Human iPSC-Derived Cardiac Myocytes: Toward an In Vitro Model of Cardiac Physiology

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Abstract

Cardiovascular Disease is a growing public health issue in the modern world, with a high incidence rate that continues to increase, and poor mortality rates. Recent technological advances have made it possible to efficiently derive cardiac myocytes from human induced pluripotent stem cells (hiPSC-CMs). These have been seen as a model for human heart disease, as well as a potential source for cellular transplantation into failing diseased heart tissue. Many laboratories have devoted substantial effort to examining the functional properties of hiPSC-CMs, including electrophysiology, intracellular calcium handling, and gene/protein expression and force. In the first part of this thesis, we utilize traction force microscopy (TFM) to determine the maximum force production of isolated hiPSC-CMs under varied culture and assay conditions. We elucidate here the relationship between cell morphology and force production, and find a significant relationship between cell size and force. HiPSC-CMs developing in culture for two weeks produce significantly less force than cells cultured from one to three months and hiPSC-CMs cultured for three months resemble the cell morphology of neonatal rat ventricular myocytes. Unexpectedly, hiPSC-CMs produce less force when assayed on increasingly stiff substrates, and generate less strain energy. Finally, hiPSC-CMs cultured in conditions of physiologic calcium concentrations are larger and produce more force than cells cultured in standard media. In the second part of this thesis, we address the concept of immaturity in hiPSC-CMs, and attempt to accelerate maturation. We use genome editing to

engineer hiPSC-CMs that contain an inducible gene expression cassette, in order to overexpress two proteins associated with maturity: SERCA2a and cardiac troponin I (cTnI). We find that we are able to overexpress both proteins in differentiated hiPSC-CMs after two weeks of treatment with doxycycline. SERCA2a-overexpressing cells showed significant alterations in physiologic function, including increased chronotropy and decreased time to peak in calcium transients following treatment with isoproterenol, a β-adrenergic agonist. Furthermore, using an impedance-measuring system to track contractility kinetics, we found that SERCA2a-overexpressing cells had shortened time to peak and time to baseline after gene induction, with continued response to isoproterenol. As a sign of maturation, SERCA cells also expressed increased cTnI, a key marker of maturity. Using RNAseq, we found that cTnI-overexpressing cells had marked, global changes in their gene expression profile. Key findings include upregulation of genes associated with cardiac contractility and development, such as cardiac myomesin and tropomyosin and ryanodine receptor, and downregulation of genes associated with pacemaker and ventricular cell types, such as HCN and GREM2, and genes associated with skeletal myocytes, such as skeletal muscle actin. Overall, our findings show that hiPSC-CMs have physiologic function similar to that of immature cardiac myocytes, but that we are able to induce maturation by overexpression of genes associated with maturity.

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Chapter 1

Introduction

Cardiovascular disease is a critical healthcare problem in the modern world, with a high incidence rate and poor mortality rates that have been improved by recent technological advances but have yet to fully address issues of death and, importantly, quality of life. Human pluripotent stem cells and stem cell-derived cardiac myocytes, recently discovered, show great promise in decreasing morbidity, mortality, inferior quality of life, and the economic burden of such a widespread chronic disease. This thesis focuses on elucidating the physiologic function of stem cell-derived cardiac myocytes, particularly in terms of force production; on elucidating the maturation status of stem cell-derived cardiac myocytes, in terms of physiologic function and protein expression; and on the use of genome editing to induce expression of physiologically significant genes in order to accelerate maturation and improve function.

To aid the reader in understanding and drawing conclusions from the data presented, and to aid in the understanding of how the ideas presented relate to another, this introduction reviews the topics of cardiovascular disease and current therapies, cardiac myocyte function and development, stem cells and stem cell-derived cardiac myocytes and current experimental uses of those cells, and genome editing as a tool for precise genetic manipulation. The current state of the field is explored, with reference to both a historical understanding of cardiac biology and a history of techniques and experiments that have led to our

current understanding, as well as the most recent, cutting-edge, in-progress results and models and future directions.

Clinical Relevance

Epidemiology of Heart Failure

Looming over the healthcare systems of the world, cardiovascular disease has been and continues to be a growing threat to public health and individuals' physical, mental, and social well-being[1]. While "cardiovascular" is a broad umbrella term that includes all major disease states relating to blood flow or lack thereof, here I will refer predominantly to another broad category of disease centered around the heart itself, which is heart failure. In terms of the history of medicine, heart failure has been used to mean any disease where the myocardium is unable to adequately produce force to result in adequate cardiac output.

This includes diseases that appear in childhood or adolescence, or the congenital cardiomyopathies; diseases that appear at various points throughout life, such as viral or post-partum cardiomyopathies; and diseases that occur in middle to old age, often referred to broadly by clinicians as congestive heart failure (CHF). In both the developed and the developing world, low levels of infectious disease and violence have resulted in an aging population with aging organs and aging cells, as well as prolonged exposure to environmental factors

that contribute to vascular disease, coronary artery disease, kidney disease, and obesity.

Globally, there are currently more than 23 million adults living with heart failure, with 6 million in the United States alone[2]. Worldwide, the prevalence of heart failure continues to increase[3], although incidence seems to have plateaued, and may be decreasing among certain populations[1], which may be due to decreased incidence among younger individuals alongside increased incidence among older individuals[4]. Mortality following a diagnosis of heart failure is high, with estimates of 30-day mortality of 10%, 1 year mortality of 20-30%, and 5-year mortality of 45-60%[5], and 75% 5-year mortality after the first hospitalization[6].

Risk factors for heart failure differ depending on subtype and clinical presentation- for example, CHF patients with preserved ejection fraction (HFpEF) are more likely to be female and have a history of hypertension[7]. However, several factors are strongly associated with adult heart failure in general, including ischemic heart disease, hypertension, diabetes, smoking, and obesity[4]. These often present as comorbidities in the same patient due to lifestyle or choices, sharing underlying common pathologies. Chronic kidney disease (CKD) shares many of these risk factors and frequently co-exists with CHF[8]. The combination of several chronic diseases such as these can complicate treatment algorithms and therapeutic regimens, sometimes confounding diagnostic and prognostic analysis.

Clinical Presentations of Heart Failure

Clinical definitions of heart failure have varied wildly over the decades, and even today different definitions are put forward by distinct academic organizations, such as the American Heart Association, the New York Heart Association, and the European Society of Cardiology[9]. However, most definitions include a combination of abnormal findings of left ventricular mass index, ejection fraction, left atrium systolic dimension, lower extremity mobility disability, summary physical performance score, and 6-minute walk test[10]. CHF is a generally insidious disease, with myocardial damage accumulating slowly over years, except in the case of damage due to myocardial infarction. Patient present to clinic with dyspnea, orthopnea, paroxysmal nocturnal dyspnea, fatigue, edema, and limitations in physical activity, which becomes worse as the disease progresses. Any combination of the above symptoms is possible. The New York Heart Association (NYHA) classifies symptoms on a functional scale of I, meaning no limitation of physical activity, to IV, meaning unable to carry on any physical activity without discomfort[11]. The assessing physician examines the patient using physical exam, exercise testing, and imaging to classify the patient on the NYHA objective scale of A, meaning no evidence of cardiac disease, to D, meaning objective evidence of severe cardiac disease[11].

Diagnosis of heart failure is made based on symptoms and evidence of myocardial dysfunction. Echocardiography gives information about diastolic and systolic dysfunction, which can be supported by information from cardiac

magnetic resonance imaging, vascular imaging, and computed tomography imaging to distinguish variations of heart failure from each other and determine underlying causes, such as valve disease or vascular disease, and provide prognostic information as well[12]. Blood tests, such as N-terminal pro-brain natriuretic peptide (NT-proBNP), which is elevated in heart failure, can provide additional information.

An important piece of the diagnosis to be made is whether ejection fraction (EF) is reduced (HFrEF) or preserved (HFpEF). This distinction has recently gained traction, as it was previously overlooked or unknown, although it is now known that the two versions should be treated slightly differently[12]. The distinction is made based on echocardiographic data; HFrEF is dominated by systolic dysfunction, while HFpEF is dominated by diastolic dysfunction, although significant overlap exists and may be present in an individual patient[13]. Heart failure can also be classified as high output, having a resting cardiac index of > 2.5-4.0 L/min/m^2 and low systemic resistance, or low output, having a resting cardiac index of < 2.5 L/min/m^2 [14].

Pathophysiology of Heart Failure

The pathophysiology of heart failure as a clinical syndrome is complex and incompletely understood. There are many precipitating events that may lead to heart failure, some of which are less common than others, including amyloidosis, radiation, constrictive pericarditis, and rare genetic disorders[15]; genetically-

encoded cardiomyopathies, including hypertrophic[16]; pregnancy[17]; and stress[18]. However, most adult heart failure is precipitated by more common, long-standing disease states such as valve disease, hypertension, obesity, renal disease, and myocardial infarction following coronary artery disease[14]. Compensated heart failure can progress to acute decompensated heart failure due to ischemia or infarction, non-adherence to therapy, uncontrolled hypertension, arrhythmia, pregnancy, alcohol intoxication, thyroid conditions, COPD exacerbation, or other causes[14].

Underlying most forms of heart failure are pathological processes common to many disease states. Inflammation has been implicated as a serious player, including upregulation of pro-inflammatory cytokines, such as TNF-α and IL-6, and downregulation of anti-inflammatory cytokines; infiltration of inflammatory cells into the myocardium, especially macrophages, is seen, and has profound effects on myocardial function[14]. Fibrosis is present in the myocardium, as well as in the lungs, is likely due to hypoxia, fibroblast dysregulation, and inflammation, and contributes to both systolic and diastolic dysfunction[13].

Metabolic dysfunction is being recognized as an important player in the pathogenesis of heart failure and involves insulin resistance, defects in substrate utilization, and defects in energy production[19]. On an organismal level, we see enhanced neurohumoral signaling and activity, as well as volume overload in the pulmonary and systemic vasculature due to inability of the heart to pump. On an organ level, we see ventricle wall hypertrophy, alterations in calcium handling,

arrhythmia, and often dilatation of the ventricle and atrium[20]. We also see angiogenesis, fibrosis, autophagy, and apoptosis.

Cell Intrinsic Dysfunction in Heart Failure

On a cellular level, vast changes occur, such as the switch to fetal gene expression, involving troponin isoform switching, myosin heavy chain isoform switching, downregulation of Akt signaling and upregulation of ERK signaling, increased PKC signaling, increased CaMKII signaling, downregulation of β-adrenergic signaling, and decreased SERCA2a and dysfunctional calcium handling[20]. Enhanced TGFβ signaling leading to SMAD2/3 activity affects many aspects of cardiac myocyte function including hypertrophy, metabolism, and contractility[21]. Stimulation of α1-adrenergic receptors plays a role in hypertrophy, but is also preventive against cell death in ischemia-reperfusion[22].

Additionally, autophagy, responding to increased levels of reactive oxygen species (ROS), as well as increased AMPK and mToR signaling, increases at first as a protective mechanism, but later decreases, seemingly unable to keep up with its task, contributing to accumulation of potentially toxic organelles and proteins[23]. Copious amounts of ROS, accumulating in the myocyte due to dysfunctional metabolism and energetics, contribute to hypertrophy and increased resting tension, and affecting mitochondrial energetics[23]. Dysfunctional calcium signaling over-activates CaMKII, which is protective during stunning, but harmful during ischemia-reperfusion[24]. The Hippo pathway can be activated by

ROS, GPCR signaling, and mechanical stress, all of which are present in failing myocardium, and can lead to hypertrophy and apoptosis, and downregulation of the pathway can improve cardiac regeneration[25].

Treatment of Heart Failure

Treatment of heart failure is mostly symptomatic, relying on medications that decrease extracellular fluid volume and decrease mechanical load on the heart through decreased fluid volume, decreased peripheral vascular resistance, and decreased cardiac chronotropy and inotropy. These are frequently used alongside medications that treat comorbidities, such as nitric oxide donors for patients suffering CAD, statins for patients with high cholesterol, diuretics for patients with hypertension, anti-clotting agents for patients with atrial fibrillation, and diabetes medications[26]; patients on many medications simultaneously means there is high probability of medication interactions. First line medications are ACE inhibitors or angiotensin receptor blockers, which decrease vasoconstriction, hypertension, and cardiac and pulmonary fibrosis, although data from clinical trials of both drugs in HFpEF patients is not entirely conclusive[26]. Beta blockers, which block activation of β-adrenergic signaling, are recommended for patients with a history of myocardial infarction, hypertension, or atrial fibrillation[14]. Other classes of drugs are available, and many others, such as those targeting dysregulated pathways mentioned above, are in clinical trials or are elsewhere in the research and development pipeline.

Mechanical devices, known as left-ventricular assist devices (LVADs), have gained importance as an end-stage treatment option; outcomes can be as good as patients who have undergone heart transplant, providing between 2-5 quality-adjusted life years (QALYs), although LVADs bring with them several major problems, including high rates of infection and prohibitive cost of device, implant procedure, and subsequent management[27]. Heart transplantation remain the final treatment in severe congenital cardiomyopathies, and is an option in other forms of end-stage heart failure in patients who are able to match with a donor. Heart transplant can add several QALYs to a patient's life, but also have several serious, persistent issues, such as infection and allograft vasculopathy[28].

Uses of Stem Cells as a Therapy in Heart Failure

More recently, a large effort has been made in both academic and industrial research laboratories to explore cardiac regeneration, as a more permanent therapy that targets the underlying pathophysiology of cardiac myocyte dysfunction by providing new, young cardiac myocytes to repopulate the myocardium and provide both systolic and systolic support. This can be done by either inducing native cell populations to divide and differentiate, or by utilizing cell therapy to transplant new cells, including stem cells and stem cell-derived cardiac myocytes or cardiac myocyte progenitors[29-30].

A landmark study utilizing carbon-14 dating showed some amount of cardiac turnover in the adult heart[31]. Several endogenous populations of cells in

the adult heart have been hypothesized to be cardiac progenitors capable of regenerating mature cardiac myocytes, including side population cells, c-kit+ cells, and other mesenchymal cell types, with potential therapies aimed at stimulating increased proliferation and differentiation of these cells. However, basic science data demonstrating clear proliferation and regeneration in native tissue of any of these cell types is severely lacking[32]. Other work has focused on reprogramming native non-progenitor cell populations into cardiac progenitors by overexpressing reprogramming factors, such as transcription factors associated with pluripotency[33].

As an alternative to native cell populations, stem cells could be transplanted into the myocardium, with the hope that they would either engraft, and then differentiate or mature (depending on cell type) and substantially contribute to mechanical function and strength of the organ, or provide paracrine signals that promote myocyte regeneration, ECM remodeling, and anti-inflammatory chemicals. Several cell types have been proposed as suitable for cell transplant; a major divide lies along whether the cells are pluripotent, such as cord blood cells, bone marrow stem cells (BMSCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs), or whether they are cardiac progenitor cells (CPCs) or cardiac myocytes (CMs) that have been differentiated from one of the above cell types[30].

Stem Cell Biology

Definitions

Stem cells are cells that differentiate into one or more terminal cell types, and self-renew during the mitotic process, distinguishing them from progenitor cells, which are also able to differentiate into terminal cell types but do not self-renew[34]. Progenitor cells are derived from stem cells or from other progenitor cells. Stem cells are abundant throughout embryonic and fetal development, and specialized niches containing stem cells exist in the adult. Adult niches containing numerous stem cells include bone marrow, hair follicles, epidermis, intestinal crypts, gonads, liver, adipose tissue, and vasculature; these tend to be organs with high rates of cellular turnover and regeneration. Niches with few stem cells as a percentage of total organ cell number include skeletal muscle, brain, and bone and cartilage, where turnover rates are low and cell replacement is infrequent[35]. The existence of stem cells in the heart is a highly-contested topic[36].

The number of different cell types a stem cell has the capacity to differentiate into defines its potency. A stem cell that can be differentiated into one cell type is unipotent, although there is argument over whether unipotent stem cells exist, or whether they are, in fact, progenitor cells; an example is a hepatoblast. A stem cell that can differentiate into a few cell types is oligopotent, such as vascular stem cells. Those that can differentiate into more cell types than an oligopotent cell, but not all cell types, is multipotent, such as cord blood stem cells, BMSCs, and MSCs. A cell that can differentiate into all cell types derived

from any of the three embryonic germ layers is pluripotent; these include ESCs, iPSCs, and embryonic cells including inner cell mass (ICM) cells and epiblast cells. Finally, a totipotent cell can differentiate into cell type of any of the three germ layers, as well as cells derived from placental cytotrophoblast and syncytiotrophoblast. The only cells capable of this in the human are the zygote, cells in the morula, and cells existing in mitotic stages between zygote and morula[37].

Embryonic stem cells are derived from the inner cell mass of blastocysts, typically from blastocysts created during in vitro fertilization procedures that are not chosen to implant into the mother's uterus[30]. They are pluripotent, capable of differentiating into all three embryonic germ layers, and have been successfully differentiated into immature cardiac myocytes. These ESC-derived cardiac myocytes (ESC-CMs) are characteristically immature, especially in terms of their ultrastructure, electrophysiological properties, and sarcoplasmic reticulum[38]. This immature phenotype presents problems for using ESC-CMs as models of human cardiac myocytes for studying disease, as well as potential problems for use of them as transplantable cell therapies, although this is not any different than hiPSC-CMs, which suffer from similar, if not the same, problems.

Furthermore, ESCs bring with them two major problems when being considered for therapeutic use: first, a substantial proportion of the general public, especially in the United States, as well as some scientists, have ethical issues with the destruction of blastocysts for science. Second, because ESCs

and cells derived from them are genetically non-identical to the host that they are transplanted into, there is an elevated risk of a host immune response to transplanted cells, which has already been shown in some models[39]. Despite this, several studies have shown benefits of transplantation of ESC-CMs into animals with infarcted hearts, with improvements in EF, wall thickness, and adrenergic response[38].

Because of the challenges of ESCs, both real and perceived, and because of the benefits of hiPSCs, both real and perceived, the field of cardiac regeneration and cardiac differentiated has moved resolutely towards hiPSC-CMs as the cell of choice. Work on reprogramming somatic cells into pluripotent cells began in in the 1960's and 1970's, when John Gurdon transferred the nucleus of a frog somatic cell into an enucleated egg, allowing the animal to be cloned[40], opening an era of vertebrate cloning that has had far-reaching effects even until today, such as in agriculture, where somatic cell nuclear transfer (SCNT) is routinely performed.

iPSCs were discovered, or rather created, by a group led by Shinya Yamanaka in 2006, ten years ago[41]. They accomplished this by overexpressing four crucial genes associated with a pluripotent state- Oct4, Sox2, Klf4, and c-Myc, although it was later shown that c-Myc was not required for reprogramming, and slightly inhibits differentiation into hiPSC-CMs[42]. Initially this overexpression was driven by viral vectors, which can integrate and persist in the cells, potentially leading to immunogenicity, and potentially disrupting differentiation to

other cell types. Viral induction of pluripotency may also lead to oncogenicity due to activation of oncogenes, as well as functional mutations caused by viral vectors inserting into the genome[43].

Alternatively, pluripotency can be induced through non-integrating methods, including chemicals that mimic transcription factors[44], adenovirus[45], plasmid[46], or recombinant proteins[47]. iPSCs have been found to be very similar to ESCs in terms of their chromatin structure and methylation patterns, morphology, cell surface markers, and differentiation capacity[48].

Differentiation of Pluripotent Stem Cells

Since the discoveries of ESCs and iPSCs, efforts have been made to differentiate these pluripotent cells into progenitor cells or terminally differentiated cell types; so far, researchers have been productive in differentiating hepatocytes, endothelial cells, fibroblasts, neuronal cells, pancreatic cells, hematopoietic cells, vascular cells, and retinal pigment epithelial cells[49]. Each cell type comes about through different developmental conditions, in response to various environmental cues including growth factors, cytokines, chemokines, chemical gradients, ionic gradients, electrical signals, haptic signals involving different textures, passive stiffness, nutrient availability, and mechanical signals including stretch, compression, shear stress, and compartments filling with fluid. Thus, differentiation of a desired cell type means that either culture conditions must be tailored to that particular cell type, and must closely recapitulate the

conditions that the cell experiences in the embryo during development, or that cells must be allowed to differentiate into all cell types at once, and desired cell types have to be selected for afterwards.

