period which is the same time required for contraction and relaxation in the adult myocardium [36]. Finally, the control group paced at 2Hz showed that it could not completely keep up with the 2Hz pacing as the total time for this group exceeded 0.5 seconds.

Again, as previously seen in the force of contraction, ET, and MCR results the optimal condition for contraction and relaxation time was the electrically stimulated group. By pacing these EHTs at 2Hz over one week, we can hypothesize that a more mature calcium handling system and a more defined action potential [115] was being stimulated within the hESC-CMs.
7.2.6 Confocal Microscopy

Figure 26 shows confocal microscopy for cardiac troponin T (cTnT), connexin 43 (Cx43), α-actinin, and actin. The regular repeating striation pattern of cTnT, α-actinin, and actin showed that myofibrils were well organized and align parallel to the direction of uniaxial tension. The uniaxial tension was generated after the EHT compacts around the two posts. After this gel compaction, the EHT becomes taut between the two posts and during contraction is further stressed in this uniaxial direction. Consequently, the myofibrils align parallel to this force to generate the greatest contractile force. Finally, Cx43 was seen (control sample not processed yet) distributed throughout the myocyte.

7.2.7 Real Time Quantitative Polymerase Chain Reaction

The real time quantitative polymerase chain reaction (RT-QPCR) data can be categorized into 4 categories

1. Calcium handling: CACNA1C, RYR2, Cx43, ATP2A2, SCN5A, Kir2.1
2. Hypertrophy: ANF, BNP
3. Structural Proteins: cTnT, MYH6, MYH7, MYL2, MYL7
4. Cardiac Markers: ISL1, NKX2.5, SIRPA

The first set of genes that were examined pertain to the calcium handling of cardiomyocytes. It was shown in section 7.2.5 that electrically stimulated EHTs displayed faster contraction and relaxation times, begging the question what is electrical stimulation doing to the cells that results in this improved calcium handling? Looking at how the action potential occurs in the adult cardiomyocyte, depolarization of the membrane is mainly dominated by Na\(^+\) channels and repolarization of the membrane is dominated by K\(^+\) channels [36, 43]. Knowing this, when examining the genes SCN5A (Na\(^+\) channel) and Kir2.1 (K\(^+\) channel), the 2Hz electrically stimulated EHTs had an upregulation of these genes compared to control EHTs. SCN5A was
expressed in relatively similar amounts in the 2Hz and EBD41 samples while Kir2.1 had a slightly higher expression in the 2Hz EHTs compared to EBD41.

The sharp and more forceful contraction of the electrically stimulated samples should be due to the higher rate and amount of calcium leaving and entering the sarcoplasmic reticulum. Calcium enters the cardiomyocyte through L-type calcium channels (CACNA1C) to induce further calcium release from the sarcoplasmic reticulum through ryanodine protein (RYR2). Both of these genes were upregulated in the 2Hz electrically stimulated samples compared to controls and EBD41. Calcium is then transported back into the sarcoplasmic reticulum through sarco/endoplasmic reticulum calcium ATPase (ATP2A2), which was upregulated in the 2Hz EHTs compared to control EHTs and was slightly greater than EBD41. Finally, Cx43 which is one of the proteins found within the gap junction protein complex that facilitates the transmission of electrical currents between cardiomyocytes was upregulated in both the control and 2Hz stimulated EHTs compared to EBD41. However, Cx43 was expressed more in the control EHTs compared to the 2Hz stimulated EHTs. One possible reason for this is that the 2Hz EHTs were being globally stimulated through a pulsed field stimulation while the control EHTs require cardiomyocyte to cardiomyocyte gap junction connections to propagate an electrical signal. Since the 2Hz EHTs were field stimulated, there was not as great a need for the presence of Cx43 resulting in its lower expression compared to the control group.

Next are the pathological hypertrophic (re-expression of fetal genes) ANF and BNP. During development ANF and BNP are highly expressed but, are rapidly downregulated during the first week after birth [116, 117]. In a normal human adult ventricle both ANF and BNP expression levels are quite low and it is during a pathological cardiac response, such as cardiac hypertrophy or heart failure, that these genes are re-expressed. We found that ANF was less expressed in both the control and 2Hz stimulated EHTs while BNP expression for the control group was downregulated and 2Hz BNP was expressed at levels similar to that of EBD20. Both physiological and pathological hypertrophy results in the re-expression of the fetal gene program, however, genes such as ANF and BNP are greatly re-expressed during pathological
hypertrophy while only slightly upregulated during physiological hypertrophy [118]. In a mouse model that looks at transverse aortic clamping, for pathological hypertrophy, and a swimming regiment for physiological hypertrophy, ANF and BNP RNA was found in the pathological group but not the physiological hypertrophy group [119]. However, in another paper looking at both pathological and physiological hypertrophy in rats, it was found that BNP mRNA was indeed greater in the pathological hypertrophy rat (spontaneously hypertensive rat 19 weeks) but the physiological hypertrophy rat (swimming) had slightly greater BNP levels compared to the control mouse (not statistically significant) [97].

Looking at the big picture in terms of the 2Hz stimulated wire having increased force of contraction, faster/stronger action potentials, and organized sarcomere structure seen in confocal microscopy, it seems that physiological hypertrophy may be occurring in the 2Hz stimulated group. With low ANF RNA levels and BNP RNA levels being comparable to that of the EBD20 but not greater, it is possible that myofibrillogenesis is occurring in the 2Hz stimulated sample resulting in physiological hypertrophy.

One of the improvements that static stress and static stretch will have on cardiomyocytes is the increase in the βMHC (β-myosin heavy chain) isoform [58]. Ideally, myofibrillogenesis will occur due to stretching the cardiomyocytes. During myofibrillogenesis, and in general physiological hypertrophy, new sarcomere units will be created resulting in larger myofibrils. An indicator that this is occurring is the upregulation of βMHC, the dominant MHC isoform found in the human ventricular cardiomyocyte. Examining the RT-qPCR data, expression of αMHC (α-myosin heavy chain) RNA decreased from EBD20 and EBD41 compared to the control and 2Hz stimulated EHTs. However, βMHC RNA expression decreased in the control compared to EBD41 while the 2Hz stimulated sample had βMHC comparable to that of EBD41. This suggested that static strain, due to the gel compaction around the two posts, along with electrical stimulation was able to maintain MHC RNA levels but not induce greater expression of βMHC compared to the time matched EB’s.
Two other structural protein RNA levels that were probed are MYL2 (myosin light chain ventricle isoform) and MYL7 (myosin light chain atrial isoform). Myosin light chain atrial isoform can be thought of as a developmental isoform as it is expressed in the ventricle cells at an early stage but, soon after birth it is completely replaced by the ventricular isoform of myosin light chain [120]. The RT-qPCR results for MYL showed a similar trend as the MHC results such that the control samples showed lower RNA values compared to EBD41 and 2Hz for both MYL2 and MYL7 while, the 2Hz stimulated EHTs had comparable RNA levels to that of EBD41. Again, this suggested that static strain along with electrical stimulation was able to maintain MLC RNA levels but not induce greater expression compared to the time matched EB`s. Furthermore, there was no reduction in MYL7 signifying a shift towards mature ventricle cardiomyocyte phenotype. However, this point is clouded by the fact that the cardiomyocyte population is mixed, ventricular, atrial and pacemaker like cells are present in the EHTs, along with the fact that RNA levels of the majority of the genes are drastically below the fetal level (data not shown) leading to all genes requiring to increase in expression.

The last structural protein RNA level that was examined was cTnT which was two times higher in both the control and 2Hz stimulated EHTs compared to EBD41.

The final group of RT-PCR genes that were examined are cardiac developmental/specific genes, ISL1, Nkx2.5 and SIRPA. As cardiomyocytes mature ISL1 should be downregulated from EBD20 to EBD41 and the EHTs while, Nkx2.5 should increase in expression and hold constant from EBD20 to EBD41 and the EHTs. In this case the opposite was seen. It has been shown that a gene profile of dropping ISL1 and increasing Nkx2.5 is consistent with cardiac muscle being generated from the second heart field while the first heart field has cardiac progenitors with an absence of ISL1 and Nkx2.5+ [8]. SIRPA is a specific cardiomyocyte surface marker and should be expressed constantly between all groups, which it was seen to do.
7.3 Discussion Future Work

The objectives of this project were threefold.