Early protocols for differentiation of cardiac myocytes from pluripotent stem cells took the second approach- adherent cells were dissociated and cultured in non-adherent dishes, where they proliferated into clusters of cells known as embryoid bodies (EBs), which contain cell types from all three embryonic germ layers[50]. Both epithelial and mesenchymal cell types appear in EBs, and tissue-like structures are also observed; without any additional manipulation of culture conditions, EB cell types have a propensity towards ectodermal lineages, such as neuronal and dermal cell types, although addition of various chemicals and growth factors can coax EBs to form predominantly endodermal or mesodermal cell types[51]. Furthermore, dissociation of EBs and replating of isolated cells onto adherent dishes or into methylcellulose, along with additional manipulations of culture media, can further direct the cells towards specified lineages.

Early cardiac myocyte differentiation protocols relied on some percentage of embryoid bodies containing spontaneously arising, spontaneously beating cardiac myocytes which expressed critical cardiac myocyte progenitor markers, such as Nkx2.5, and had crucial ionic currents, although they lack certain currents as well[52]. The protocol has been improved significantly, with additions to

the media, such as Wnt inhibitors and nitric oxide, enhancing the percentage of cells that express cardiac markers[53-54].

Alternatively, embryoid body formation can be bypassed, and pluripotent stem cells can be treated with environmental factors that recapitulate the embryonic milieu as closely as possible. The goal is to direct the cells towards differentiation into the most cardiac myocyte-like cells as possible, while using the simplest culture conditions possible; simpler culture conditions have the benefits of reproducibility between labs and individuals, leading to standard cells that can be experimented on by different groups while still being able to compare results between them, as well as reduced cost, allowing more cells to be generated for increasing numbers of experiments or transplant. If they are to be used for transplant, simpler culture conditions and lower costs mean reduced barriers to clinical implementation for greater numbers of individuals. The simplest way to do this is by using chemicals that can be added to the medium at prescribed time points during the differentiation process; growth factors, or small molecules that inhibit growth factors, are easily additive and do not require the technical skill of genetic overexpression.

This, however, is rather complex, as the development of the heart and of cardiac myocytes in the embryo relies on enormously complicated networks of signaling from various neighboring cell types[55]. The first step in producing cardiac myocytes is differentiation of mesoderm, which happens during gastrulation of the epiblast, which in turn is formed from the inner cell mass (ICM). This is

important because human embryonic stem cells and induced pluripotent stem cells resemble epiblasts in terms of gene expression patterns[56], although mouse embryonic stem cells resemble ICM cells, and mouse epiblast cell lines can be derived[57]. Furthermore, human pluripotent stem cells can be pushed towards the naïve ground-state of ICM pluripotent cells through culture conditions which are distinct from those required for maintaining the ground state of murine ground-state cells; ground-state pluripotent cells may differentiate more efficiently to cardiac myocytes than cells that retain the epiblast-like signature[58]. The conversion between the two appears to depend on the presence (in ground-state) or absence (in epiblast-like cells) of Wnt signaling[59].

Cardiac Development

Induction of mesoderm at the primitive streak during gastrulation depends on many factors including Nodal, bone morphogenic protein (BMP), Wnt, and fibroblast growth factor (FGF)[60]. Thus, differentiation protocols that intend to produce mesodermal lineages must start with a step that activates this induction. Thus, early protocols to differentiate ESC-CMs or iPSC-CMS began with addition of BMP4, bFGF, and Activin A to culture[61-63]. Variations on this protocol included one or two of the three, aforementioned growth factors, or combinations of these added at various times over the course of the first one to four days, enough time for mesoderm to fully develop.

Next, the mesoderm must differentiate to a cardiac myocyte; in the embryo, it does this through several steps and cell types- first, cardiogenic mesoderm progenitors develop, which express MesP1[55]. These cells migrate to an anterior lateral position caudal to the head folds and form the cardiac crescent, which contain the first and second heart fields (FHF and SHF). The first heart field will contribute to the heart tube and will eventually give rise to cardiac myocytes and smooth muscle cells; FHF cells express Nkx2.5 and Tbx5. Second heart field cells will also contribute to the heart tube, and will give rise to cardiac myocytes, smooth muscle cells, and endothelial cells; they express Nkx2.5 and Isl1. The second heart field will also give rise to proepicardium, which will develop into the epicardium and some cardiac muscle tissue. Increased levels of BMP and FGF contribute to both the first and second heart fields; however, the first heart field is associated with decreased canonical Wnt, and the second heart field is associated with increased canonical Wnt signaling.

Development of both the first and second heart fields is also associated with vascular endothelial growth factor (VEGF) and Dickkopf-1 (DKK1)[55]. So, early defined culture conditions for differentiation of cardiac myocytes from pluripotent cells by treatment with VEGF, DKK1, and FGF, or some combination of the three, in order to push the mesodermal cells towards a cardiac progenitor cell type[61]. From there, the first and second heart fields develop into the heart tube, which folds and becomes a primitive organ; further signaling, both chemical and otherwise, promotes development of the myocardium. For most

differentiation protocols, these signals are too complex and unknown to effectively contribute to further myocyte development, and so they rely on the passage of time to take the cells from cardiac progenitor to cardiac myocyte[61].

Small Molecules and the Matrigel Sandwich Method

However, because growth factors are peptides or proteins, they are time-consuming to produce, and expensive. Organic small molecules that mimic the effects of growth factors are significantly cheaper, and cheaper reagents mean that more differentiated cells can be produced, which is ideal for a lab proposing to study many conditions, or many drugs, or for a transplant patient that may require hundreds of millions or billions of cells[64]. Accordingly, several groups have developed protocols that utilize small molecules; they differ by the pathways activated, the specific drugs used to activate them, and timing of addition of small molecules to culture media (after 24 hours vs after 48 hours, etc.). The most widely accepted protocol has been the one called either the “matrix sandwich method” or “Matrigel sandwich method,” due to the first step of dissolving Matrigel (a collection of ECM proteins derived from mouse tumors) into the differentiation media on the first day of the protocol; this protocol was developed by researchers at the University of Wisconsin, Madison[65].

This protocol begins with iPSCs that are nearly confluent, having been passaged approximately 4 days beforehand. Cells are treated with a chemical called CHIR99021, which inhibits GSK3, thereby allowing beta-catenin to be

released and act as a signaling molecule, effectively activating the canonical Wnt pathway. This is given in conjunction with the Matrigel as mentioned above. The media used here is RPMI with a supplement called B27, which was originally designed for use in differentiation and culture of neuronal cell types, and which contains nutrients and is serum-free (Thermo Fisher). For the first several days, the media is insulin-free. Three days later, one of several molecules used to inhibit Wnt signaling pathways is given. Thus, the protocol uses activation of Wnt to differentiate iPSCs into mesoderm, and later inhibition of Wnt to push the cells towards a cardiac lineage. After that, cells are allowed to develop in culture until they begin to beat spontaneously, at which point they are fed with media containing insulin, which has been shown to inhibit differentiation of cardiac mesoderm[66], but which promotes cardiac growth and hypertrophy later[67-68].

Once the stem cells are differentiated into cells that contract spontaneously and morphologically resemble cardiac myocytes, the issue of what to use the cells for remains. The two major schools of thought are that they could be transplanted into failing hearts, or hearts suffering cardiomyopathy, or hearts that have suffered infarction, with the hope that they could engraft and contribute mechanically to wall motion and strength[64]. Those that are unenthusiastic about the immediate use of iPSC-CMs as a transplantable therapy, as well as some that are, believe that they are better used as a model for cardiac myocyte function, or at least for myofibril function[69].

Uses of Stem Cell-Derived Cardiac Myocytes

Stem Cell-Derived Cardiac Myocytes as Therapy

Pluripotent stem cells and derived cardiac myocytes have excited researchers with the prospect of being used as a clinical therapy. In the field of cardiology, there is great interest in using these cells as a potential treatment for heart failure and post-infarction cardiomyopathy. Pluripotent stem cell-derived cardiac myocytes injected directly into the myocardium are an obvious starting point. If these cells can engraft and electrically couple to existing myocardium they might provide sufficient contractile force to improve cardiac output. On the other hand, the cells may not survive the procedure, or may fail to couple to native myocytes, or may even be detrimental, as the procedure may cause deadly arrhythmias. These potential complications warrant extensive basic research before translation to human studies.

Several groups have published results from experiments injecting stem cells into animal models. It was demonstrated that hESC-CMs transplanted into immunocompromised NOD-SCID mice that had undergone myocardial infarction, engrafted and transiently improved cardiac function[70]. Transplanted cells were tracked by GFP and anti-human-protein antibodies and the GFP+ cells were detected in the heart at least 12 weeks after transplant. The authors proposed functional coupling of transplanted and native cells based on connexin-43 and desmoplakin staining. Four weeks after transplant, transplanted mice had greater

EF than non-transplanted controls; however, this difference in EF was lost 12 weeks after transplant.

Two important caveats to these results should be considered: first, the population of transplanted cells, taken from beating EBs, was only 20–25% CMs. Second, connexin and desmoplakin staining are incomplete evidence of functional coupling in the absence of electrical or ionic data, especially since, as the authors noted, gap junctions and desmosomes were seen between hESC-CMs and themselves, but not between hESC-CMs and native myocytes. The same group later published results after injecting 3 times as many cells as previously, but noting, again, no functional improvement 12 weeks post-transplant[71].

In a similar study, EB-derived hESC-CMs were transplanted into male Sprague–Dawley rats that had undergone LAD and were treated with cyclosporine A and methylprednisolone to prevent rejection[72]. They reported evidence of engraftment of cells by imaging GFP or by staining for human markers, noting engraftment took place mostly in the border zone of the infarct region. Echocardiography showed decreased LV dilatation, greater fractional shortening (FS), and decreased pulmonary congestion in animals transplanted with hESC-CMs.

In an effort to improve engraftment, human ES-CMs were transplanted into infarcted rat hearts along with a “pro-survival cocktail (PSC)” that included Matrigel, a peptide from Bcl-XL, cyclosporine A, pinacidil, IGF-1, and a caspase inhibitor ZVAD-fmk[63]. This group detected engraftment based on human specific immunostaining and qPCR. Based on echocardiography, hearts transplanted with hESC-CMs showed decreased left ventricular end systolic diameter (LVESD) and increased FS, as well as increased thickening of the left ventricular wall in the infarct region.

Later, they published a similar study[73], this time injecting cells 1 month after infarction. This treatment improved cardiac function, but did not alter dimensions or geometry of the myocardium. In 2012, this group published a study[74] using transplanted cells in immunosuppressed guinea pigs, wherein they sought to demonstrate electrical coupling between hESC-CMs and host myocytes using the genetically encoded calcium sensor GCaMP3. To do this, they correlated fluorescent transients to ECG to determine synchrony. They also showed fewer arrhythmias in the form of premature ventricular contractions (PVCs) and ventricular tachycardia (VT) in transplanted animals versus controls. However, in their isolated heart studies, they demonstrated heterogeneous calcium transients and incomplete coupling, as well as a loss of coupling at higher pacing frequencies.

In a technical tour de force, this group transplanted hESC-CMs into a non-human primate model of infarction[64]. The hESC-CM transplanted animals responded differently to treatment; while some had improved EF after transplant, some had no improvement. Perhaps most concerning, all hESC-CM transplanted animals displayed increased arrhythmias following transplant. This important study represents the current state of stem cell engraftment. It will be important to see follow-up studies in coming years as the issues of stable, physiological engraftment and functional restitution are established.

Stem Cell-Derived Cardiac Myocytes as a Model of Human Disease

Pluripotent stem cells derived from individuals with genetic diseases can be used to illuminate disease phenotypes in an in vitro model. Here, iPSC-CMs derived from patients with known genetic mutations, especially monogenic mutations, that lead to cardiac phenotypes can be used to probe gene function and potential therapies. With the electrical assays readily available as mentioned above, an early target for disease modeling with iPSC-CMs was Long QT syndrome, a genetic disorder characterized by delayed repolarization of cardiac myocytes, a disease that places patients at risk of deadly arrhythmias including Torsade de Pointes and ventricular fibrillation[75]. In hiPSC-CMs from patients with Long QT Syndrome Type 3 (LQTS-3) (caused by mutations in the sodium

channel SCN5A) there is faster recovery from inactivation of Na+ current in mutant cells versus wild-type cells, and a larger tetrodotoxin (TTX)-sensitive current in the mutant cells[75]. Both of these findings are consistent with data from adult mouse myocyte studies[76]. The group also found prolonged AP duration in SCN5A mutant cells.

Similar observations were made using iPSC-CMs from LQTS-3 patients, however with different mutations in the SCN5A gene[77]. Using patch clamp to study Na+ and Ca^{2+} currents, as well as whole-cell current clamp to measure AP, they found that LQTS cells have tendencies toward prolonged AP duration, smaller Na+ current density, slower time to inactivation, and increased time to peak.

At least three groups have studied LQTS using pluripotent cells from patients with LQTS Type 2, caused by mutations in KCNH2 (hERG channel, responsible for inwardly rectifying potassium current I_{Kr}). One group, using cells from a single patient, utilized whole-cell patch clamp and found AP duration prolongation and reduced amplitude of peak I_{Kr} activation and tail currents[78]. Using MEA, they reported prolonged field potential duration (FPD). Importantly, by looking at single cell AP as well as MEA, they found significant arrhythmogenicity in the form of EADs and ectopic activity. The second[79] and third groups[80] found similar prolongation of APD and FPD in KCNH2 mutant iPSC-CMs. Furthermore, these studies demonstrated significant reduction in I_{Kr}

density and enhanced arrhythmogenic potential. In hiPSC-CMs from a patient with Timothy syndrome [caused by a mutation in the L-type Ca^{2+} channel (CaV1.2)] the QT interval was prolonged[81]. This study reported prolonged APD, arrhythmic activity, and abnormal Ca^{2+} transients in the patient-derived cells compared to control cells.

Other models of arrhythmia have been studied using patient-derived cells. Catecholaminergic polymorphic ventricular tachycardia (CPVT), caused by a mutation in the ryanodine receptor (RYR2), is characterized by aberrant Ca_{2+} release from the SR and ventricular arrhythmia. Cells derived from patients with this disease indeed show elevated diastolic Ca^{2+} concentrations, reduced SR Ca_{2+} content, and susceptibility to DAD in patient derived cells compared to control[82]. Apart from arrhythmic disease models, other hereditary conditions have been studied in patient-derived iPSC-CMs. Using cells derived from a patient with Barth Syndrome, a mitochondrial disorder, it was observed that the cells closely recapitulated several hallmarks of the disease, including irregular sarcomere formation, irregular mitochondria, and weak contractility[83]. Furthermore, they were able to elucidate mechanisms of pathophysiology of the disease involving excess reactive oxygen species.

Others have studied cells from a patient with Pompe disease, a glycogen storage disorder caused by mutations in acid alpha-glucosidase (GAA)[84]. Here the patient-derived hiPSC-CMs showed abnormally high levels of glycogen and mitochondrial dysfunction, and ultrastructural abnormalities, consistent with

findings from myocytes. In LEOPARD syndrome, a developmental disorder characterized by a cluster of abnormal findings caused by mutations in the Ras-MAPK signaling genes, a common abnormality is hypertrophic cardiomyopathy. Differentiated iPSC-CMs derived from patients with a mutation in a protein tyrosine phosphatase encoded by PTPN11, display abnormal Ras-MAPK signaling, cell hypertrophy and abnormal sarcomere organization[85].

Models of cardiomyopathies, while more difficult to study due to the immature phenotype of iPSC-CMs, have also shown some progress. For example, cells have been taken from a patient with arrhythmogenic right ventricular cardiomyopathy (ARVC), a poorly-characterized disease associated with arrhythmia and sudden cardiac death, as well as fibrofatty replacement of the right ventricular myocardium[86]. This patient had a mutation in PKP2, encoding plakophilin 2, a desmosomal protein. The hiPSC-CMs from this patient showed decreased expression of desmosomal proteins and high levels of lipid storage.

A separate in vitro model of dilated cardiomyopathy (DCM)[87], characterized by eccentric ventricular hypertrophy, decreased Ca_{2+} sensitivity, and impaired force production, was derived from iPSCs from patients carrying a mutation in cardiac troponin T (TNNT2) and their unaffected family members. Mutant iPSC-CMs showed comparable cell size to control, but had deranged sarcomere organization. They also had smaller Ca^{2+} transient amplitudes and smaller SR Ca^{2+} stores, and lower force production based on an atomic force

microscopy assay. Overall, the immaturity of the iPSC-CMs makes more challenging the ability to fully assess a disease phenotype normally associated with mature myocytes.

Genome Editing to Model Human Cardiovascular Disease

A recent area of great research interest involves the use of genome editing techniques. These include such technologies as transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9. Both cleave DNA in a site-specific manner, which may be repaired by non-homologous end-joining (NHEJ), potentially disrupting the gene, or, if a template is provided, by homology-directed repair (HDR)[89]. These can be used to introduce disease-specific mutations into an otherwise healthy cell line, reducing noise from genetic variability between lines. It may also be used to correct disease-causing mutations.

Hematopoietic diseases such as Fanconi Anemia, β-thalassemia, and myelodysplastic syndrome have been modeled and corrected[89], but the technology has not yet been extensively applied to cardiovascular disease. Together, these studies demonstrate the feasibility and wide-reaching capacity for modeling genetic diseases using iPSC-CMs. Emerging and advancing technologies will allow this capacity to increase even further, and significant advances may come from these studies that may not be found in rodent and animal models.

Stem Cell-Derived Cardiac Myocytes for Drug Discovery

HiPSC-CMs express ion channels, including sodium channels, potassium channels, the hERG (Kv1.1) channel, and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel (funny current), that have the potential to interact with many pharmaceutical compounds[90]. Pharmaceutical researchers have a strong interest in identifying interactions between these channels and potential clinical drugs due to the severe risks that such interactions entail. As such, iPSC-CMs present an attractive alternative for other models, such as isolated adult myocytes, or human embryonic kidney cells or Chinese hamster ovary cells that have been used to force expression of hERG channels. These earlier techniques, where a single channel in a non-myocyte is probed, can miss a potential drug interaction. A better approach is to use a cell expressing many types of ion channels, in order to more accurately assess all potential interactions.

One group, using MEA, tested the effects of eleven reference compounds on electrical activity of iPSC-CMs[91]. Of these, 5 were hERG blockers, 2 were Ca^{2+} channel blockers, 1 was a nonselective Ca^{2+} channel/hERG blocker, 1was a K_{ATP}-channel blocker, and 2were I_{Ks} blockers. The hERG blockers all prolonged the FPD, as expected, and the Ca^{2+} blockers shortened the FPD, as expected. The I_{Ks} blockers had only minor effects on FPD, but there was no expected response for either, and the authors were able to use the iPSC-CMs to evaluate the potential role of these drugs as well as the role of the I_{Ks} current in cardiac

myocyte function. Furthermore, as mentioned above, iPSC-CMs can be useful models for channelopathies, including Long QT syndrome, where the risk of a fatal drug interaction is much higher than in a healthy individual.

Using cells from a LQTS-2 patient, effects of three compounds including nifedipine, a Ca^{2+} channel blocker; pinacidil, a K_{ATP}-channel opener; and ranolazine, a Na+ channel blocker were also examined[78]. All three drugs had predictable effects on LQTS iPSC-CMs, namely antiarrhythmic effects, demonstrating the ability of these cells to recapitulate the in vivo effects of anti-arrhythmic drugs in an in vitro system. A second group, used another LQTS-2 iPSC line, and showed the ability of an I_{Kr} blocker, E4031, to prolong APD/FPD, and of a K_{ATP}-channel opener, nicorandil, to shorten APD and abolish EADs[79]. Furthermore, they demonstrated counteracting effects of isoprenaline, a β-adrenergic receptor agonist, and nadolol or propranolol, both β-adrenergic antagonists. On the other hand, the arrhythmogenic effects of the β antagonist sotalol were demonstrated at high concentrations when applied to LQTS iPSC-CMs, but not control iPSC-CMs[80]. Moreover, other research groups[82] have been able to show positive effects of dantrolene on aberrant Ca^{2+} handling in cells from a patient with CPVT.

Taken together, these studies in healthy as well as diseased cells show that iPSC-CMs can robustly examine the effects of current-affecting drugs on myocytes, and may be able to predict effects in future studies. Additionally, as shown above[91], iPSC-CMs can be useful for examining the mechanism of action

of untested compounds. In this context, there is a report of altered hypertrophic signaling via the α-adrenergic pathway in hiPSC-CMs that should be addressed by detailed further study when pursuing iPSC lines for drug discovery[92].

Physiologic Function of Stem Cell-Derived Cardiac Myocytes

In order for researchers to effectively use hiPSC-CMs as either a platform for modeling cardiovascular disease, or as a platform for drug discovery and testing, it is necessary to examine the physiologic function of the cells. Several methods have been used, to examine areas of function such as calcium handling, electrophysiology, and contractility.

Electrical Function of Stem Cell-Derived Cardiac Myocytes

Due to the essential role of ionic currents and electrical conduction in the functionality of cardiac muscle, deciphering electrical function of iPSC-CMs allows researchers to characterize mature versus immature, healthy versus diseased cells, and ventricular versus atrial cell types. However, at such an immature state, the last categorization may be difficult to distinguish, and attempting to do so may not provide realistically useful information[93]. Generally, electrical function is measured using extracellular field potential recordings, sharp electrode recordings, or patch clamp recordings. Perhaps the easiest electrical assay to perform is the recording of extracellular field potentials using a

multielectrode array (MEA). MEA recording does not require recording from a single cell, but rather measures the voltage from a population of cells in a dish[94].

In cardiac myocytes, the field potential duration (FPD) corresponds to the action potential duration (APD) of a single cell, which in turn corresponds to the QT interval on the electrocardiogram, an important parameter for researchers using iPSC-CMs to model arrhythmic diseases or as a platform for drug discovery. This technique has been well validated in hESC-CM models[94-96]. It has recently been further validated in iPSC-CMs, demonstrating that it can be used to reliably detect drug induced arrhythmias and repolarization delay, even across distinct facilities[97]. Recent work characterized and optimized field potential recordings, including in response to a drug, and included some limitations of the method that should be taken into consideration when performing experiments and data analysis[98].

In order to dissect out the role of specific currents and ions, single cell electrophysiology techniques are useful, including patch clamp and sharp electrode recording. A patch-clamp pipette can be attached to the cell in several configurations, including cell-attached, whole cell, and perforated-patch modes. Patch clamp has been used to evaluate currents in hiPSC-CMs derived from patients with long QT syndrome type 2. This technique was employed to examine the effects of various drugs on those cells, demonstrating its value in studying models of arrhythmia[79]. Patch clamp electrophysiology has also been used to

evaluate individual currents of iPSC-CMs, including I_{Na}, I_{Ca}, I_{Kr}, and I_{Ks}, as well as their ability to be blocked by known channel blockers[99]. It was concluded that use of iPSC-CMs for electrophysiology studies is feasible. Similar experiments using an automated patch clamp technology came to similar conclusions[100].

However, a separate group, using patch clamp to study APD, AP frequency, AP shape, I_{Na}, and I_{Ca}, as well as effects of channel blockers TTX and lidocaine, noted significant variability between cells from different sources, and suggest utilizing these techniques with caution[101]. Sharp electrode electrophysiology has been used in several published iPSC-CM studies. Intracellular recordings of APs of murine iPSC-CMs[102] and human iPSC-CMs[103] have been made to examine whether the cells differentiated into atrial, ventricular, or nodal types. However, due to the immature nature of these cells, cell types can be difficult to distinguish based on AP shape alone, and caution should be used when drawing conclusions from these types of experiments. Overall, these studies show that iPSC-CMs express appropriate ion channels and have electrical activity similar to human cardiac myocytes, and can be reliably tested for action potential duration, ion currents, and drug interactions if appropriate precautions are taken when drawing conclusions.

Calcium Handling in Stem Cell-Derived Cardiac Myocytes

The adult ventricular cardiac myocyte displays a well-defined sequence of events with regard to Ca^{2+} cycling. Ca^{2+} influx via L-type Ca^{2+} channels serves as

an initial trigger, initiating Ca^{2+} release from the sarcoplasmic reticulum by activating Ryanodine receptor 2 (RyR2) via a process called calcium-induced calcium release (CICR)[104-105]. Phosphorylation of these channels increases calcium flux, increasing contractility. In general, little is known about the excitation contraction coupling (ECC) and Ca^{2+} handling properties of hiPSC-CMs. Gene expression and immunostaining studies showed that key Ca^{2+} handling proteins are expressed in hiPSC-CMs[106].

Furthermore, hiPSC-CMs are dependent on both trans-sarcolemmal Ca^{2+} entry via L-type Ca^{2+} channels and on RYR2-regulated SR Ca^{2+} release and functional SERCA2a pump-based Ca^{2+} reuptake[106]. The majority of Ca^{2+} in the cytoplasm during systole is released from and then taken back up into the sarcoplasmic reticulum (SR)[107]. Release is triggered by depolarization of the cell membrane, and, as such, is intricately connected to AP activity of the cell as well as sarcomere contraction. However, it can be uncoupled from both, and it is necessary to measure Ca^{2+} activity separately. Calcium transients in myocytes are typically measured using fluorescent Ca^{2+}-binding dyes on a fluorescent microscope[107].

Satin et al. laid the groundwork using hESC-CMs[108]. They loaded cells with the dye Fluo-4 AM and recorded intracellular Ca^{2+} transients with a confocal microscope. Using this technique, they observed both entire-cell AP-driven transients, as well as localized SR Ca^{2+} release events (sparks). They then used

caffeine to mobilize Ca^{2+} release from the SR, as well as ryanodine to inhibit release from the ryanodine receptor RyR. They were able to provide evidence that hESC-CMs have large stores of Ca^{2+} in the SR, a hallmark of mature cardiac myocytes. Moving to iPSC-CMs, several labs[106,109] have imaged Ca^{2+} transients and sparks in healthy cells by loading them with fluo-4 under a confocal microscope, and used caffeine to probe SR stores. Fura-4F, a ratiometric calcium dye, has been used to observe the propagation of Ca^{2+} transients through iPSC-CM monolayers, while simultaneously measuring intracellular voltage with the voltage-sensitive fluorescent dye, di-8-ANEPPS[110].

Using hiPSC-CMs derived from patients with catecholaminergic polymorphic ventricular tachycardia (CPVT), a genetic arrhythmic defect, differences were shown in Ca^{2+} handling in diseased versus healthy cells based on Fluo-4 fluorescent imaging[111]. Furthermore, they used voltage clamping to directly measure the L-type Ca^{2+} current (I_{Ca}) and the Na^{+}–Ca^{2+} exchanger (INCX). A combined fluorescent Ca^{2+} imaging with voltage-clamped I_{Ca} measurement can also demonstrate changes in how iPSC-CMs handle Ca^{2+} as they mature[112].

Recently, Ca^{2+} handling characteristics across cells derived in different laboratories have been compared[113], using the ratiometric Ca^{2+} dye Fura-2 AM and recording transients while the cells were electrically stimulated. Using caffeine, SR Ca^{2+} stores were estimated, and I_{Ca} was measured by whole-cell

patch clamp. Overall, there were comparable results between these laboratories. To summarize, iPSC-CMs have functional Ca^{2+} stores, and release and reuptake Ca^{2+} via the SR, and these events can be detected by Ca^{2+}-sensitive fluorescent dyes such as a Fura or a Fluo derivative.

Force Production in Stem Cell-Derived Cardiac Myocytes

The most important parameter of a myocyte's function is its force production, as this determines how effectively the organ can circulate blood. An adult myocyte, with its well-organized sarcomeres and rectangular shape, can relatively easily be studied using video microscopy or force transducers[114]. In comparison, the morphology of a stem cell-derived myocyte presents some distinct challenges. Early on, groups used video edge detection to track movement of stimulated beating EBs as a percent of baseline length[115]. They were able to show increased contractility in response to β-adrenergic stimulation, a hallmark feature of cardiac myocytes. HiPSC-CMs derived by a monolayer method can also be monitored by video microscopy. Motion tracking software can be used to assess beating frequency, amplitude, and kinetics of a 3D tissue-like construct formed by seeding iPSC-CMs onto a filamentous polymer matrix[116].

While video-based edge detection of contractility is very useful, the ability to measure force production against a load provides a more direct measure of

myocyte function. One approach to measuring force involves seeding beating clusters from iPSC-derived EBs onto 300 μm thin strips of neonatal murine ventricular myocardium, and then attaching the myocardial strip to force transducers for measurement[102].

Sun et al. used single cell-dispersed iPSC-CMs derived from a patient with dilated cardiomyopathy to assess single-cell force production using atomic force microscopy. This approach relies on the microscope for detecting changes in force at the cell surface caused by changes in stiffness within the cell, presumably due to rearrangement of sarcomeric and cytoskeletal proteins during contraction[87]. However, it should be noted that this is a technique that has not yet been validated in pluripotent cell-derived cardiac myocytes, neonatal cardiac myocytes, or adult myocytes. In the paper, the authors cite a paper exploring use of the technique on embryonic chicken cardiac myocytes, where the authors note that parameters such as beat period and pulse amplitude were observed to be unstable[117].

The micropost array, a technique that has been well validated in other myocyte cell types, has been used to measure contractile force of iPSC-CMs[118]. In this assay, an array of microposts is fabricated from polydimethylsiloxane (PDMS) using photolithography and the tops of the posts are coated with ECM proteins, and cells are seeded on top of that. As cells contract, the posts deflect with the cell, and video microscopy with subsequent analysis is able to convert

movement into force needed to move the microposts. The authors were able to examine different force production of cells that had been seeded on distinct types of ECM protein.

In a similar vein, Sheehy et al. utilized the muscular thin film (MTF) assay, wherein thin strips of PDMS are fabricated and coated with ECM proteins. The iPSC-CMs are then seeded and stimulated[119]. As cells contract, the MTFs bend and curvature is analyzed with video microscopy, and force production is extrapolated from MTF displacement. These studies show that iPSC-CMs produce a force that is able to be measured; however, the techniques for force measurement are not well developed, and there is an urgent need for improved methods.

Cardiac Myocyte Structure Contributing to Function

The Sarcomere

The main module of the myocyte that allows the cell to contract and produce force is the sarcomere. Sarcomeres connected in series form myofibrils, which run parallel to the long contractile axis of the cell. They are made up of thick filaments, which consist of myosin heavy chain and light chain and which are centered around the M line, and thin filaments, which consist of actin, troponin, and tropomyosin, and which connect at the Z disc, which contains sarcomeric actinin and several other proteins[114]. There are various other proteins involved, such as titin and nebulette, which control things such as Z disc spacing,

and which contribute to elasticity. During contraction, calcium released from the sarcoplasmic reticulum binds to troponin, which causes movement of tropomyosin, allowing myosin to bind to actin and myosin ATPase activity; myosin moves along actin, pulling Z discs nearer each other, resulting in contraction of the cell when all sarcomeres in the myocyte move simultaneously[114].

Actin

Actin plays a crucial role in the contraction of the myocyte, as well as in cytoskeletal structure, cell morphology, membrane protein localization, and transduction of force from the sarcomere to the cell's environment. Actin is a globular protein that can be found as a monomer (G actin) or a filamentous polymer (F actin), which forms following nucleation when G actin has ADP bound. F actin is dynamic, meaning that it is elongating at the (+) end and shortening at the (-) end, unless there are proteins to prevent growth or shortening, such as CapZ, which caps the (+) end. Otherwise, for an F-actin filament to remain a constant length, elongation and shortening must be in equilibrium[120].

In the thin filament, actin is of the isoform alpha-sarcomeric actin, coded for by the ACTC1 gene; although the sarcomere is a fairly constant length, actin dynamics and CapZ capping dynamics occur in response to physiologic stimuli such as exercise and mechanical stimulation, which increases dynamics[121].

Levels of alpha-sarcomeric actin in the myocyte also influence hypertrophy and cell size, and are influenced by regulators of hypertrophy[122].

Actin in the cytoskeleton lives just below the cell membrane and plays a key role in maintaining and altering the size and shape of the cell, and in motility in motile cells, such as epithelial cells and leukocytes. In the cardiac myocyte, control of cytoskeletal actin dynamics has been shown to be crucial for cell hypertrophy and maintenance[123]. Furthermore, because of connections made between actin and some membrane-bound proteins including channels and receptors, cytoskeletal actin plays a role in subcellular localization of receptors and channels, as well as their functional state and open-closed status, allowing specific microenvironments to form within the cytoplasmic compartment with differential concentration of ions, signaling proteins, and cytoskeletal components[124].

Cytoskeletal actin, along with other cytoskeletal proteins, provide a crucial link between the cell membrane, extracellular proteins, and contractile proteins, and there is considerable evidence that changes in one of those compartments, such as changes in extracellular matrix proteins, contribute to changes in contractile protein expression, with actin as an important signaling protein[125].

Mechanosensing Mechanisms

As all cells live in a physical environment with discrete three-dimensional spatial arrangements and constant, various physical forces being exerted on them, such as compression, stretching, or shear forces, it is crucial for cells to be able to respond appropriately to those stimuli, whether that entails building cellular structures that resist those forces, or building cellular structures that allow the cell to take advantage of or move with those forces, or possibly conversion of one cell type to another. This ability to sense requires a conversion of a mechanical signal to a chemical signal, and ultimately activation or inactivation or proteins through phosphorylation or other post-translational modifications or through gene upregulation or downregulation. Cells can do this through several methods, including mechanosensitive ion channels, which open and allow influx or efflux of a depolarizing or hyperpolarizing ion in response to a threshold stress[126].

Cells also utilize adhesions between the cell and its surrounding extracellular matrix, called focal adhesions; in myocytes, the costamere, or dystrophin-glycoprotein complex (DGC), which connects the contractile machinery of a striated muscle cell to the ECM, may also be involved. Focal adhesions contain proteins such as integrins, actin, filamin, vinculin, talin, and focal adhesion kinase (protein tyrosine kinase 2). In one well studied model of mechanosensing, ECM proteins form connections with integrins, which are transmembrane proteins with both extracellular and intracellular domains. Movement of ECM pulls on integrins, which activate talin or paxillin intracellularly.

Activation of paxillin can lead to activation of FAK, Rho- and ARF-GTPases, and the ILK/pinch/parvin complex. Talin is connected to cytoskeletal actin, which can activate the ILK/pinch/parvin complex, as well as membrane channels, or nuclear cytoskeletal proteins[127].

In a cardiac myocyte, mechanotransduction from the environment to the cell can open ion channels that can lead to stretch-induced arrhythmia, and can lead to upregulation and secretion of atrial natriuretic peptide[128]. Mechanical stress in the heart can come from various places including stretch due to increased ventricular or atrial blood volume, stiffness due to fibrosis and increased ECM deposits, or changes in the passive or active tension of neighboring cardiac myocytes. In the myocyte, other mechanosensing pathways than the focal adhesion pathways mentioned above come into play- opening of stretch-activated calcium channels creating calcium influx, which can in turn cause calcium-induced calcium release (CACR); or activation of Angiotensin II release, which feeds back by binding to AT receptors on the myocyte surface. Activation of these pathways in the myocyte can lead to altered gene expression, fibrosis, and cell and organ hypertrophy[129].

Force Transmission from the Cell to the Environment

The production of force by a cell on its environment is in some ways the reverse of mechanosensing- the cell must create an internal force that is transferred to its environment. In motile cells, such as migrating cells during embryonic development, or during dendritic or axonal growth of neurons, or in non-motile contractile cells such as myofibroblasts or endothelial cells whose contraction depends on stress fibers, this transmission of force occurs at the focal adhesion, through the actin-integrin-extracellular protein connection. It depends to a significant extent on actin motility[130] and relies on feedback from both stretch-activated channels and mechanotransduction through the same focal adhesions[131].

In the myocyte, transmission of force from the sarcomere to the environment depends on lateral transmission through the costamere[132]. There are several steps in between- first, sarcomeric actin pulls on actinin in the Z disc; actinin transmits this force to the major intermediate filament protein holding Z discs together- in the adult cardiac myocyte this is desmin, although in the developing cardiac myocytes and in immature cardiac myocytes this is vimentin, which has different biophysical properties than desmin[133].

Disruption of desmin during extreme mechanical stress also plays a role in sarcomerogenesis and myofibrillogenesis, leading to cardiac myocyte hypertrophy[134]. Desmin can transmit force to a number of protein complexes at the cell membrane including focal adhesions; spectrin, ankyrin, crystallin, sodium

channels, and Na-K ATPase; and the DGC. The DGC includes proteins such as the transmembrane proteins sarcoglycan and dystroglycan, the extracellular protein laminin, and intracellular proteins dystrobrevin, syntrophin, plectin, utrophin, and dystrophin, which have roles in structural support, modulation of force transmission, and maintenance of cell and membrane elasticity and integrity[132].

Force Production in the Cardiac Myocyte

Contributors to Contractility- Calcium

When it is necessary for the cell to produce more or less force, modulation of contractility takes place. A cell producing more force against the same mechanical load will contract more in terms of length of shortening, and a cell with more sarcomeres will produce more force than a cell with fewer sarcomeres, if the individual sarcomeres are producing equal amounts of force. On the sarcomeric level, increasing contractility requires increasing activation and recruitment of filaments; meaning, more frequent attachments between myosin and actin. Since this attachment relies on troponin moving tropomyosin away from actin so that myosin can bind, increased contractility can be achieved either through increased calcium, which binds to troponin C and activates the complex, or through increased phosphorylation of the troponin complex. Increased calcium can occur through increased flux across the cell membrane through the L-type calcium channel (LTCC; dihydropyridine receptor DHPR), increased release from

the sarcoplasmic reticulum; or decreased uptake into the sarcoplasmic reticulum[135].

The amount of calcium released from the SR depends largely on the amount loaded into the SR during diastole through the Sarcoplasmic/endoplasmic reticulum ATPase (SERCA). SERCA activity is regulated by phospholamban (PLN); binding of PLN inhibits SERCA function, but when it is phosphorylated it exists in a pentameric state and cannot inhibit SERCA. Thus, adrenergic signaling acting through PKA and cAMP inhibits PLN and increases SERCA activity, thereby increasing contractility through increased calcium. PKA also increases the open probability of ryanodine receptors (RyR), which release calcium from the SR into the cytoplasm[136]. Several drugs aimed at improving cardiac function take advantage of this by inhibiting phosphodiesterase (PDE), which breaks down cAMP[137]. There is also good evidence for a role in myosin binding protein C (MyBP-C) in cardiac contractility. MyBP-C associates with the thick filament; it has PKA phosphorylation sites, and affects crossbridge cycling kinetics[138].

Contributors to Contractile Kinetics- Troponin

While absolute force production is important to a myocyte's function, contractile kinetics are also important- a heart that beats faster must contract and relax faster in order to maintain ejection fraction and increase cardiac output. Increasing the number of activated SERCA pumps through regulation of PLN can

increase the rate of removal of calcium from the cytoplasm, shifting the equilibrium of unbound calcium and calcium bound to TnC towards the unbound side and increasing the rate of deactivation of troponin and detachment of myosin from actin[135]. We have also noted that MyBP-C and its phosphorylation through the adrenergic-PKA pathway plays a role in crossbridge kinetics[138]. Phosphorylation of the troponin complex, such as troponin I, by PKA and adrenergic signaling, also plays a very significant role in a sarcomere's ability to contract and relax quickly in response to sympathetic activation. Of note, phosphorylation of troponin I enhances the speed of both contraction and relaxation[139].

The isoform of troponin I expressed also influences contractility kinetics; cardiac troponin I (cTnI), which is expressed in adult cells, is faster than slow skeletal troponin I (ssTnI), which is expressed in immature cells. However, ssTnI is a positive inotrope, especially under acidic/hypoxic conditions, and has increased calcium sensitivity, although it lacks the PKA-mediated phosphorylation site that causes calcium desensitization in cTnI. Mutant isoforms, and engineered isoforms, of TnI can enhance or diminish some of these characteristics. Importantly, the A164H isoform of cTnI, where the alanine and position 164 is replaced with a histidine, maintains the increased lusitropic effects of cTnI, while rendering the protein more resilient to acidotic/hypoxic conditions[140].