1. Design and create a platform that allows for functional screening of engineered heart tissues (EHTs) by measuring EHT force of contraction.

2. Augment the platform to facilitate mechanical and electrical stimulation to be simultaneously applied to EHTs.

3. Optimize a regiment of mechanical and electrical stimulation to obtain the greatest amount of maturation in the EHTs.

Objectives one and two were completed such that a platform that can provide electrical field stimulation and mechanical static stretch to EHTs was created. However, an optimized regiment was not completed. Below are suggestions for future work on this project.

A baseline for the system has been presented in this document (control group) along with one condition, electrical stimulation at 2Hz. The next step is to:

- Perform experiments at 5% strain and 2Hz electrical stimulation
- Perform experiments at 10% strain and 2Hz electrical stimulation
- Perform experiments at a combined 5-10% strain and 2Hz electrical stimulation

Once an ideal mechanical and electrical stimulation regiment has been determined, a more detailed assessment of the EHTs can be conducted. These assessments may include:

- Ultrastructure characterization through transmission electron microscopy
- Calcium handling functionality determined through optical mapping and calcium transients
7.4 Mechanical-Electrical Platform Figures

Figure 18: Refined myograph PDMS post force vs. distance graph.
The force required to move a PDMS post as determined by the force transducer within the refined myograph system is shown.

Table 3: Percent error between myograph system and analytical solution.
Using Equation 1 to determine the amount of force required to deflect the free end of the PDMS post in comparison to the actual force required to deflect the PDMS post as determine by the force transducer is presented. The final column show the percent error between the two methods.

<table>
<thead>
<tr>
<th>Distance (mm)</th>
<th>Myograph Force (mN)</th>
<th>Analytical Solution Force (mN)</th>
<th>Percent error (%)</th>
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<td>0.206</td>
<td>0.706</td>
<td>0.646</td>
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</tr>
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Table 4: Percent error between the expected (stretch) distance between posts and the actual distance between posts.

The PDMS platform was stretched using the constructed device. Images were taken of the PDMS platform at each of the four stretch settings of 5, 10, 15 and 20%. The actual distance between the posts was measured and percentage error between the desired stretch and the average actual stretch is shown.

<table>
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<th>Average Stretch (%)</th>
<th>Desired Stretch (%)</th>
<th>Percent Error (%)</th>
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</thead>
<tbody>
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<tr>
<td>16.91</td>
<td>20</td>
<td>18.28</td>
</tr>
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Figure 19: Sensitivity analysis of post free end deflection with varying tissue height along post.

A tissue of 0.4mm thickness situated at the bottom of the post with an average point force of contraction of 0.2mN was the base scenario (0% height change) in this graph. The tissue was then moved up the post and the deflection at the free end was calculated again based on the same force of contraction. This graph shows that the free end deflection is highly sensitive to the tissue’s placement along the post as a parabolic relationship was found.
Figure 20: Sensitivity analysis of post free end deflection with varying distributed load.
A tissue of 0.4mm thickness situated at the bottom of the post with an average point force of contraction of 0.2mN (0.5N/m) was the base scenario at 0% change in distributed load. The distributed load was then varied while assuming the tissue remained at the bottom of the post. A linear trend between deflection at the free end and change in load is seen. Also of note is that below a 0.2mN point force, the displacement of the post drops below 1μm.
Figure 21: Majority of EHT gel compaction occurs over two weeks.

Time laps of EHT over 21 days. Images show the top view of the EHT in a single post deflection well with two posts. Over the first two weeks, the majority of the gel compaction occurs resulting in a suspended EHT fixed at the two ends by gel compaction around each post.
Figure 22: EHT compaction occurs at the bottom of the post deflection system in a reproducible manner.