Contributors to Contractility- Mechanical Load

The heart alters contractility in response to mechanical loads, while attempting to maintain homeostatic levels of cardiac output. The heart as an organ experiences two types of mechanical load- preload and afterload. Preload affects the amount of stretch of cardiac myocytes before systole, and the amount of passive tension that the cardiac myocytes experience, and encompasses all the factors that contribute to passive wall stress, including end-diastolic volume, central venous pressure, end-diastolic filling pressure, chamber wall thickness and wall compliance. Afterload affects the amount of active tension that a cardiac myocyte must generate in order to produce sufficient cardiac output, and encompasses the factors that contribute to wall tension during systole, including systolic volume, systolic pressure, chamber wall thickness, outflow tract resistance, and systemic arterial resistance (systolic arterial pressure, diastolic arterial pressure, arterial compliance)[141].

In order to maintain adequate cardiac output, cardiac myocytes must appropriately respond to preload and afterload; by using the mechanosensing pathways noted above, a cardiac myocyte increases contractility in response to increased preload (increased stretch and sarcomere lengthening[142]. In response to increased afterload, cardiac myocytes may increase contractility due to adrenergic signaling through the sympathetic nervous system, or, through mechanosensing, cells may automatically increase force production in order to maintain equivalent contractile amplitude[142].

Measuring Contractility in Stem Cell-Derived Cardiac Myocytes

Traction Force Microscopy

In order to explore these aspects of contractility and response to load in hiPSC-CMs, an appropriate assay is needed. The total force produced by an adult cardiac myocyte can be measured using piezoelectric force transducers; the unique geometry and dense sarcomeric protein structure allows quantification of a precise nature[143]. Contractile kinetics of an adult cardiac myocyte can be measured optically, by tracking sarcomere length and shortening, taking advantage of regular light-dark patterns of registered sarcomeres; this can be done on the same force transduction apparatus, or on a modern system such as the Ionoptix [144]. These assays have so far been poorly suited to use in iPSC-CMs.

Traction force microscopy is a valuable method that utilizes the known mechanical properties of a deformable substrate coupled with high-resolution microscopy to accurately measure the developed force of contractile or motile cells. Traction force microscopy allows investigation of the physiologic function of a single isolated hiPSC-CM by measuring force produced by a single cell against a load produced by a stiff substrate. Measuring the force of a single cell, rather than a sheet of cells, allows rigorous determination of the effects of cell geometry on contractility, and removes several potentially confounding effects that might come from sheet-based assays, including number of cells, relative

orientation of cells to each other, and presence of non-cardiac myocyte cell types that may have differentiated in culture alongside the myocytes.

Traction force microscopy has been utilized to measure the amount of stress produced by cells including fibroblasts[145], keratocytes[146], osteoblasts[147] and NRVMs[148] and has been used to calculate the strain energy, which is the energy required to deform a material[149]. Determining stress and strain energy in response to changes in substrate stiffness allow modeling of both increased stiffness changes that may occur in the heart under various conditions, as well as changes in afterload that ventricular myocytes experience during systole under conditions of valvular disease or increased systemic mean arterial pressure.

The rigorousness and versatility of traction force microscopy also allows examination of the effects of other influential factors in the development and differentiation of cardiac myocytes. For example, one can look at the effects of prolonged time in culture on contractility and morphology, or the effects of extracellular calcium concentrations, as it has been recently shown that calcium concentrations play a vital role in the development and hypertrophy of cardiac myocytes during development[150-151]. Taken together, traction force microscopy provides a reliable and rigorous method of determining contractile force of iPSC-derived cardiac myocytes.

Traction Force Microscopy- Methodology

The technique begins with micropatterning stamps[152]. Single cell shapes are placed far enough apart to ensure contraction of one cell would not affect substrate deformation of neighboring cells. Stamp masters are created using photolithography by applying photomasks to silicon wafers coated with photoresist and exposing to light. Stamps are created by curing polydimethylsiloxane (PDMS) on the patterned silicon master (for patterned stamp) or on an unpatterned silanized silicon wafer (for blank stamps). Both patterned and unpatterned stamps are made new each time they were used. Stamps are coated and stamped according to the stamp-off protocol laid out by Desai et al[153]. Briefly, blank stamps are coated with laminin (50 µg/ml in molecular biology grade water) and incubated for 60 minutes, then dried and inverted onto patterned stamps which have been UV-activated. Blank stamps are immediately peeled off and placed onto 15 mm coverslips which have been plasma-activated by running through a blue flame. Cover slips are then ready for use with gels.

Polyacrylamide gels are made using ratios of Acrylamide to N,N'-methylenebisacrylamide that allow various substrate stiffness to be examined[154]. Ahead of time, 25 mm glass coverslips were UV treated, then treated with 3-aminopropyltriethoxysilane for 3 minutes, and rinsed with ethanol. Polyacrylamide was made with 1x phosphate-buffered saline, and 0.2 µm fluorescent beads are mixed into the unpolymerized acrylamide solution. Ammonium persulfate and N-hydroxysuccinimide ester are added to solution.

Laminin-coated coverslips are inverted onto the solution and allowed to polymerize. hiPSCs are dissociated and replated onto polyacrylamide gels. Cells adhere overnight, then assayed on day 3 after plating.

Images of fluorescent beads moving within the substrate that the cells are exerting a stress on are acquired using a high-resolution microscope at the fastest rate possible while still acquiring images suitable for analysis. Cells can be paced in the single cell state, or allowed to pace at their intrinsic rate, although pacing cells allows for rigorous comparison of cells at the same frequency and voltage. Images may be analyzed using any suitable TFM analysis software, though many people are using ImageJ code developed by Tseng et al[155-156].

Stacks of images of the cell and fluorescent beads before and during contraction are compared; the software calculates a displacement field using a particle image velocimetry program, by comparing movement of areas of interrogation with surrounding areas. It then calculates stress vectors from displacement vectors using the Fourier transform traction cytometry method[157]. A smoothing parameter of 1x10-9 may be applied for improved accuracy, as described in Stricker et al[158]. Stress vector magnitudes may be integrated over the area of interest and reported as total force. Strain energy can be calculated according to the equation laid out in Oakes et al[149], by taking the integral of the product of the displacement and traction stress vectors of each cell.

Traction Force Microscopy on Stem Cell-Derived Cardiac Myocytes- Current Understandings and Results

At least two or three research groups have performed this assay on hiPSC-CMs, with differing results. Most follow the steps listed above with slight variations in their protocols; they share themes of micropatterning and isolated cardiac myocytes, but they choose to examine the effects of different conditions of contractility and force production. One group focused heavily on the quantitative aspects of the data analysis; they show that iPSC-CMs have contractile curves with similar shapes to those of adult cardiac myocytes. They also increased force in response to isoproterenol, and decreased force in response to verapamil, which blocks the LTCC. They also use the assay to show cardiotoxicity of the known cardiotoxic drug dofetilide[159].

Other researchers have used the assay to show that force production increases with increased time in culture; they also showed a weak response to isoproterenol on soft substrates but not stiff ones[160]. In this paper, they showed that force increases as cells are plated on increasingly stiff substrates, although in their next paper they show the opposite. A collaborating group showed that higher aspect ratios (7:1 vs 3:1 or 5:1, which is the ratio of the length of the long, contractile axis over the short, perpendicular axis) produce higher force, presumably due to increased myofibril orientation[161].

Gene Expression Throughout the Life of a Cardiac Myocyte

Gene Expression and Isoform Switching in Fetal Development

During the cardiac development process, as has been described in detail above, there are significant changes in the genes that are expressed, and particularly in differential expression of isoforms of proteins, such as sarcomeric proteins myosin heavy chain (MHC) and troponin I (TnI), as well as in beta-adrenergic receptors, ion channels, and transcription factors; there are also changes in the localization of some of these proteins. In striated muscle, there are three isoforms that are transcribed from three separate genes in a muscle fiber type-specific manner[162-164]. These isoforms are fast skeletal troponin I (fsTnI/TNNI2), expressed in fast skeletal muscle fibers; slow skeletal troponin I (ssTnI/TNNI1), expressed in slow skeletal muscle fibers and fetal cardiac muscle; and cardiac troponin I (cTnI/TNNI3), expressed only in adult cardiac muscle. SsTnI and cTnI are the two key myofilament isoform proteins in the cardiac myocyte and are antithetically expressed during the transition from fetal to adult life[164-165].

The ssTnI isoform is expressed during neonatal or fetal life and is then stoichiometrically replaced by the cTnI isoform, which is exclusively expressed in mature adult cardiac myocytes[164-169]. The cTnI isoform has a unique N-terminal extension containing serine residues that are phosphorylated in response to adrenergic stimulation of the heart, making it PKA-responsive. This PKA responsiveness is critically required for the fast relaxation that is necessary for

optimal adult cardiac function[139,170-171]. Thus, acquisition of this key adult signature maturation marker by hiPSC-CMs is indispensable.

Cardiac myosin has two cardiac myosin light chain 2 (MLC2) isoforms, MLC2a and MLC2v. In the developing human and mouse heart, MLC2a expression is detected in all chambers[172]. In the postnatal heart, MLC2v is confined to the ventricle, and this chamber specificity persists to adulthood[172-173]. MLC2v expression is also considered to be a marker of cardiac myocyte maturity. Thus, several groups have used the expression patterns of MLC2a and MLC2v to define cardiac myocyte identity and stages of development.

hiPSC-CMs primarily express MLC2a at early time points; at later time points, expression of MLC2v increases and expression of MLC2a decreases, with the majority of hiPSC-CMs co-expressing both MLC2a and MLC2v[174]. Here, hiPSC-CMs expressing MLC2v most likely represent ventricular-like cells, while those expressing MLC2a may represent a range of immature cardiac myocytes, including atrial-like cells. Earlier reports demonstrated prevalent MLC2v/MLC2a double-positive cardiac myocytes, with disorganized sarcomeres and weak hERG channel responses, to be immature, resembling human fetal cardiac myocytes[175].

Changes in Gene Expression During Heart Failure

During heart failure, there are significant changes in gene expression, including some reversions to fetal gene expression. Many genes that encode for

contractile proteins have a characteristic pattern of expression during development, and many of these are also altered by disease conditions[176]. The failing heart reactivates fetal genes and reverts to a fetal pattern of energy substrate metabolism[176-177]. For instance, in mice, levels of fetal genes such as ANP, BNP, βMHC, skeletal actin, and metabolic genes such as GLUT1 are reactivated in the failing adult heart[176-178]. Moreover, αMHC, SERCA2a, ion channels, and metabolic genes such as GLUT4 are reduced during heart failure[178-179.] So, based on what is understood about cardiac development and the key proteins in cardiac function, this thesis focuses on three major proteins: TnI, MHC, and SERCA2a.

Crucial Genes for Cardiac Development and Function

Troponin

Troponin exists in a complex of three distinct proteins- inhibitory troponin I (TnI), troponin C (TnC), and troponin T (TnT), which binds tropomyosin. In the activated state, calcium binds to troponin C, causing a conformation change, moving TnI and tropomyosin away from actin so that myosin can bind. During diastole, when calcium is taken back up into the SR, the troponin complex returns to its inhibitory state, releasing myosin from actin and causing relaxation. TnC has a fast isoform (TNNC2 gene) and a slow isoform (TNNC1 gene); cardiac muscle only expresses the slow isoform, though skeletal muscle expresses both, and there is no developmental switch[180]. Troponin T has a slow

skeletal (TNNT1), cardiac (TNNT2), and fast skeletal (TNNT3) isoform, and there is a possibility that cardiac muscle may express some slow skeletal TnT[181]. As noted above, TnI exists in slow skeletal, fast skeletal, and cardiac isoforms. The mutant A164H isoform shows increased calcium sensitivity in the face of hypoxia or hypercapnia by a histidine in the switch region between actin binding and TnC binding domains[182].

Myosin

Myosin heavy chain is part of the myosin group of proteins; it falls under myosin class II, which contains 15 members. It has three domains- a head, which binds actin; a neck, which acts the main force transducing domain; and a tail, which binds other myosins, including a regulatory MLC and an essential MLC. During systole myosin goes through crossbridge cycling; in the relaxed state ADP and inorganic phosphate (Pi) are bound to the myosin head, which allows it to bind actin when troponin is activated, forming a crossbridge. When it releases ADP and Pi, the myosin head bends forward producing a power stroke and contraction; when ATP binds to myosin it releases from actin, the myosin ATPase function breaks down ATP into ADP and Pi, and the cycle can start over, or the filament can relax[183]. In cardiac myocytes, there are two isoforms expressed; the alpha isoform has 3-fold higher ATPase activity and higher actin filament sliding velocity. It is expressed at low levels in the adult heart, but not in the developing heart, and cardiac disease such as heart failure and myocardial infarction decreases expression, though this may be somewhat reversed with

exercise. Expression is dependent on activation of thyroid hormone receptors[184]. The isoform of MHC expressed has significant effects on the function of troponin as well[185].

SERCA

As has been noted above, SERCA2a, the dominant isoform of SERCA expressed in cardiac myocytes, plays a key role in cardiac contractility, e.g. in the amplitude of contractility, by regulating the amount of calcium taken up into the SR during diastole. Phospholamban inhibits SERCA ATPase activity, and phosphorylation of PLN increases SERCA activity[78]. Sarcolipin regulates SERCA activity in atrial myocytes but not ventricular[186]. Thyroid hormone upregulates SERCA expression and decreases PLN inhibition of SERCA, and adiponectin enhances SERCA activity, presumably by relieving oxidation and causing free-radical scavenging, since free radicals inhibit SERCA function[187]. SERCA can also be glutathionylated or SUMOylated, both of which enhance function; glycosylated or O-glcNAcylated, both of which decrease function; or acetylated or nitrosylated, which have unknown effects on function and protein amount[187].

It is also known that heart failure decreases expression of SERCA, and exercise increases expression of SERCA, and there have been attempts to increase SERCA expression levels or decrease SERCA inhibition for therapeutic purposes[187]. It is also known that calcium handling is crucial for the development of cardiac myocytes, and that the amplitude and kinetics of intracellular calcium

transients change as cells mature, along with a shift from dependence on T-type calcium channels to L-type calcium channels, decreased NCX expression with greater dominance of Forward NCX mode, decreased response to calcium, increased buffering of cytosolic calcium due to increased TnC, and increasing SERCA expression which is paralleled by increasing SR calcium content[188].

Hypertrophy as a Marker of Cardiac Development and Contractility

Pathways to Hypertrophy

Hypertrophy is a key marker of various intracellular processes, both physiologic and pathologic. In a cardiac myocyte, hypertrophy essentially means an increased overall number of sarcomeres, although this does not always translate to increased force if other pathological factors are involved. There are several major pathways involved in cardiac myocyte hypertrophy; most of them are interconnected in some way, with significant cross-talk between pathways; some involve calcium signaling and proteins with calcium-binding sites, such as the nuclear factor of activated T-cells (NFAT)/calcineurin pathway, and the calcium/calmodulin-dependent protein kinase II (CaMKII) pathway, making them significant for cardiac myocytes which rely on calcium handling for physiologic function and which display altered calcium handling in development and pathology[189].

In the calcineurin/NFAT pathway, activation of calcineurin, either through calcium binding or through non-calcium-mediated mechanisms such as direct binding of the sodium/hydrogen exchanger (NHE)[190] leads to dephosphorylation of NFAT and translocation from the cytoplasm to the nucleus, where it interacts with other transcription factors such as NFκB to enhance expression of genes associated with hypertrophy, such as sarcomeric and cytoskeletal genes[191]. This pathway can be activated by signals such as testosterone[189], Angiotensin II[192], and beta-adrenergic signaling mediated by GPCR Kinases (GRKs)[193]. Sustained levels of increased calcium concentrations in myocytes has been shown to be mediated through transient receptor potential cation channels (TRPC channels), which are mechanosensitive and may be activated in pressure overload situations[194].

CaMKII is an important signaling molecule in cardiac myocytes, in that activation and upregulation of it leads to arrhythmia and cardiomyopathy, suggesting a role in homeostasis of ionic function, and leading to interest in it as a therapeutic target, although a role for CaMKII in the healthy heart has yet to be definitively found[195]. It is activated by calcium, although phosphorylation of the autoinhibitory site allows it to be active in the absence of calcium and calmodulin, and it can form hexameric structures that can autophosphorylate[196]. It depends on activation by calmodulin, which is a 4 EF-hand containing calcium-binding protein that is expressed in all eukaryotic cells[197].

CaMKII can phosphorylate calcium-activated potassium currents[198], ryanodine receptors[199], and sodium channels[200], and can be triggered by oxidative stress[201]. CaMKII can alter transcription of hypertrophy-associated genes by phosphorylating histone deacetylases HDAC4 and 5, exporting them out of the nucleus and inhibiting their inhibition of hypertrophic transcription factors such as MEF2, and CaMKII can work in tandem with calcineurin[24]. CaMKII can also interact with the MAPK/ERK pathway by phosphorylating ERK, and it has been shown that targeting CaMKII for inactivation can ameliorate pathologic hypertrophy and heart failure in some animal models[202].

Other paths to hypertrophy exist in the cardiac myocyte, which are not specific to the cardiac myocyte and have often been studied in more depth in other cell types; for example, the mitogen activation phosphatase (MAPK)/ERK pathway, which can be activated by growth factors, and can interact with NFAT, as well as MEK1 and GATA-4 and MEF2[203]. The mToR/Akt pathway is involved; here mToR is a downstream target of Akt (protein kinase B), which activates protein synthesis and inhibits autophagy, but is inhibited by low levels of ATP through AMP Kinase[194]. This pathway can be activated by such diverse signals as insulin, cholesterol[204], inflammation[205], and angiotensin II[206]. Finally, sirtuins (SIRT) play a role, including SIRT3 and SIRT6 which inhibit hypertrophy by acting through Akt, and SIRT1 which has a more complex role[207].

Pathologic versus Physiologic Hypertrophy

The differences between pathological organ hypertrophy and physiological organ hypertrophy can be seen clinically; for example, a patient with pathological left ventricular hypertrophy has impaired diastolic ventricular filling, including delayed relaxation and a decrease in maximal early velocity of diastolic filling, while a patient with physiological hypertrophy has normal filling patterns[208]. Tissue Doppler also shows decreased systolic velocities in patients with pathologic hypertrophy, but not in athletes with physiologic hypertrophy[209]. Molecular and cellular differences are less well-known; but several genes are associated with pathologic but not physiologic hypertrophy- for example, BNP, angiotensin converting enzyme (ACE) and vascular cell adhesion molecule-1 (VCAM1) are overexpressed in pathologic hypertrophy but not exercise-induced physiologic hypertrophy[210].

Genome Editing to Induce Cardiac Myocyte Maturation

Early Technological Developments

In order to induce expression of these proteins of interest, which are crucial in the development, maturation, and physiologic function of cardiac myocytes, this project takes advantage of a fairly recent technological innovation known as genome editing. Before genome editing, expression of a particular gene or protein relied on viral vectors, which come in many flavors and are still

extremely useful- some viruses are able to express genes transiently, such as adeno-associated virus (AAV); some viruses are able to express genes for several months to years, such as adenovirus (AV); some viruses are able to integrate into the host genome and express genes long-term, such as lentivirus[211]. Issues with viral delivery include short expression times, limited size of DNA that viruses are able to carry, host immune response, lack of viral receptors on target cells, or random integration of genes into potentially disruptive sites in the host genome. There also exist other technologies and means of introducing nucleic acids into cells, such as engineered nanoparticles that can carry DNA or RNA into the host cell; these also result in short periods of expression[212].

Genome editing, however, utilizes enzymes that make either double- or single-stranded breaks at very precise locations in the genome of the host cell- this allows the cell to repair it the DNA using non-homologous end-joining (NHEJ), which may lead to nullification of the gene, or, if a template is provided, the cell may use homology-directed repair (HDR), which may lead to the cell incorporating the template-provided sequence into the genome permanently- this can be used for short changes in the sequence of a gene, including single-nucleotide changes, or for introduction of entire genes and promoters[213].

The earliest technology used for this was zinc-finger nucleases (ZFNs), used since the early 2000s, which are restriction enzymes engineered to

recognize a specific DNA sequences through the zinc finger domain of the protein, and can recognize longer sequences of bases when zinc finger domains are tandemly repeated; a ZFN with four fingers can recognize a 24-bp sequence. They contain a FokI domain for DNA cleavage. ZFNs suffer from a need for selection of specific binding domains for a given sequence, rather than the ability to engineer it exactly- they rely on bacterial selection to find the most specific sequence, which can be a lengthy process[213].

Following on the success of ZFNs, several researchers began engineering DNA-binding enzymes using transcription activator-like effectors (TALEs), proteins from a bacterial plant pathogen *Xanthomonas*, which bind specific sequences of DNA using domains that are essentially loops with two amino acids at the outside of the loop that bind a particular base; so that up to 18 loops in a row will bind a sequence of 18 amino acids[214]. By attaching a FokI domain for nuclease activity, a TALEN is created, and TALENs can be used to efficiently cleave genomic DNA- they require two TALENs, one on either side of the cleavage site, for FokI to activate, thus improving specificity[215].

TALENs are more easily engineered than ZFNs, with a higher rate of success, and with comparable mutagenesis[216]. Protocols and kits for rapidly building locus-specific TALEN cDNA using bacterial cloning have also been developed[217-218]. TALENs have advantages of being modular, easy to use, and effective; they suffer from the fact that because each nucleotide is recognized by

an entire protein domain, the cDNA can be large ~3 kb compared to ~1 kb for a ZFN, potentially inhibiting its ability to be packaged into a viral vector or transfected into a cell[215].

Recent Developments: CRISPR/Cas9

The most recent advance in genome editing has come in the form of clustered interspace short palindromic repeats (CRISPR) and CRISPR-associated systems (Cas); these are bacterial defense systems that have been discovered in several species of bacteria, including *Streptococcus pyogenes* and *Neisseria meningitides*. They are comprised of a strand of mRNA that recognizes the locus of interest, and an enzyme with nuclease activity that binds the mRNA (guide RNA, gRNA) and cleaves the DNA at that site[219].

The CRISPR system has the advantages of being smaller than TALENs, and thus more deliverable, as well as faster and cheaper to engineer and build, so that several gRNAs can be tested against a particular locus to find the one with highest cleavage efficiency and fewest off-target effects. Additionally, many modifications to the Cas enzyme have been made- some optimization has been done in order to improve efficiency, but modifications have also been made that reduce nuclease activity to a single strand, or abolish it completely so that the system acts as a way of guiding transcription factors to the locus, or they have been used as designer transcription factors or repressors[220].

Genome Editing in Stem Cells and Stem Cell-Derived Cardiac Myocytes

Disease Modeling

Genome editing as a way of permanently altering genomic DNA has been used and envisioned as a way of more effectively modeling certain diseases, either by making precise deletions, insertions, or point mutations to model genetic disease; by long-term overexpression or deletion in cell lines, including stem cells and stem cell-derived cells; or by efficient editing of animal model genomes to produce animal models more rapidly and without potentially disruptive random insertion of genes into the genome[221]. For example, TALENs have been used to model Hemophilia A in iPSCs by creating an inversion in the factor VIII gene, a genotype which is commonly seen in Hemophilia A patients, and furthermore, TALENs were able to correct that same inversion[222]. ZFNs were used to remove GAA repeats from the frataxin gene (FXN), since GAA repeats in that gene are the genotype associated with Friedrich's Ataxia; when these cells were differentiated into neuronal cells, the Friedrich's Ataxia phenotype was reversed, with rescued expression of frataxin[223].

In the field of cardiovascular disease, genome editing has been used to model Long QT syndrome; one group used ZFNs to insert voltage-gated potassium channels KCNQ1 and KCNH2 with dominant-negative mutations into the AAVS1 safe-harbor site of iPSC-derived cardiac myocytes. Their edited cells

displayed characteristic phenotypic markers including prolonged action potential duration and amelioration with LTCC blocker nifedipine[224]. As noted in their paper, isogenic genome editing allows edited cells to be compared with nonedited cells that have the exact same genetic background, thus removing potentially confounding factors of SNPs and mutations found elsewhere in the genome, meaning that phenotypic changes are due to the genomic edit alone. Elsewhere, a group has used TALENs to introduce a mutation in PLN to iPSC-CMs in order to recapitulate dilated cardiomyopathy (DCM) associated with that mutation, and then TALENs were used to isogenically correct it as well. They showed that cells edited to contain the mutation produced low amounts of force, similar to cells from patients already harboring the mutation, which was corrected with isogenic correction[225].

Genome Editing for Therapeutic Purposes

Beyond modeling, genome editing has begun to be studied for use in therapeutic settings, including cell therapy. The immunology world has quickly moved to take advantage of this, with the hopes of altering immune cells that can be phoresed from the patient's blood, edited, and then re-transplanted back into the patient. A popular strategy has been the engineering of T cells to express chimeric antigen receptors (CARs) that recognize epitopes specific to cancer cells[226]. Many groups have continued down the path of gene editing from merely expressing CARs to downregulating or modifying other proteins and pathways

that interfere with the efficacy of CARs, such as the elimination of endogenous T cell receptors (TCRs)[227].

In the world of cardiovascular and muscular disease, Duchenne muscular dystrophy (DMD) has been an attractive target for genome editing. Several groups have explored using it in animal models or cell-based models to not correct the underlying mutation, but to excise entire groups of exons in a way that allows a truncated but still functional dystrophin protein to be expressed, thereby allowing a single set of nucleases to be effective against a group of patients with a range of different mutations[228-229]. It has been used to correct mice with the arrhythmic PRKAG2 cardiac syndrome phenotype by packaging the CRISPR/Cas9 system into AAV9 vectors to disrupt the mutant allele while leaving the healthy allele intact, leading to improved phenotype[230].

Conclusions

There is significant room for growth and improvement in the fields of cardiovascular disease, stem cell biology, iPSC-CMs, and genome editing for the use of disease modeling and cell therapy. Cardiovascular disease, including congestive heart failure with its myriad of contributing and precipitating factors, remains a significant problem in the developed world and is becoming more of a problem in the developing world. Stem cells, including induced pluripotent stem cells, and cardiac myocytes derived from them have shown enormous potential in the treatment and research of cardiovascular disease, although there remain

important barriers to using them fully, including their immature physiological function, lack of adequate physiologic assays to probe their function, and immature gene expression profile.

This thesis uses several cutting-edge technologies, including traction force microscopy and genome editing, to rigorously measure hiPSC-CM function under important conditions such as increased mechanical load and altered extracellular ionic concentrations. Furthermore, this thesis explores attempts to alter the isoforms of crucial proteins expressed, including calcium handling proteins and mature isoforms of sarcomeric proteins, in order to affect physiologic function including calcium handling and contractile kinetics, and to ultimately affect the gene and protein expression profile in a cell-wide level.

Chapter 2

Elucidation of the Functional Maturation and Physiological Performance of Cardiac Myocytes

Author Contributions:

- Matthew Wheelwright performed all experiments except those noted below
- Zaw Win (Department of Biomedical Engineering, University of Minnesota) designed and made silicon wafers for stamp creation, and taught many of the protocols related to traction force microscopy.
- Kamilah Amen (Department of Biomedical Engineering, University of Minnesota) tested elastic modulus of polyacrylamide gels.
- Patrick Alford (Department of Biomedical Engineering, University of Minnesota) provided expertise and imaging equipment for traction force microscopy.
- Joseph Metzger (Department of Integrative Biology and Physiology, University of Minnesota) provided intellectual guidance and expertise.

Abstract

Recent technological advances have made it possible to efficiently derive cardiac myocytes from human induced pluripotent stem cells (hiPSC-CMs). These have been seen as a model for human heart disease, as well as a potential source for cellular transplantation into failing diseased heart tissue. Many laboratories have devoted substantial effort to examining the functional properties of hiPSC-CMs, including electrophysiology, intracellular calcium handling, and gene/protein expression and force. Here we utilize traction force microscopy (TFM) to determine the maximum force production of isolated hiPSC-CMs under varied culture and assay conditions. We elucidate here the relationship between cell morphology and force production, HiPSC-CMs developing in culture for two weeks produce significantly less force than cells cultured from one to three months and hiPSC-CMs cultured for three months resemble the cell morphology of neonatal rat ventricular myocytes. hiPSC-CMs produce less force when assayed on increasingly stiff substrates, and generate less strain energy. Finally, hiPSC-CMs cultured in conditions of physiologic calcium concentrations are larger and produce more force than cells cultured in standard media. Collectively, these findings establish single cell TFM as a valuable approach to illuminate the quantitative physiological maturation of force in hiPSC-CMs. The demonstration here of an inverse relationship between force and substrate stiffness has implications for translating hiPSC-CMs into ongoing efforts to remuscularize diseased myocardium in vivo.

Introduction

Over the past several years it has become possible to efficiently derive from human induced pluripotent stem cells (hiPSCs) robust spontaneously contracting cardiac myocytes (hiPSC-CMs)[65-66,231]. Regarded as a potential source of virtually unlimited human cardiac muscle tissue, researchers and clinicians have begun utilizing these hiPSC-CMs as sources of therapeutic cell-based repair via transplantation into host[63] and for cellular and tissue models of cardiac disease [232-233]. Since their discovery, considerable efforts have been underway to assess and quantify hiPSC-CM contractile function and development using culture conditions that may better mimic the physiological cues imposed on native cardiac cells and tissues in vivo. Importantly, substantial effort has been made to address the developmental state and physiological maturity of the hiPSC-CM[112,174,234].

The central measure of the physiologic function of a cardiac myocyte, and the essential purpose of the cell, is force production. To date, several groups have optimized assays to measure force production of hiPSC-CMs, either as a syncytium[102,235] or population of cells on a thin film[148], or as single cells using micropost arrays[236] or using traction force microscopy[160]. Specific force production is an important quantitative index of cardiac myocyte maturity but thus far has been difficult to ascertain in hiPSC-CMs. It has been shown that human fetal cardiac myofibrils have low force compared to adult cardiac myofibrils, and that this increases over time in human development[69]. Furthermore, isometric

force in skinned myocytes from mice and sheep increase as gestational age increases[237-238].

Closely related to the myocyte's ability to produce force is its morphology, including total cell area, length and width. The use of micropattern printing to manipulate the shape of neonatal ventricular cardiac myocytes, shows a range of aspect ratios that result in maximal force production, presumably by improved sarcomere and myofibril alignment[148,239]. Similar studies in hiPSC-CMs demonstrate increased force in longer cells compared to shorter ones[161]. Investigations of the relationship between cell size and force is important, as cardiac myocyte size changes dramatically during cardiac development with the transition from immature to mature cardiac myocyte involving a significant increase in cell area[240].

Force production in both adult and fetal cardiac myocytes is highly dependent upon the load against which the cell is contracting, which includes the stiffness of the immediate microenvironment[241]. This allows the heart to adjust cardiac output and this may be compromised in cases where tissue stiffness changes drastically, as in fibrotic diseases of the heart[242]. The stiffness of the human heart changes during development in utero; however, the elastic modulus of the native myocardium is still being debated and varies markedly depending on the method of measurement. To date, most studies report that the elastic modulus of the myocardium increases with age[243-247]. Thus, the ability to produce

force against varying levels of stiffness is another important marker of heart muscle physiologic maturity.

HiPSC culture media composition is another critical factor in guiding hiPSC-CM maturation. It is well known that the physiologic extracellular calcium concentration in mammalian interstitial spaces is between 1.5-2.0 mM[248], providing a strong electrochemical gradient opposite a much smaller intracellular calcium concentration in heart muscle[104,249]. However, the calcium concentration in RPMI, which is the basal medium used in differentiation and growth of hiPSC-CMs in most laboratories, is sub-physiological at 0.42 mM[250]. It has been shown by numerous groups that extracellular calcium and calcium signaling play a significant role in cardiac development, namely in cardiac myocyte hypertrophy[150-151]. With this information, we hypothesized that physiological extracellular calcium concentration is necessary for the development of force production in hiPSC-CMs.

In the present study, we investigated the developmental maturation status of hiPSC-CMs using single cell traction force microscopy to measure force production of isolated myocytes. We examined force and contractility in the context of several physiologically relevant environmental parameters. First, we tested hiPSC-CMs contractile maturation by comparing them to neonatal rat ventricular myocytes (NRVMs), with a focus on cell morphology and geometry. Then, we measured force in response to substrates of varying stiffness. Finally,

we tested hiPSC-CM development during culture in varied physiologic extracellular calcium conditions.

Methods

Culture and Differentiation of Human iPSC-Derived Cardiac Myocytes

Human iPSC line DF 19-9-11T, which was derived from healthy donor fibroblasts using a vector-free episomal induction method[251], was graciously provided to us by the laboratory of Dr. Timothy Kamp at the University of Wisconsin-Madison. HiPSCs were cultured according the protocol outlined in that paper; briefly, cells were grown in TESR-E8 media (Stemcell, Vancouver, CA), on Matrigel-coated (Corning, Corning, NY) 35 mm 6 well plates, and passaged every 4 days via EDTA with a dilution factor of 1:12.

HiPSCs were differentiated using a small molecule Wnt/GSK3 inhibition protocol[66]. Briefly, hiPSCs were cultured to approximately 90% confluency, then treated with a GSK3 inhibitor, CHIR99021 (Stemgent, Cambridge, MA) in RPMI supplemented with B-27 minus insulin (Thermo Fisher, Waltham, MA) and Matrigel, for 24 hours. Media was replaced for 48 hours. Cells were treated with IWP-4 (Stemgent, Cambridge, MA) in RPMI with HEPES with B-27 minus insulin for 48 hours, and media was replaced every 48 hours until cells began to beat spontaneously, at which point they were grown in RPMI with HEPES supplemented with insulin-replete B-27. Media was changed every 2-3 days until ready for assays. Higher-calcium (physiological) media was prepared by

supplement RPMI + B27 growth media to 1.8 mM Ca^{2+} using 1M CaCl, and then stirred to dissolve any precipitations that form. Cells grown in physiological calcium media had media changed every 1-2 days, as these cells produce acidic media more rapidly.

Isolation and Culture of Neonatal Rat Ventricular Myocytes

All methods for handling laboratory animals were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. One day-old Sprague Dawley rat pups were sacrificed via decapitation and hearts excised through the chest. Cardiac myocytes were isolated using sequential trypsin and collagenase treatments according to the protocol provided with the Worthington Neonatal Cardiomyocyte Isolation System (Worthington Biochemical, Lakewood, NJ). NRVMs were plated directly onto patterned polyacrylamide constructs at a density of 100,000 cells per well and cultured in DMEM + 4% FBS. Media was changed after 24 hours, and cells were allowed to continue to adhere for 48 more hours until assaying. NRVMs were treated the same way as hiPSC-CMs during the assay procedure.

Micropatterning of Polyacrylamide Constructs

Micropatterning stamps were created according to protocols outline by Wang et al[152]. Briefly, photomasks were designed in AutoCAD (AutoDesk, Mill Valley, CA) and printed by Fineline Imagine (Colorado Springs, CO). Single cell

shapes were placed far enough apart to ensure contraction of one cell would not affect substrate deformation of neighboring cells. Stamp masters were created using photolithography by applying photomasks to silicon wafers coated with photoresist and exposing to light. Stamps were created by curing polydimethylsiloxane (PDMS) (Dow Corning Sylgard 184, Ellsworth Adhesives) on the patterned silicon master (for patterned stamp) or on an unpatterned silanized silicon wafer (for blank stamps). Both patterned and unpatterned stamps were made new each time they were used. Stamps were coated and stamped according to the stamp-off protocol laid out by Desai et al[153]. Briefly, blank stamps were coated with laminin (50 µg/ml in molecular biology grad water) and incubated for 60 minutes, then dried and inverted onto patterned stamps which have been UV-activated. Blank stamps were immediately peeled off and placed onto 15 mm coverslips which had been plasma-activated by running through a blue flame. Cover slips were then ready for use with gels.

Polyacrylamide Gel Construction

Polyacrylamide gels were made using ratios of Acrylamide to N,N'-methylenebisacrylamide according to Tse et al[154] and then actual gel stiffness was measured using uniaxial stress testing. Ahead of time, 25 mm glass coverslips were UV treated, then treated with 3-aminopropyltriethoxysilane for 3 minutes, and rinsed with ethanol. Polyacrylamide was made with 1x phosphate-buffered saline, and 0.2 µm red FluoSpheres fluorescent beads (Thermo Fisher,

Waltham, MA) were mixed into the unpolymerized acrylamide solution at a concentration of 0.005% (diluted 1:200). The solution was degassed for 15 minutes, and to the unpolymerized solution was added tetramethylethylenediamine (final dilution 1:1500), and the solution was brought to a pH of 7 via HCl. Ammonium persulfate (final concentration 0.017% w/v) and N-hydroxysuccinimide ester (final concentration 0.0083 mg/ml) were added to solution. 15 µl of solution were quickly pipetted onto APS-treated coverslips. Laminin-coated coverslips were inverted onto the solution and allowed to polymerize at room temperature for 60 minutes. Top coverslips were removed from the polymerized gels, and gels were incubated in 4% BSA at 37° for 45 minutes, then rinsed 3 times with 1x PBS.

HiPSCs were dissociated in Accutase (Thermo Fisher, Waltham, MA) for 20 minutes, then resuspended in warm RPMI + B27 and replated onto polyacrylamide gels at 100,000 cells per well. Cells adhered overnight, then media was changed the following morning. Cells were allowed to adhere for two more days, then assayed on day 3 after plating.

Traction Force Microscopy and Analysis

Experiments were performed on an Olympus X81 Inverted Microscope using a 40x UPLSAPO40X2, NA 0.95 objective in an environmental control chamber at 37° C. Images were acquired using MetaMorph software (Molecular Devices, Sunnyvale, CA) at a rate of 3.5 frames per second. Cells were paced at

0.5 Hz with a 35 mV square pulse using a MyoPacer field stimulator (IonOptix, Westwood, MA) in RPMI + B27 with HEPES. Cells were paced for 3-5 minutes before recording, and data was recorded from each dish for no more than 30 minutes to avoid recording from dying cells. Cells were given fresh media 60 minutes before data acquisition. 3-4 contractions were acquired from each cell. Images were analyzed using ImageJ code developed by Tseng et al[155-156]. Stacks of images of the cell and fluorescent beads before and during contraction were oriented vertically and cropped to an area of 64.4 μm wide by 128.8 μm tall before analysis.

Particle image velocimetry using iterative interrogation windows of 128-64-32 pixel width was completed between the matched bead images from the same cell location at different time points. The noise-filtered displacement field was used to calculate traction stresses with a Fourier transform traction cytometry (FTTC) ImageJ plugin using the Fourier transform traction cytometry method[157]. A regularization factor of 1x10-9 was applied for improved accuracy, as described in Stricker et al[158]. Stress vector magnitudes were integrated over the area of interest and reported as total force. Strain energy of the substrate was calculated for each individual hiPSC-CM as[149].

$$U = \frac{1}{2}\int \mathbf{T} \cdot \mathbf{u} dx dy$$

where **T** is the traction stress and **u** is the displacement.

Statistical Methods

All statistical analysis was performed using Prism software (GraphPad, San Diego, CA). Correlation analysis was done by linear regression, and estimation of significant non-zero slopes was determined. Significant differences between groups for all other experiments was estimated by one-way ANOVA with a Tukey's post hoc test. Significant differences in Figure 5 were estimated with a Student's T test. Spline plots were created using MATLAB (Mathworks, Natick, MA).

Results

HiPSC-CMs Align and Contract Along a Single Axis

Human iPSC-CMs were transferred to polyacrylamide (PA) gels that had been micropatterned with laminin rectangles with an area of 2000 μm^2 and an aspect ratio of 7:1, which has been reported as an ideal aspect ratio for NRVM force production[148]. This allowed individual hiPSC-CMs to adhere to the substrate and occupy an area of up to 2000 μm^2. Here, hiPSC-CMs formed a rectangular shape aligned along the direction of the long axis of the patterned area (Figure 2.1A). Most hiPSC-CMs formed geometries with an area smaller than 2000 μm^2 and an aspect ratio slightly smaller than 7:1 (Figure 2.1F).