Stereomicroscope images on the PDMS electromechanical platform without tissue (A-C) and with tissue (D-E). A) Top view of PDMS well with two posts, B) shows a zoomed in view of a single post, C) side view of a single post. Image D) shows a side view of a post with fixed EHT tissue (3 week time point) wrapped around the post. E) shows again a fixed EHT (3 week time point) side view of the tissue spanning the two posts.
Figure 23: Force of heart tissue contraction increases through gestation and after birth until heart reaches full adult maturity.

Figure A shows the force of contraction per area of a sheep’s heart during gestation and into adulthood. Arrow indicates birth with x-axis representing number of days [111]. Figure B shows EHT force of contraction for control and electrically stimulated (2Hz) samples. Force of contraction was measured at 1Hz and 2Hz pacing at the end time point of three weeks. 1gm = 10mN. N=1 with 3 wires per group.
Figure 24: Electrical stimulation of EHTs leads to better functional properties.

Electrically stimulated (2Hz) EHTs display a lower excitation threshold and higher maximum capture rate in comparison to the control EHTs. Excitation threshold performed at constant 2Hz while maximum capture rate was performed at constant 4V/cm. N=1 with three wires per group.
Figure 25: Electrically stimulated EHTs display a more adult like contraction and relaxation time period.
Figure A) shows the percentage of time the EHT is both contracting and relaxing during a single action potential. Since the samples were paced at 1Hz or 2Hz, the action potentials were instigated every second or every 0.5 seconds. Of note are the electrically stimulated EHTs displaying faster duration transients compared to the control EHTs. In other words, the electrically stimulated EHTs were at rest for a longer period during each cycle. The breakdown of the time required for contracting, relaxing and tissue resting time is shown in figure B). Note that the electrically stimulated samples displayed faster and evenly split contraction and relaxation times while the controls are slower and do not display such an even split between contraction and relaxation.
Control

cTnT and Cx43

2Hz Stimulated

α-actinin and Actin

Control
2Hz
Stimulated

Figure 26: Myofibril structure and alignment is enhanced by the post deflection system.
Confocal microscopy images of control and 2Hz stimulated samples at 3 weeks. EHTs show aligned sarcomeres parallel with the uniaxial tensile force generated by the EHT contracting around the two posts. The top set of images show cardiac troponin T in green and connexion 43 in red. The bottom set of images show αActinin in green and actin in red.
Group 2

**ANF**

- EBD20
- EBD41
- CTRL
- 2Hz

**BNP**

- EBD20
- EBD41
- CTRL
- 2Hz
Group 3

![Bar chart for Group 3 showing cTnT/GAPDH ratios for EBD20, EBD41, CTRL, and 2Hz conditions.](chart.png)
Group 4

**ISL 1**

**Nkx 2.5**

[Bar charts showing expression levels of ISL 1 and Nkx 2.5 for different conditions: EBD20, EBD41, CTRL, 2Hz.]
Figure 27: Post deflection system in conjunction with electrical stimulation promotes the up-regulation of structural and calcium handling related genes.

Real time polymerase chain reaction (RT-QPCR) was performed at the 3 week end time point on control and electrically stimulated EHTS. Four subgroups of genes are shown: 1) calcium handling, 2) ultrastructure, 3) hypertrophy, and 4) cardiac marker related genes. N=1 with 2-4 samples per group.
8 Statistics and Sources of Variability

A major concern with using hPSC-CMs was the heterogeneity of the differentiated cells. The work presented in this thesis was based upon obtaining cells from a directed differentiation protocol that yielded immature cardiomyocytes (section 2.2) with batch to batch variability. The variability was seen in the yields of myocyte versus non-myocyte populations and within the myocyte population itself. This meant that there was a fluctuating ratio of myocyte versus non-myocyte populations in each experiment. Furthermore, using directed differentiation protocols did not produce defined myocyte populations. In each batch of differentiated cells there was a mixture of ventricular, atrial and pacemaker like cells. Ideally, the approximate ratios of these different myocyte populations would be similar from batch to batch leading to similar EHTs generated each experiment; however, the larger problem was in trying to model ventricular myocardium with the presence of atrial and pacemaker cells.