Patterned single hiPSC-CMs were paced via field stimulator at 0.5 Hz and 35 mV. Paced myocytes contracted along their long axes, creating visible deformations in the fluorescent bead-containing substrate. Particle image velocimetry analysis showed greatest substrate displacement towards the ends

of the hiPSC-CMs, as well as in areas surrounding the ends of the cells (Figure 2.1B). Traction force analysis showed that the largest traction stress was developed in the substrate at these same locations (Figure 2.1C). Averaged contractions showed a force development curve resembling that of adult cardiac myocytes (Figure 2.1D, E)[252]. hiPSC-CM calculated force measurements were on the order of 10^{-8} N, which is in line with measurements previously reported by others[161].

Heterogeneous Cell Geometry Affects Contractility

In general, current practice differentiation protocols result in the development of a heterogeneous population of contractile cells, as evidenced by varied electrophysiological parameters[253], calcium handling[254] and gene expression profiles[255]. We thus sought to examine the potential effects of geometric heterogeneity on physiologic force production. Micropatterned PAA constructs were designed as a rectangle with a 7:1 aspect ratio and a 2000 μm^2 surface area. HiPSC-CMs that have been cultured on the constructs are able to occupy an area of up to 2000 μm^2, in their preferred aspect ratio, which ranged from 4:1 to as long as 10:1, with a mean of 6.6:1 (Figure 2.1F).

Based on this outcome, we examined the effects of cell geometry on contractility. We first measured total force production of d90 hiPSC-CMs on a 5 kPa substrate. There was a significant positive correlation between cell size and total force produced (Figure 2.2A, $R^2 = 0.21$, $P = 0.02$). However, we found no

correlation between long axis (length), short axis (width), or aspect ratio and force. Based on these findings, for the remainder of this paper we report total force, as well as force per unit area, which we refer to here as normalized force.

Development of hiPSC-CMs under Prolonged Culture Conditions

It is widely accepted that hiPSC-CMs phenotypically resemble immature cardiac myocytes and, depending on the studied characteristics, resemble embryonic cardiac myocytes[256], fetal cardiac myocytes[69,234,] or neonatal cardiac myocytes[119]. Furthermore, with increased culture time, a more mature phenotype can be obtained. Accordingly, we examined TFM-based force production of hiPSC-CMs after 14 days, 30 days, or 90 days in culture and compared to NRVMs.

At d14, hiPSC-CMs produce small but detectable amounts of force, whereas d30 cells produce significantly more total force and normalized force (one-way ANOVA $p < 0.0001$) (Figure 2.3A-C). At d90, single hiPSC-CMs produce more total force than at d30. However, as they are also larger, normalized force is not significantly different (Figure 2.3D). However, d90 cells produce significantly more total force ($p < 0.0001$) and normalized force ($p < 0.0001$) than d14 cells. D90 cells were significantly larger than d14 cells ($P = 0.01$). NRVMs showed similar cell size and total force produced compared to d90 hiPSC-CMs; however, they had significantly higher normalized force ($P < 0.05$).

Effects of Substrate Mechanics on Contractility

Cardiac myocyte work adapts significantly during development[20]. Furthermore, cardiac myocytes produce different amounts of traction in response to altered mechanical environments[160,257]. To investigate whether this holds true for hiPSC-CMs, we cultured hiPSC-CMs for 30 days under normal growth conditions, then transferred isolated hiPSC-CMs to PAA gels with a defined modulus of 3.1, 9.8, or 13.5 kPa. hiPSC-CMs produced decreased total force (one-way ANOVA, $P < 0.0001$) (Figure 2.4A) and normalized force (one-way ANOVA $P < 0.0001$) (Figure 2.4C) as a function of increased substrate stiffness. HiPSC-CMs on 3.1 kPa modulus substrate produced significantly more force than on 9.8 or 13.5 kPa (Figure 2.4A, C). Cell area was not significantly different between conditions, indicating the range of substrate stiffness tested was not enough to induce changes in cell spreading, and that cell spreading was not the cause of differential force production (Figure 2.4B). At a substrate modulus higher than 13.5 kPa, bead displacement was very small, resulting in a poor signal-to-noise ratio (data not shown). We also calculated strain energy generated by each cell during a full contraction and found a decrease in strain energy with increasing stiffness. This correlated to decreased force production, with cells on a substrate with a modulus of 3.1 kPa substrates generating significantly more energy than those on substrates with a modulus of 9.8 or 13.5 kPa substrates (one-way ANOVA $P < 0.0001$).

Effects of Extracellular Calcium on hiPSC-CM Development

Typical hiPSC-CM growth medium contains sub-physiological levels of calcium (~0.42 mM), whereas physiologic extracellular calcium concentrations are much higher, ranging from 1.3 mM[258] to 2.0 mM[254,259-260]. To examine the effects of media calcium concentrations on hiPSC-CMs, we cultured hiPSC-CMs in growth media that had been supplemented with CaCl up to 1.8 mM Ca^{2+}, beginning on the day that they began spontaneously contracting (d7), and continuously until they were tested (d30). hiPSC-CMs in media with physiologic calcium levels produced greater total force than cells grown in standard growth medium, when assayed in standard growth medium ($P = 0.0073$) (Figure 2.5A). Additionally, these hiPSC-CMs were significantly larger ($P = 0.0004$) (Figure 2.5B). However, specific force was not significantly different between the two groups ($p = 0.75$) (Figure 2.5C).

Discussion

Human induced pluripotent stem cell-derived cardiac myocytes are an attractive model system for experimental therapeutic discovery and as a potential cell/tissue source for regenerative therapy in diseased hearts. However, a significant obstacle to realizing this potential is the physiologic immaturity of hiPSC-CMs relative to adult cardiac myocytes. In this study, we utilized traction force microscopy to rigorously investigate absolute force production in isolated single hiPSC-CMs. Relative to other methods of assaying cardiac myocyte

contractility, single cell traction force microscopy eliminates potential confounding effects of neighboring cells, including myocytes and fibroblasts. Here, our study has several main findings, including establishing an inverse relationship between force and substrate stiffness. This is potentially significant for ongoing and future studies attempting to translate hiPSC-CMs for regenerative therapies for the diseased myocardium in vivo.

Additional new findings include guiding hiPSC-CM area and aspect ratio to adopt a rectangular shape with a single contractile axis force vector to demonstrate that force production correlates with overall cell area but not length, width, or aspect ratio. This led us to normalize all force measurements to cell area in order to obtain a more accurate representation of cellular contractile performance, termed here as normalized force. While aspect ratio did not correlate to total force, aspect ratios were distributed normally around with a mean between 6:1 - 7:1, which is very close to the aspect ratio that other groups have determined for hiPSC-CMs and NRVMs to produce maximal force[148,161].

Data further shows that hiPSC-CMs progress along a timeline similar to the natural embryonic development of cardiac myocytes, wherein normalized force increases as myocytes mature[69,238]. Results show hiPSC-CM normalized force increases as a function of time in culture, which is in line with results from other research groups[160]. Analysis of single hiPSC-CMs, as compared to neonatal rat ventricular myocytes using identical assay conditions, show that hiPSC-CMs produce comparable total force as NRVMs. While instructive, these

results also show that it is not yet possible to achieve physiologic maturation in terms of force output approaching that of adult cardiac myocytes.

The stiffness of the human heart increases during development[243-244], prompting us to investigate the ability of hiPSC-CMs to produce force against substrates of varying elastic moduli. We implemented here three moduli that are in line with the range that a cardiac myocyte encounters as the heart develops from an embryonic state to adult[244]. Data show that both total force and normalized force decrease as substrate modulus increases from 3.1 kPa to 9.8 kPa - 13.5 kPa, in agreement with a recent report[161]. These findings are in contrast to another earlier study; however, it is difficult to directly compare findings due to the higher force variability in that work[160]. This discrepancy may be due to different methods of creating polyacrylamide constructs, or potentially to different methods of measuring the stiffness of the hydrogel.

Another new finding of this study is the elucidation of strain energy applied by hiPSC-CMs to the surrounding environment. Strain energy characterizes the work done by the cell on the underlying substrate, which is important to contractile cells, including hiPSC-CMs. It has been shown that fibroblasts generate similar amounts of strain energy on substrates of different stiffness[149]. However, as shown here, hiPSC-CMs generate less strain energy in the deformation of stiffer substrates. We posit that in hiPSC-CMs, due to their immaturity, they are better able to produce force against a substrate that more closely mimics the stiffness of an embryonic heart than that of an adult or fibrotic

heart. We speculate that an understanding of strain energy in hiPSC-CMs can help decipher the basis of the difficulties encountered by groups attempting to transplant immature hiPSC-CMs in diseased adult myocardium[63].

Finally, we discovered that ionic content of media for long-term hiPSC-CM culture has important outcomes in terms of cellular maturation and force output. The effects of extracellular calcium on the developing heart has been explored recently[150-151], with evidence that cells with sub-physiological calcium influx are smaller than those with normal calcium gradients and signaling. With hiPSC-CMs in vitro, a unique opportunity is present to track force development while modifying extracellular calcium levels directly. Data show increases in hiPSC-CMs normalized force during culture with physiologic calcium levels (1.8 mM) compared to widely used standard RPMI calcium levels (0.4 mM). This is important because calcium signaling is crucial for cardiac myocytes in terms of excitation-contraction coupling[104,261] as well as signaling through calcium binding proteins, such as calmodulin[262] and calcineurin[263]. Collectively, this is evidence that differentiation and development protocols that attempt to recapitulate embryonic development should take into consideration the concentrations of all electrolytes, including calcium.

Taken together, these new data show that the functionality of hiPSC-CMs, as determined by their ability to produce force against a substrate via single cell TFM, closely resembles that of an immature cardiac myocyte. Specifically, in terms of the impact of geometry on hiPSC-CM contractility, total normalized force

production by single myocyte TFM is similar to that of neonatal rat cardiac myocytes. As hiPSC-CMs perform more optimally working on less stiff substrates, we report hiPSC-CMs function is comparable to neonatal cardiac myocytes. Ultimately, quantitative analysis of hiPSC-CM contractile performance as done here via traction force microscopy will be critical toward optimization of culture content and cell environment, including matrices, to advance the maturation state of hiPSC-CM toward human adult myocardium.

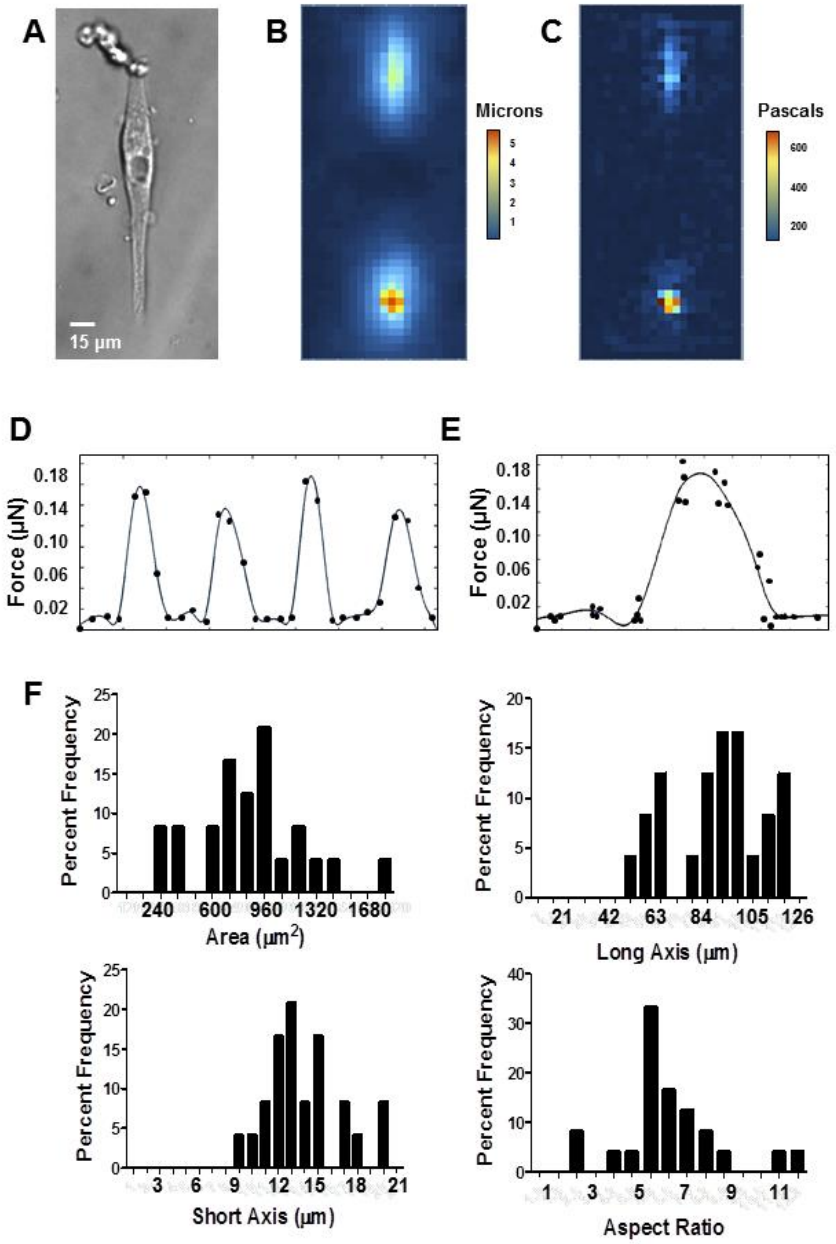


Figure 2.1. HiPSC-CMs and measurement of force by TFM. A, Representative cell, 30 days post-differentiation on a 9.8 kPa substrate. B, Heat map showing

magnitudes of deformation strain of the substrate under the representative cell. C, Heat map showing magnitudes of stress of the representative cell calculated from strain of the substrate. D, total force of a single cell over time with respect to baseline at the point t = 0 seconds, over four contractions paced at 0.5 Hz, fitted with a smoothed spline curve. E, total force of a single cell over time, average of four contractions, fitted with a smoothed spline curve. F, histograms showing distribution of cell geometries.

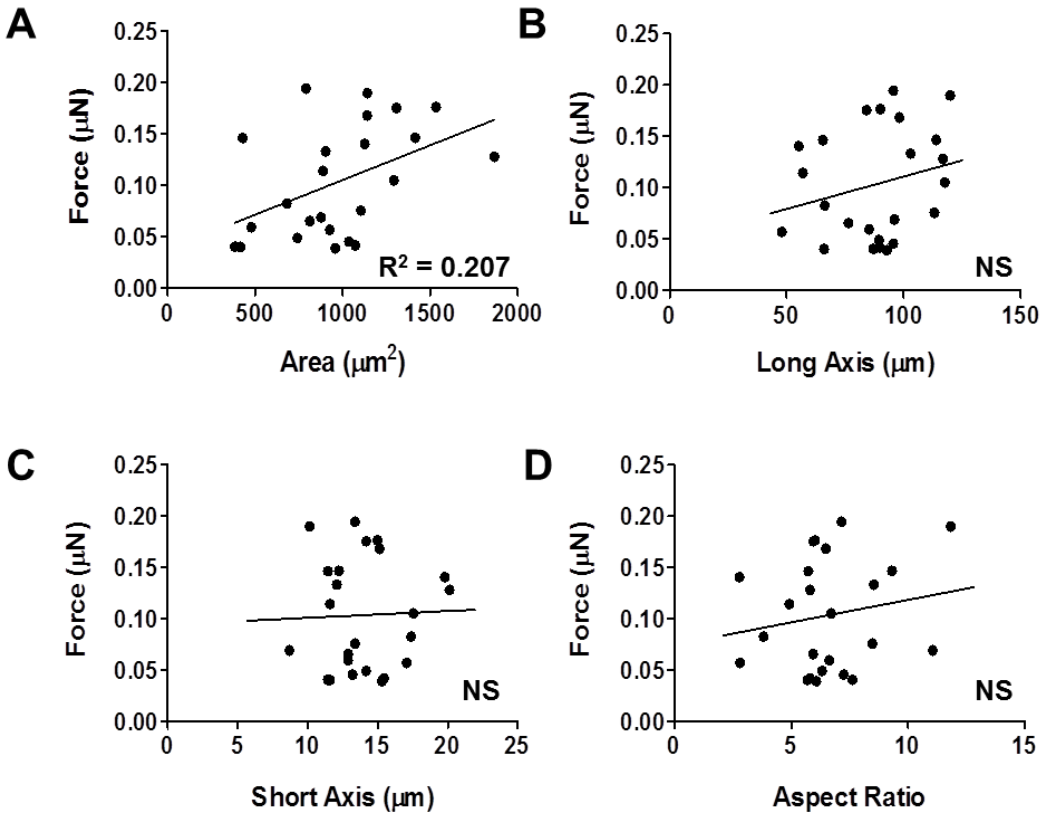


Figure 2.2. Effects of single cell hiPSC-CM morphology on force production. A, peak force versus cell area, $R^2 = 0.21$, $P<0.03$ B, peak force versus long axis (axis of contraction). C, peak force versus short axis (perpendicular to axis of contraction). D, peak force versus aspect ratio (long axis/short axis).

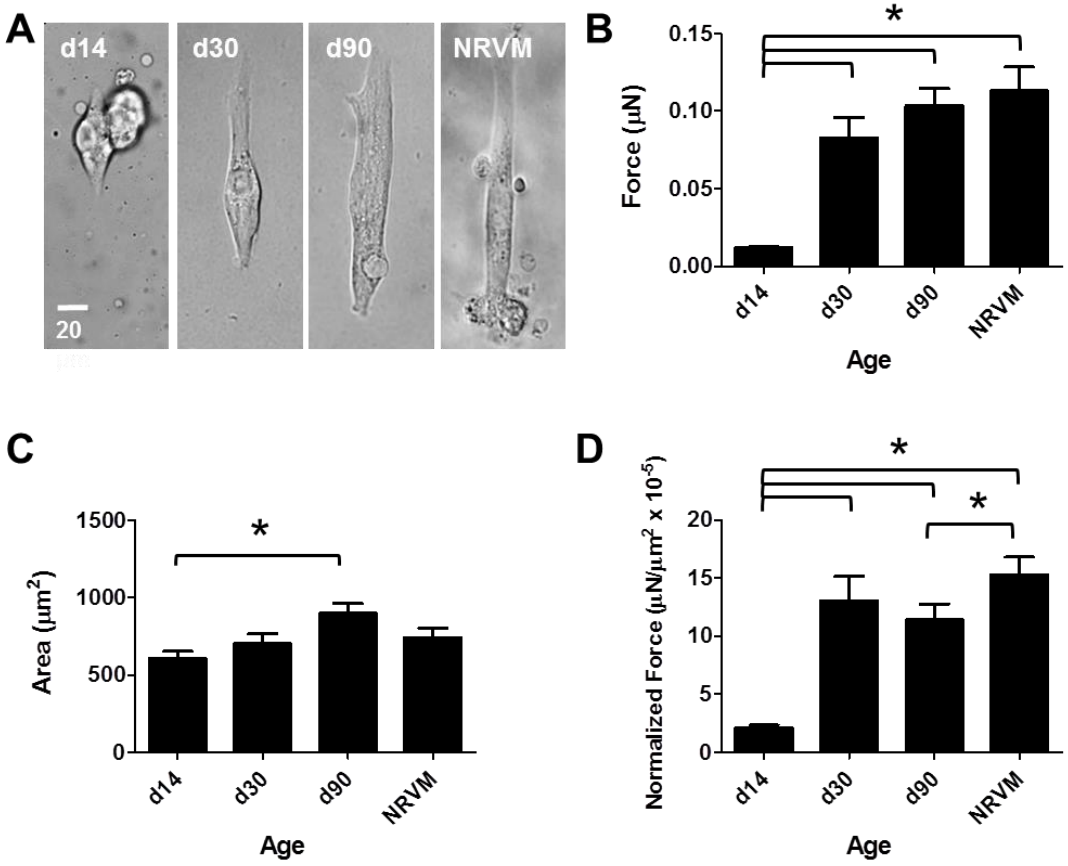


Figure 2.3. Effects of length of hiPSC differentiation on force production. A, representative hiPSC-CMs from day 14, day 30, and day 90 post-differentiation, and representative NRVM. B, total force versus length of differentiation (mean = 0.012 ± 0.001 µN, n = 17; 0.083 ± 0.013 µN, n = 15; 0.103 ± 0.011 µN, n = 24; 0.113 ± 0.016 µN, n = 12). C, cell area versus length of differentiation (mean = 605.7 ± 47.1 μm^2, 702.3 ± 63.2 μm^2, 898.2 ± 64.4 μm^2, 741.9 ± 61.4 μm^2). D, normalized force versus length of differentiation (mean = 2.12 ± 0.23 mN/mm2 x 10^{-5}, 13.0 ± 2.14 mN/mm2 x 10^{-5}, 11.4 ± 1.4 mN/mm2 x 10^{-5}, 15.3 ± 1.5 mN/mm2 x 10^{-5}).