Since all analysis of the EHTs was done on the entire tissue, it was not only ventricular-like cardiomyocytes being analyzed but also atrial and pacemaker like cells. For instance, during RT-qPCR since the entire EHT was processed for RNA, we were essentially looking at a gene expression profile of the “whole heart” instead of just the ventricle. When examining gene regulation for α and βMHC, atrial cells should express high levels of αMHC while ventricular cells should predominantly express βMHC as they are maturing and once they reach an adult phenotype. However, when examining a mixed population both genes would be present and possibly upregulated in comparison to age matched EBs. Since both α and βMHC could be upregulated, it would make it difficult to distinguish whether or not ventricular cells were truly maturing or if they were still expressing high levels of fetal genes (αMHC) or if it was atrial cells that were expressing greater amounts of αMHC.

Since it was not fully understood how the side population of cells affected cardiomyocytes, when these populations vary between experiments greater error was generated. This error was seen in
the variability of proliferation rates or in the gene expression profiles. In the pathological hypertrophy project, when a disease state was induced, the cells would revert to a fetal gene profile. However, the control (untreated hESC-CMs) still expressed certain aspects of the fetal gene profile which made it difficult to distinguish the absolute effects of the pathological treatment. For instance, to examine an isoform switch between β and αMHC during healthy and diseased states requires CMs to be expressing the dominant ventricular βMHC isoform. However, the CMs were not expressing βMHC as the dominant isoform due to their immature state making it difficult to determine certain characteristic features of pathological hypertrophy.

When examining the force of contraction in the post deflection platform certain aspects of the set-up could be modified to enhance or diminish the force of contraction generated by the EHT. This included the number of non-myocytes, the number of CMs, the volume of gel, the collagen concentration of the gel, duration of culture and geometry of the set-up. All of these factors contributed to optimizing EHTs force of contraction and while a cross sectional area was used as a normalization factor, the passive tension force of the matrix and non-myocytes were not taken into consideration. Possibly, the best way to determine force of contraction would be to disrupt the actin cytoskeleton of the non-myocytes and determine the elastic modulus of the matrix with cells present. After this, when force of contraction is examined it would represent the myocyte population alone contracting the matrix and the posts. By ensuring an exact input population of myocyte versus non-myocyte and knowing the cell number per construct, a force of contraction per myocyte could be produced.

While the variability of differentiated hESC-CMs was high (and can be compounded by differences in cell lines [26]), it was apparent that generating EHTs and culturing the cells in a 3D matrix with stimulation cues helped to mitigate this variability. When examining the RT-qPCR data, fetal genes such as BNP and ANF have a large standard deviation in the EB samples in comparison to the biowire samples. This was also seen in the proliferation assay where the EBs had a larger variance between groups in comparison to the biowire samples. Moving forward, a mindful and more carefully managed approach to the generation of EHTs, using
defined side populations with a fixed ratio of myocyte versus non-myocyte, could aid in generating hPSC-CMs that are better matured and also generating a more homogenous cardiomyocyte population.
9 Project Conclusions

hPSC-CMs hold the potential to further understand the fundamental mechanics of how the heart works. The common theme running through all three projects has been cardiomyocyte hypertrophy. At the heart of this cellular phenomenon is a shroud of mystery as the hypertrophic response can teeter between pathological and physiological under similar stresses. The increase in biomechanical stress that leads to this hypertrophic response is accompanied by a plethora of different signaling events leading to either pathological or physiological hypertrophy. It is with hPSC-CMs and bioengineering techniques that these uniquely orchestrated events were attempted to be recapitulated \textit{in vitro} to further understand how cardiomyocyte hypertrophy occurs.

Generating hPSC-CM based EHTs in the biowire or post deflection platform lead to improved ultrastructure. A common technique between all the platforms was the utilization of gel compaction around a stiff substrate (biowire suture) or anchoring points (posts) to create a uniaxial stress to signal hPSC-CM elongation and alignment of sarcomere units. Mechanical stress leads to improved cardiomyocyte ultrastructure.

Once we were confident in the function and ultrastructure of the EHTs being generated using the biowire platform, we attempted to explore how pathological hypertrophy could be induced using Et-1, Ang II or Iso. We found that certain characteristics of pathological hypertrophy could be induced, such as disrupted ultrastructure and re-expression of the fetal gene program. However, due to the immaturity of the hPSC-CMs we could not truly say that an adult \textit{in vitro} model of pathological hypertrophy had been generated. This sparked the pursuit of electrical and mechanical stimulation in hopes of inducing a physiological hypertrophic response to induce maturation of the hPSC-CMs.

When examining the effects of the 3Hz and 6Hz ramp-up regiments using the biowire platform, electrical stimulation was able to induce half of the desired maturation/hypertrophic response we were expecting. Electrical stimulation lead to improved ultrastructural features, but, did not
improve structural protein gene expression. To address this we created a platform that would be able to both electrically and mechanically stimulate our EHTs. Although, mechanical stimulation has not been implemented, the post deflection system inherently has a mechanical stimulation effect through the EHTs ability to contract the posts. When EHTs in the post deflection system were electrically stimulated many of the structural proteins and calcium handling genes were upregulated on the RNA level. Ideally, once static stretch is applied in conjunction with electrical stimulation a further improvement will be seen.

In all cases the adult cardiac phenotype was never achieved. Consequently, there is still plenty of room for improvements such as: improved device design for cyclic stretch, further probing of electrical stimulation and time of culture using the biowire platform to encourage physiological hypertrophy. To truly replicate the niche environment of a ventricular cardiomyocyte both electrical and mechanical stimulation are required. While field stimulation is a good means to provide the electrical stimulation aspect, static stretch is not. An next step for device design would be to provide not only cyclic stretch but to also provide a cyclic stretch that provides that appropriate stretch to the tissue, diastole, and allow the tissue to contract rapidly and fully to mimic systole. This improvement could be based upon the current post deflection design utilizing pneumatic pistons as the means to stretch the tissue. Since the PDMS petri dish is an elastomer, it would retract to its original dimensions once stretched, however, this would not allow for the tissue to contract more than its original starting length and the rate at which the PDMS contracts may not be conducive to faster stretching regiments. Furthermore, a computer program would be required to sync the electrical and mechanical stimulation.

During electrical stimulation we found that the ramp-up regiment lead to significant improvements of the hESC-CMs. We also examined, not shown here, what happened when the biowires were stimulated for a week using the 6Hz stimulation protocol and then cultured for an additional week at 1Hz and found that continued improvements to ultrastructure were found. A more detailed examination of electrical stimulation and culture time on hESC-CM improvements should be explored. For instance, what happens if the 6Hz regiment is capped at 5.2Hz, the
highest frequency possible to be capture by the biowires, and then left at 5.2Hz for one addition week at the end of the 6Hz regimen? Would the continued high frequency stimulation of the biowires lead to continued rapid maturation of the ultrastructure or does a continued high frequency of pacing eventually lead to a disease model of cardiac failure? If the biowries were kept at 6Hz for an additional week after the 6Hz regiment would this induce a pathological state? Since the biowires are unable to keep pace with 6Hz, by pacing the biowires at this frequency a constant state of fibrillation would be induced with erratic calcium handling. This may possible be a favourable set of conditions to induce a generic pathological cardiac state that revolves around cardiomyocytes not being able to cycle calcium properly. Finally, culturing the biowires for long periods of time after the 6Hz stimulation protocol could lead to continued cardiomyocyte improvements and could be examined further. Hopefully with a greater understanding of how mechanical and electrical stimulation effect hESC-CMs we can one day mature these cells to an adult phenotype to aid in the generation of high fidelity *in vitro* cardiac tissues for modeling human cardiac diseases and to test novel cardiac therapeutics.
References


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Appendix A: Publications and Presentations Summary

Published Publications


Publications in preparation

Conference Poster Presentations