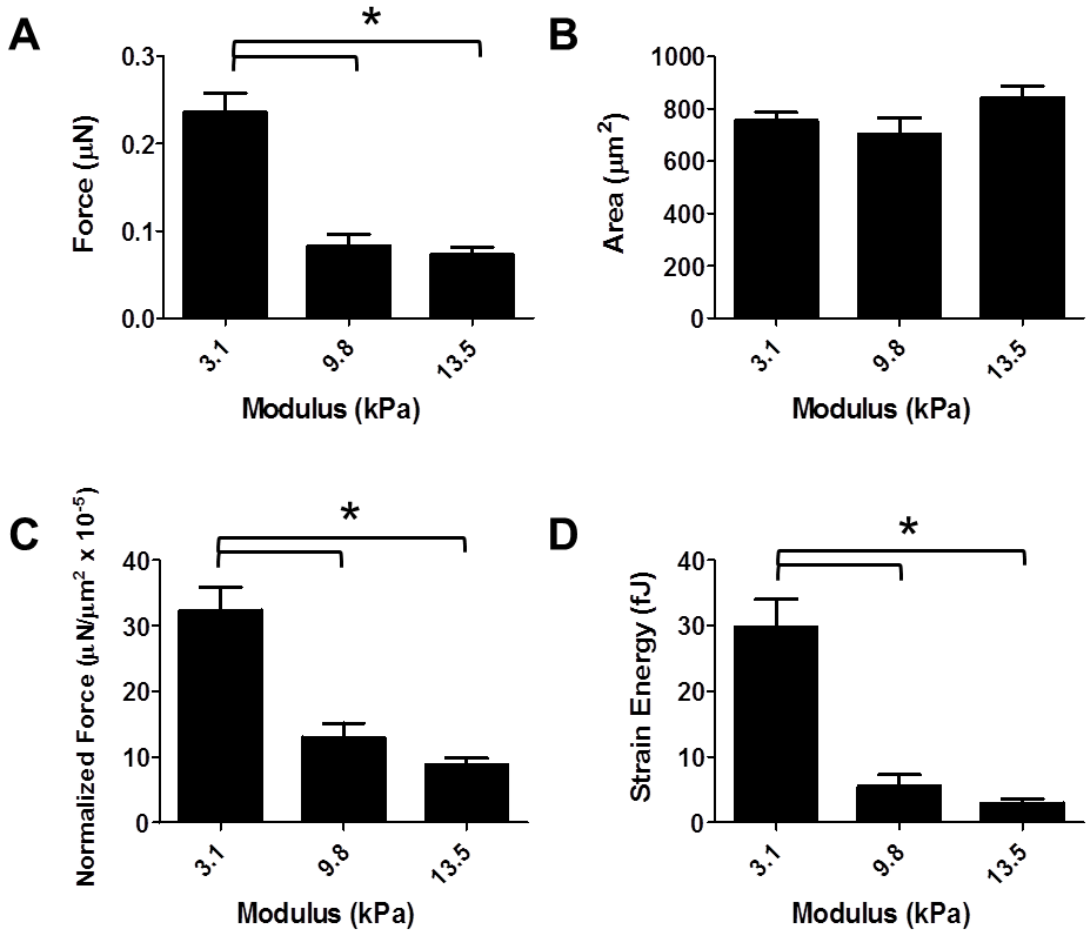


Figure 2.4. Effects of substrate stiffness on hiPSC-CM force production. A, total force versus substrate elastic modulus (mean = 0.236 ± 0.02 µN, n = 21; 0.083 ± 0.01 µN, n = 15; 0.075 ± 0.01 µN, n = 22). B, area versus substrate elastic modulus (mean = 754.9 ± 31.5 μm^2, 702.3 ± 63.2 μm^2, 851.6 ± 43.7 μm^2). C, normalized force versus substrate elastic modulus (mean = 32.4 ± 3.5 mN/mm2 x 10^{-5}, 13.0 ± 2.1 mN/mm2 x 10^{-5}, 9.1 ± 1.0 mN/mm2 x 10^{-5}). D, strain energy versus substrate elastic modulus (mean = 29.7 ± 4.3 fJ, 5.5 ± 1.7 fJ, 3.1 ± 0.6 fJ).

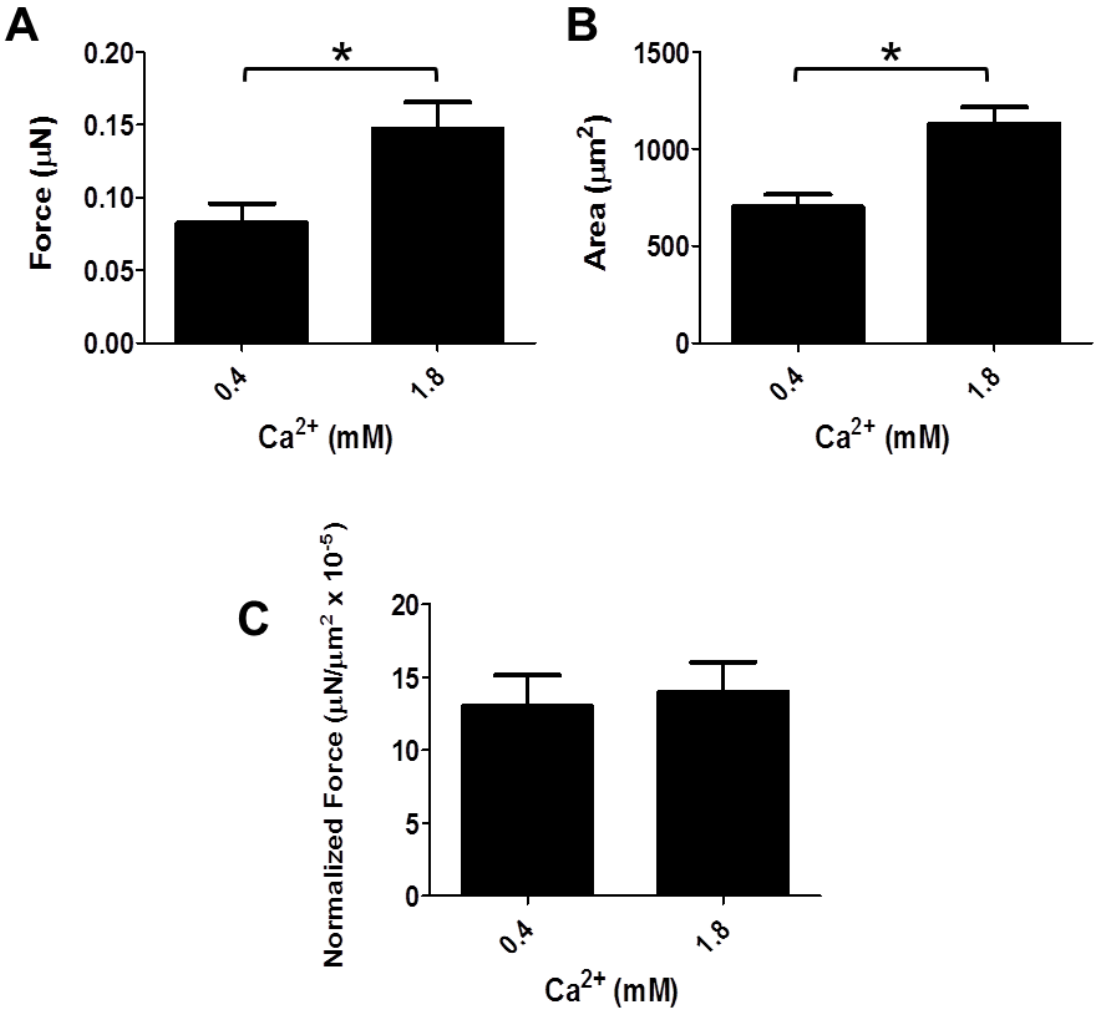


Figure 2.5. Effects of calcium concentration in growth media on hiPSC-CM force production. A, total force versus calcium concentration (mean = 0.083 ± 0.013 μN, n = 15; 0.147 ± 0.02 μN, n = 14). B, cell area versus calcium concentration (mean = 702.3 ± 63.2 μm^2, 1130.3 ± 86.2 μm^2). C, normalized force versus calcium concentration (mean = 13.0 ± 2.14 mN/mm2 x 10^{-5}, 14.0 ± 2.0 mN/mm2 x 10^{-5}).

Chapter 3

Inducible Expression of Mature Cardiac Markers Improves the Maturation Status of hiPSC-CMs

Author Contributions

- Matthew Wheelwright carried out all experiments other than those noted below
- Fikru Bedada (Department of Integrative Biology and Physiology, University of Minnesota) performed genome editing of iPSCs
- Bruce Conklin and Mo Mandegar (Department of Molecular Pharmacology, University of California San Francisco) designed the TALENs and template DNA
- RNAseq was performed at the Morgridge Institute at the University of Wisconsin, Madison
- Emily Keuhn (Department of Integrative Biology and Physiology, University of Minnesota) performed Western blots of cTnI overexpression
- Jennifer Mikkila (Department of Integrative Biology and Physiology, University of Minnesota) performed some Western blots and qPCR
- McKayla Carlson (Department of Integrative Biology and Physiology, University of Minnesota) cultured iPSC-CMs for experiments
- Joseph Metzger (Department of Integrative Biology and Physiology, University of Minnesota) provided intellectual guidance and expertise

Abstract

Cardiovascular medicine and regenerative biology have benefited greatly from recent advances in the ability to differentiate cardiac myocytes from human induced pluripotent stem cells (hiPSC-CMs). hiPSC-CMs have the benefit of being human cells, being virtually unlimited in supply, and being readily propagated in a short period of time. They have the potential to be a valuable model for studying cardiovascular physiology. However, hiPSC-CMs have been shown to exist in an immature state, more closely resembling embryonic cardiac myocytes than adult cardiac myocytes, limiting the amount of information that can be applied from hiPSC-CM studies to adult cardiac physiology. Here, we used genome editing to create hiPSC-CMs that can be induced to express one of two genes closely associated with cardiac maturation- SERCA2a or cardiac troponin I (cTnI). We found that overexpression of SERCA2a leads to enhanced physiologic function in terms of calcium handling and contractility kinetics, especially in response to isoproterenol. Additionally, SERCA2a expression leads to upregulation of other markers of maturation, such as cTnI. Expression of cTnI in iPSC-CMs leads to global changes in the gene expression profile as measured by RNAseq, including upregulation of important cardiac and developmental genes, and downregulation of genes associated with non-ventricular phenotypes. Overall, expression of proteins associated with maturity results in enhanced maturation of hiPSC-CMs.

Introduction

The fields of cardiovascular physiology, regenerative biology, and clinical medicine have benefited significantly from recent advances in the ability to differentiate cardiac myocytes from pluripotent cells. Notably, the ability to reprogram a human somatic cell to an induced pluripotent stem cell state (hiPSC)[41], and then to differentiate these cells into cardiac myocytes (hiPSC-CMs)[66]. In this setting, the investigator has exclusive control over the environment of the cell in its earliest stages of differentiation. This approach provides an almost unlimited source of human cardiac cells for experimentation and for potential use as a therapeutic agent.

One promising use of hiPSC-CMs has been as a model for myocardial disease and dysfunction. To date, research groups have used hiPSC-CMs to study electrophysiological parameters, including studies examining the effects of mutations in sodium channels[75,77]. In addition, investigations of calcium handling defects have been conducted by examining the effects of mutations in calcium handling proteins[82]. Futhermore, mitochondrial dysfunction has been studied by examining the effects of mutations in mitochondrial proteins[83]. These examples emphasize a significant strength of hiPSC-CMs, which is, they express human isoforms of proteins implicated in disease states. Furthermore, mutations in these proteins can be precisely isolated, and either introduced or potentially corrected by genome editing, given the pliable developmental state of the pluripotent cells[224-225].

However, it has been demonstrated by several groups that hiPSC-CMs, differentiated according to current protocols, exist in an immature developmental state, and that they more closely resemble neonatal myocytes than adult cardiac myocytes. For example, action potentials and pharmacology show increased susceptibility to arrhythmia[93], ultrastructure shows less-developed sarcoplasmic reticulum and myofibrillar patterns[264], isoforms of critical sarcomeric proteins are predominantly immature[174], and calcium currents show immature contributions of extracellular versus intracellular transporters[113]. Thus, attempts to study effects of dysfunctional proteins in hiPSC-CMs lead to conclusions that must necessarily be interpreted in the context of an immature myocyte.

To address this limitation, we investigated here the roles of two genes associated with a mature developmental state on the function of hiPSC-CMs. First, we addressed the role of induced overexpression of the sarco/endoplasmic reticulum calcium ATPase SERCA2a, the calcium pump of the sarcoplasmic reticulum (SR) membrane. SERCA2a is the major protein responsible for removal of calcium from the cytoplasm during diastole and reuptake into the SR to be stored for the next calcium release. It is known that the amplitude and kinetics of intracellular calcium transients change during cardiac development, with increasing SERCA2a expression in more mature cardiac myocytes[188]. In addition, it is known that SERCA2a expression declines in failing myocardium[187].

Second, we investigated troponin I, the key molecular switch of the troponin complex responsible for regulating myosin binding to actin for

contraction and relaxation of the sarcomere. Specifically, since hiPSC-CMs express almost exclusively the immature isoform of troponin I, slow skeletal troponin I (ssTnI), we studied the effects of expression of the adult isoform, cardiac troponin I (cTnI), which has important effects on sarcomere mechanics and kinetics[140,165]. Collectively, by employing genome editing platforms with inducible functionality, we tested here whether targeted expression of key adult genes can advance maturation in hiPSC-CMs.

Methods

hiPSC Culture and Differentiation

The human induced pluripotent stem cell line used in this paper was the DF 19-9-11 line, graciously gifted to us by the Timothy Kamp lab, which is derived from foreskin dermal fibroblasts and is transgene- and vector-free[251]. hiPSCs were cultured as previously described; cells were grown in TeSR-E8 media (Stemcell, Vancouver, CA) in plastic dishes coated with Matrigel (Corning, Corning, NY) and passaged by 0.5 mM EDTA in PBS when they reached 90% confluency, and replated with Rho kinase inhibitor 10 μM Y-27632 (Selleckchem, Houston, TX).

hiPSCs were differentiated at 90-95% confluency in 35 mm dishes according to the small molecule Matrigel sandwich method published previously[66]. At day 0, cells were treated with Matrigel dissolved in RPMI + B27 supplement minus insulin (Thermo Fisher, Waltham, MA) with 10 μM CHIR99021

(Stemgent, Lexington, MA), which is a GSK3 inhibitor. On day 2, media was changed without addition of small molecules. On day 3, cells were treated with 10 µM IWP-4 (Stemgent, Lexington, MA), an inhibitor of Wnt signaling, in RPMI + B27 supplement minus insulin. On day 5 and every 2 days after that, media was changed until cells started to beat vigorously, approximately day 7-10, at which point media was switched to RPMI plus B27, insulin-replete.

For all assays that involved dissociation and replating of cells, cells were dissociated with Accutase (Thermo Fisher, Waltham, MA) for 30 minutes at room temperature, followed by mechanical dissociation with a P1000 pipet and centrifugation for 3 minutes at 800 rpm.

Genome Editing

Genome editing was carried out by a pair of TALENs specific to the AAVS1 locus on chromosome 19, with a template plasmid containing homology arms specific to the targeted site and a tetracycline-inducible promotor-effector system[265]. The genes of interest (SERCA2a or cTnI) human cDNAs were designed with restriction sites to fit into the template plasmid and constructed by Integrated DNA Technologies (Coralville, IA), and then cloned into digested template plasmids.

All three plasmids (forward TALEN, reverse TALEN, and template) were transfected into undifferentiated hiPSCs at a concentration of 15 µg per 400 µl cuvette volume by electroporation using the Bio-Rad Gene Pulser Xcell (Bio-Rad,

Hercules, CA), using an exponential waveform with parameters of 250 V, 400 µF, and infinite resistance. hiPSCs were replated onto 25 mm wells coated with Matrigel and allowed to recover for 3-4 days. Puromycin selection was done starting with 0.2 µg/ml and increasing to 1 µg/ml over the course of 5-7 days. Individual surviving colonies were dissociated and replated, and continued to be passaged as in the above methods. Undifferentiated hiPSCs collected for RNA samples were treated with doxycycline on days 1 and 2 after passaging. In order to express the gene of interest in hiPSC-CMs, differentiated cells were treated with doxycycline from day 42 to day 60, so that experiments were carried out on d60 cells after 2 weeks of gene induction.

RNA Isolation and Real-Time PCR

RNA was collected from undifferentiated hiPSCs before doxycycline and after 24 or 48 hours of treatment with doxycycline. hiPSCs were isolated with EDTA as in the passaging protocol mentioned above, then spun at 800 RPM for 3 minutes and treated with the RNEasy Plus kit (Qiagen, Hilden, Germany) and quantified by NanoDrop spectrophotometry (NanoDrop, Wilmington, DE). In parallel studies, RNA was collected from differentiated hiPSC-CMs by dissociation with Accutase (Thermo Fisher, Waltham, MA) for 25 minutes at room temperature, then spun at 800 RPM for 3 minutes and treated with the RNEasy Plus kit and quantified by NanoDrop spectrophotometry.

Next, cDNA libraries were created using the Superscript Vilo kit (Thermo Fisher, Waltham, MA) and quantitative real-time PCR was performed using the Bio-Rad SYBR Green qPCR Master Mix Bio-Rad, Hercules, CA) on an Eppendorf Mastercycler machine (Eppendorf, Hamburg, Germany). Primers were designed using the Integrated DNA Technology PrimerQuest tool (IDT, Coralville, IA) and synthesized by IDT. Analysis of expression and fold change was carried out using the ddCt method[266], with GAPDH used as a housekeeping gene.

Western Blot and Protein Quantification

Protein was extracted by mechanical dissociation in RIPA buffer and quantified by Pierce BCA protein assay (Thermo Fisher, Waltham, MA). All samples were denatured by boiling and β-mercaptoethanol. 20 µg of protein in Laemmli buffer was loaded into each lane, and samples were run on 12% SDS-PAGE gels at 120 V until the dye front reached the end of the gel. Protein was transferred to PVDF membranes. Membranes were stained for SERCA2a (2A7-A1, Abcam, Cambridge, UK), ssTnI (MAB1691, Millipore, Billerica, MA), pan-TnI (1E7, Novus, Littleton, CO), and cardiac actin (5C5, Santa Cruz Biotechnology, Dallas, TX). Membranes were imaged on the LI-COR Odyssey (LI-COR, Lincoln, NE).

Calcium Imaging

Differentiated hiPSC-CMs grown in a monolayer were loaded with Fura-2 AM (Thermo Fisher, Waltham, MA) at 1 μM in RPMI + B27 supplement with 1.8 mM calcium for 10 minutes at room temperature, then allowed to de-esterify for 10 minutes at 37°C. Isoproterenol was used at a concentration of 10 nM, as previously described[267]. Experiments were carried out at 37°C using the IonOptix myocyte calcium and contractility system (IonOptix, Westwood, MA). Ratiometric calcium transients were curve-fitted and analyzed using the IonOptix software. We measured spontaneous transient rate, time to peak, and time to 75% baseline in d14 unedited cells (soon after spontaneous beating begins in culture), d60 unedited cells treated with doxycycline, and d60 SERCA2a overexpressing cells treated with doxycycline for 2 weeks. These measurements were made at baseline and in response to 100 nM isoproterenol, a β-adrenergic agonist.

Impedance Measurement and Contractility

Here, 35 day old differentiated hiPSC-CMs were dissociated and replated at a density of 80,000 cells per well into a 96-well Nanion Sensor Plate (Nanion, Munchen, Germany) that had been coated with Matrigel, and allowed to adhere 18 hours. Media was changed after 18 hours and then every 2 days after that. Cells generally began beating again 7 days after replating, at which point baseline impedance measurements were recorded. hiPSC-CMs were treated with doxycycline for 2 weeks after that, so that cells were 2 months old in culture at the time of post-induction measurements. Impedance was measured on the

Nanion CardioExcyte 96. Measurements were taken at 5 minute intervals over the course of 1 hour in an environmentally-controlled chamber at 37°C and 5% CO_2. Isoproterenol was used at a concentration of 10 nM. hiPSC-CMs formed syncytia after several days in culture and contracted simultaneously. hiPSC-CMs were not electrically stimulated, as attempts to do so interfered with the ability of this platform to collect accurate data. We measured at baseline and in response to isoproterenol, before and after 2 weeks of treatment with doxycycline. We measured time from 10% to 90% peak and time from 90% to 10% return to baseline

Data was exported to a txt file and transients were analyzed using Clampfit™ software (Molecular Devices, Sunnyvale, CA) to normalize to baseline and to fit a sum of exponents curve to raw impedance data.

RNA Sequencing

RNA sequencing was performed by the Bioinformatics group at the Morgridge Institute for Research, at the University of Wisconsin Madison. hiPSC-CMs were cultured and dissociated as above and RNA was extracted from dissociated cells using the RNEasy Plus kit (Qiagen, Hilden, Germany) and quantified by NanoDrop spectrophotometry (NanoDrop, Wilmington, DE). Samples were processed for quality control on an Agilent Bioanalyzer (Agilent, Santa Clara, CA) and a Qubit Fluorometer (Thermo Fisher, Waltham, MA). Libraries were created using Ligation Mediated RNA sequencing (LM-seq)[268] and

sequenced on a HiSeq 2500 (Illumina, San Diego, CA). Transcript counts reported as transcripts per kilobase million (TPM) were compared between groups for each gene and Bonferroni statistical tests were performed using CLC Sequence Viewer (Qiagen, Hilden, Germany).

Statistical Analysis

All statistical analysis other than that done on RNA sequencing data was performed using Prism (GraphPad, San Diego, CA). All groups of data were analyzed by ANOVA. Paired data was analyzed by Student's t test. RNA sequencing data was analyzed as above.

Results

Genome Editing Results in Long-term Expression of Inducible Genes

Undifferentiated hiPSC-CMs were edited, as outlined in the methods, using a pair of TALENs directed against the AAVS1 locus on chromosome 19 (Figure 3.1a). The gene of interest, either SERCA2a or cTnI, was cloned into a template plasmid containing sequences homologous to the AAVS1 site, allowing the gene of interest and tetracycline-inducible promoter system to be introduced to the site via homology-directed repair. Cells that had undergone HDR were selected for by puromycin-resistant selection.

In order to establish the kinetics of the tetracycline-inducible promoter system, expression of SERCA2a RNA transcripts was examined in

undifferentiated hiPSCs before doxycycline treatment and after 24 and 48 hours of doxycycline treatment. Here, qPCR showed increased expression after 24 and 48 hours of treatment, indicating rapid responsivity of the system (Figure 3.1b). In hiPSC-CMs that had been edited to contain an exogenous SERCA2a gene, Western blot analysis showed a 5.6-fold increase in SERCA2a expression compared to edited cells without doxycycline at the same time point ($P = 0.05$) (Figure 3.2a). In hiPSC-CMs that had been edited to express the adult cTnI gene, Western blot analysis showed increased of expression of cTnI after induction with tetracycline for 2 weeks; quantification shows a 4.3-fold increase in the ratio of cTnI: ssTnI ($P = 0.16$) (Figure 3.2b).

Induced expression of SERCA2a Enhances Calcium Handling and Adrenergic Responsivity

To examine the role of induced SERCA2a on the physiology of hiPSC-CMs, we used the ratiometric calcium indicator Fura-2 AM to visualize and quantify spontaneous calcium transients (Figure 3.3). At baseline, spontaneous transient rate was not significantly different between among unedited and edited cells with or without doxycycline. However, while d14 and d60 unedited cells do not display a chronotropic response to isoproterenol, the d60 SERCA cells had a significant ($P < 0.05$) response, indicating increased adrenergic responsivity.

The d60 unedited cells did not have a response to isoproterenol in time to peak; however, d60 SERCA cells show marked decrease in time to peak after isoproterenol treatment compared to baseline ($P < 0.05$).

Induced Expression of SERCA2a Leads to Improved Contractility Kinetics and Adrenergic Responsivity in hiPSC-CMs

Induction of SERCA2a expression in hiPSC-CMs would be expected to display altered cell contractility. We utilized here an impedance measurement system to track contractility of a syncytium of spontaneously beating d60 hiPSC-CMs (Figure 3.4). Prior to doxycycline, there were no significant differences between unedited and edited hiPSC-CMs, wherein each exhibited similar decreased time to peak and decreased time to baseline in response to isoproterenol. However, after treatment with doxycycline, SERCA2a hiPSC-CMs showed shortened time to peak at baseline compared to doxycycline-treated unedited cells (Figure 3.4a).

Induced Expression of SERCA2a Results in Increased Expression of Mature Cardiac Genes

By Western blot, markers of improved maturity or physiologic function were examined in hiPSC-CMs. In the SERCA edited iPSC-CMs, we tested whether increased expression of SERCA2a could induce developmental maturation of the hiPSC-CMs, including proteins not directly associated with

intracellular Ca^{2+} handling. We found that upregulation of SERCA2a in hiPSC-CMs for two weeks with doxycycline treatment significantly increased the cTnI: ssTnI ratio compared to hiPSC-CMs of the same age without doxycycline ($P < 0.05$) (Figure 3.5).

Induced Expression of cTnI Alters hiPSC-CM Gene Expression Profiles

TnI is a physiologically crucial protein that shows a robust switch from ssTnI to cTnI in the development of immature to mature cardiac myocytes[165]. There is some evidence that in some tissues, some TnI isoforms may localize to the nucleus and facilitate gene transcription[269]. Thus, we speculated that turning on production of cTnI may have far-reaching effects on the gene expression profile of the cell. To study this, we performed RNAseq on cTnI overexpressing cells that had been induced with doxycycline, and compared results with unedited cells that had been treated with doxycycline. In order to account for potential effects of heterogeneity of differentiated cell populations (cardiac myocyte versus other cell types) as well as proliferation or death of cardiac myocytes, we normalized transcripts per kilobase million (TPM) values of each gene to TnC TPM values for that group.

After filtering for genes with a Bonferroni test statistic $p < 0.05$ and normalizing to cardiac troponin C (cTnC), we found 586 genes upregulated and 41 genes downregulated in cTnI hiPSC-CMs compared to unedited cells at the same time point after treatment with doxycycline (Figure 3.6a, b). Importantly, we

found that the mRNA ratio of cTnI: ssTnI was higher in cTnI overexpressing cells compared to unedited cell, or edited cells without doxycycline, indicating appropriate induction of cTnI (Figure 3.6b).

Using DAVID pathway analysis software, we analyzed pathways and gene clusters that were upregulated or downregulated in cTnI hiPSC-CMs. Downregulated clusters included genes associated with early development and BMP signaling, as well as genes associated with non-cardiac or early-cardiac calcium signaling and muscle contraction (Figure 3.7a). Upregulated pathways included many clusters associated with cardiac muscle contraction, calcium signaling, and late development. Additionally, genes associated with cell migration, morphogenesis, cell-cell contact, and regulation of ROS were highly expressed (Figure 3.7b).

The list of upregulated genes included several genes crucial to the development and maturation of cardiac myocytes, and ventricular cardiac myocytes in particular, including MYH6, MYOM3, TTN, and MYL9, which are cardiac-specific sarcomeric genes. Calcium handling genes such as RYR2, CACNB2, and CALML4, which are important for cardiac calcium handling were upregulated; also, developmental genes including GATA4 and HAND2 were expressed. Downregulated genes included Grem2[270], coding for the protein Gremlin, which is important for atrial myocyte development; HCN4, which is associated with pacemaker cells; NKX2.5, which is an early cardiac development

gene; and several skeletal muscle-specific genes, including ACTA1 and MB (Figure 3.7).

Discussion

In this paper, we examined the effects of induced overexpression of two key proteins involved in cardiac myocyte maturation, SERCA2a and cardiac TnI. We found that induced expression of SERCA2a led to altered physiologic function of hiPSC-CMs, including increased responsiveness of calcium transients to adrenergic signaling, and faster contractile kinetics. Furthermore, induced expression of cTnI resulted in widespread changes in the gene expression profile of hiPSC-CMs as seen by RNAseq, including upregulation of genes and pathways associated with ventricular myocyte development and regulation.

SERCA2a is critical for removal of calcium from the cytoplasm during diastole[104], allowing the cell to relax while loading the SR with calcium for release during the next contraction. It communicates with other calcium handling proteins and sarcomeric proteins, and its function is modulated by adrenergic signaling pathways[187]. Expression of SERCA2a increases as cardiac myocytes mature into adult myocytes; decreased SERCA2a is also associated with myocardial failure, and restoration of SERCA2a can improve myocardial performance[188]. Furthermore, hiPSC-CMs have altered calcium handling properties compared to adult myocytes[188], making SERCA2a an attractive target for overexpression.

cTnI is an important marker of the switch from immaturity to maturity, as immature cardiac myocytes express nearly 100% ssTnI, and mature cardiac myocytes express nearly 100% cTnI, and this does not change in heart failure[238]. cTnI has profound effects on the contractile kinetics of the myocyte, namely in its ability to respond to adrenergic signaling and allow the sarcomere to experience increased chronotropy while maintaining sarcomere length shortening[140].

The choice to introduce genes that are associated with cell maturity, but that have important roles in the physiologic function of the myocyte, allows us to probe the ways that these proteins have effects in both areas. Both are crucial for researchers using hiPSC-CMs to study human myocardial disease and function. Additionally, our data showed important feedback and communication between the two genes of choice- induced expression of SERCA results in increased expression of cTnI, as well as changes in contractility kinetics. Also, RNAseq showed that induced expression of cTnI resulted in increased expression of genes associated with cardiac calcium handling, including CALML4 and RYR2. Thus, both systems, sarcomeric and calcium handling, seem to be both necessary and sufficient for induction of maturation in hiPSC-CMs.

The maturation status of the hiPSC-CM affects proteins that are involved in calcium handling, contractility, and other physiologic function, as well as proteins that are less directly involved, such as proteins involved in development, metabolism, hypertrophy, transcription, and cell motility and survival. Here, our RNAseq data demonstrated that overexpression of cTnI can lead to global

changes in gene expression in line with increased maturity. This includes a shift towards ventricular cardiac myocytes, as seen by the increased expression of cardiac genes such as MYOM3, MYL9, CALML4, RYR2, and TPM2. Furthermore, there was a shift away from atrial cardiac myocytes, which predominate at earlier developmental time points, shown by the downregulation of Grem2, and from pacemaker cells, shown by the downregulation of HCN4. Interestingly, the data showed evidence of a shift away from skeletal muscle lineages, seen by the downregulation of CASQ1, ACTA1, and ENO3, suggesting that either these genes are important in the early development of cardiac myocytes, or that the current differentiation protocol may not be specific to cardiac myocytes.

Our data demonstrates that overexpression of mature cardiac proteins addresses both the immediate- and long-term physiologic functionality of hiPSC-CMs. With only 2 weeks of overexpression of SERCA2a, we saw changes in the calcium handling and contractile properties of hiPSC-CMs. SERCA-overexpressing hiPSC-CMs calcium transients, while unchanged at baseline, had significantly increased responsivity to isoproterenol, including shortened time to peak and increased chronotropy. On the other hand, SERCA-overexpressing cells at baseline had improved contractile kinetics in terms of time to peak at baseline, as well as in response to isoproterenol, compared to unedited cells. Our results suggest that overexpression of a critical regulator of physiologic performance, especially one involved in the transport of calcium, which is an

important signaling ion, may have additional downstream effects on other calcium handling, sarcomeric, and adrenergic signaling proteins, either through direct activation or inactivation, or through altered transcriptional regulation of those genes.

These results emphasize the importance of studying the function and effects of wild-type cardiac proteins in hiPSC-CMs, especially those involved with a mature cardiac myocyte phenotype. A complete understanding of their effects on maturation and physiology may lead to a better understanding of the basic physiology of hiPSC-CMs, and may allow us to more rigorously study disease-causing mutations in cardiac genes, as well as pathology-inducing environmental conditions. Ultimately, this will lead to a better and more efficient model of heart disease and failure that may lead to improved therapies and clinical benefit.

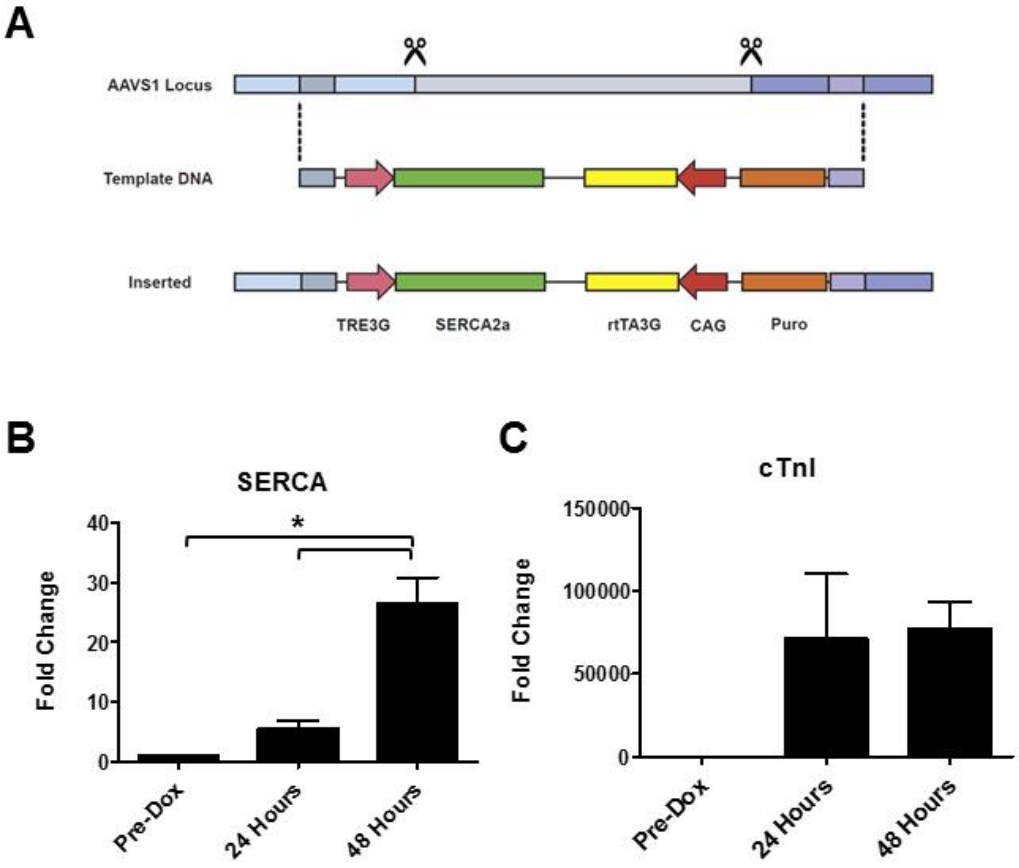


Figure 3.1. A, genome editing schematic. Forward and reverse TALENs make a DSB at the AAVS1 safe harbor site, and template DNA containing the inducible gene of interest is inserted by HDR. B, quantification of SERCA2a mRNA in undifferentiated hiPSCs in response to doxycycline (mean 1.0-fold, 5.57 ± 5.84-fold, 26.49 ± 15.02-fold, $p < 0.05$). C, quantification of cTnI mRNA in undifferentiated hiPSCs in response to doxycycline (mean 1.0-fold, 71221 ± 68248-fold, 77543 ± 68208-fold).

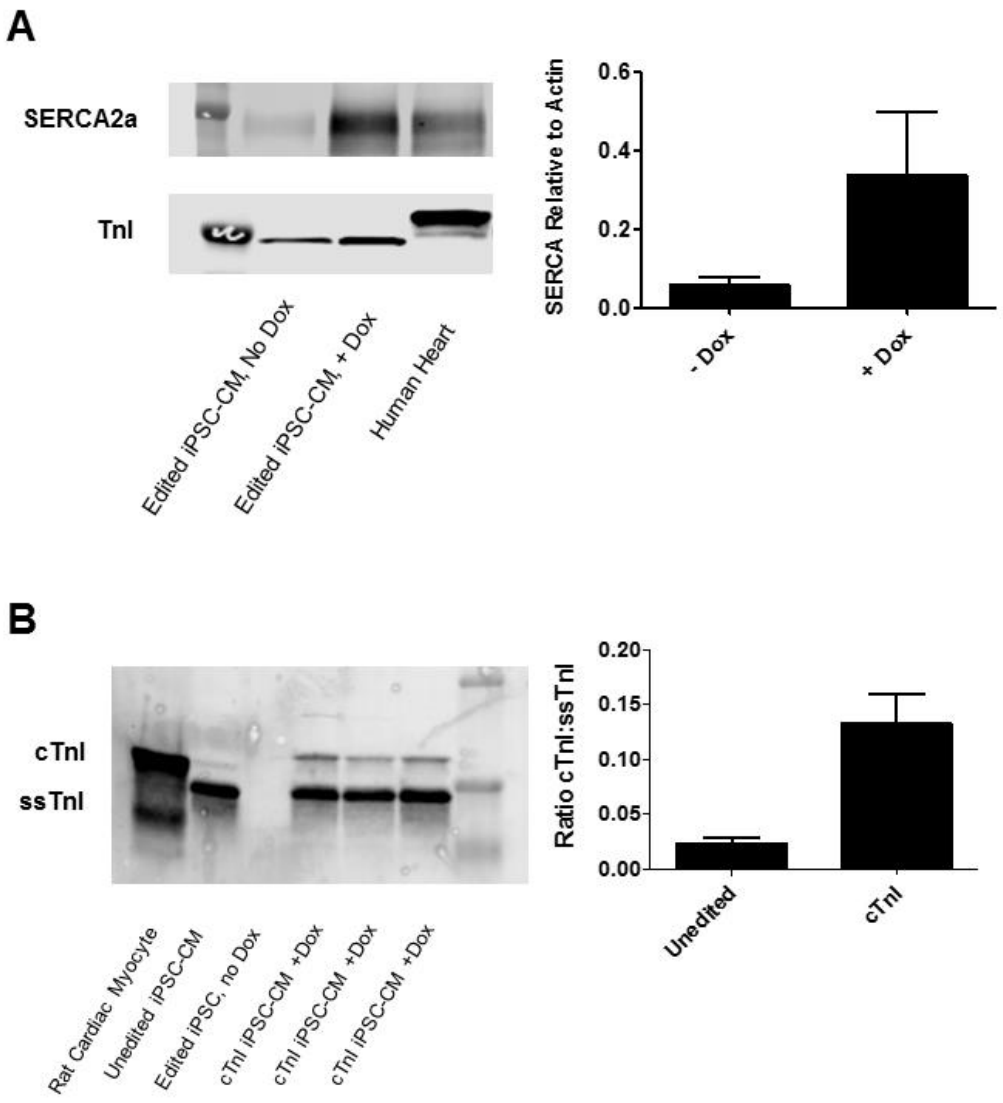


Figure 3.2. A, Western blot and quantification of SERCA2a in SERCA2a-overexpressing cells in response to doxycycline, normalized to sarcomeric actin (mean 0.06 ± 0.09, n = 4, 0.34 ± 0.6, n = 4). B, Western blot and quantification of cTnI in cTnI-overexpressing cells in response to doxycycline, expressed as a ratio of cTnI: ssTnI (mean 0.03 ± 0.02, n = 4, 0.13 ± 0.10, n = 4).

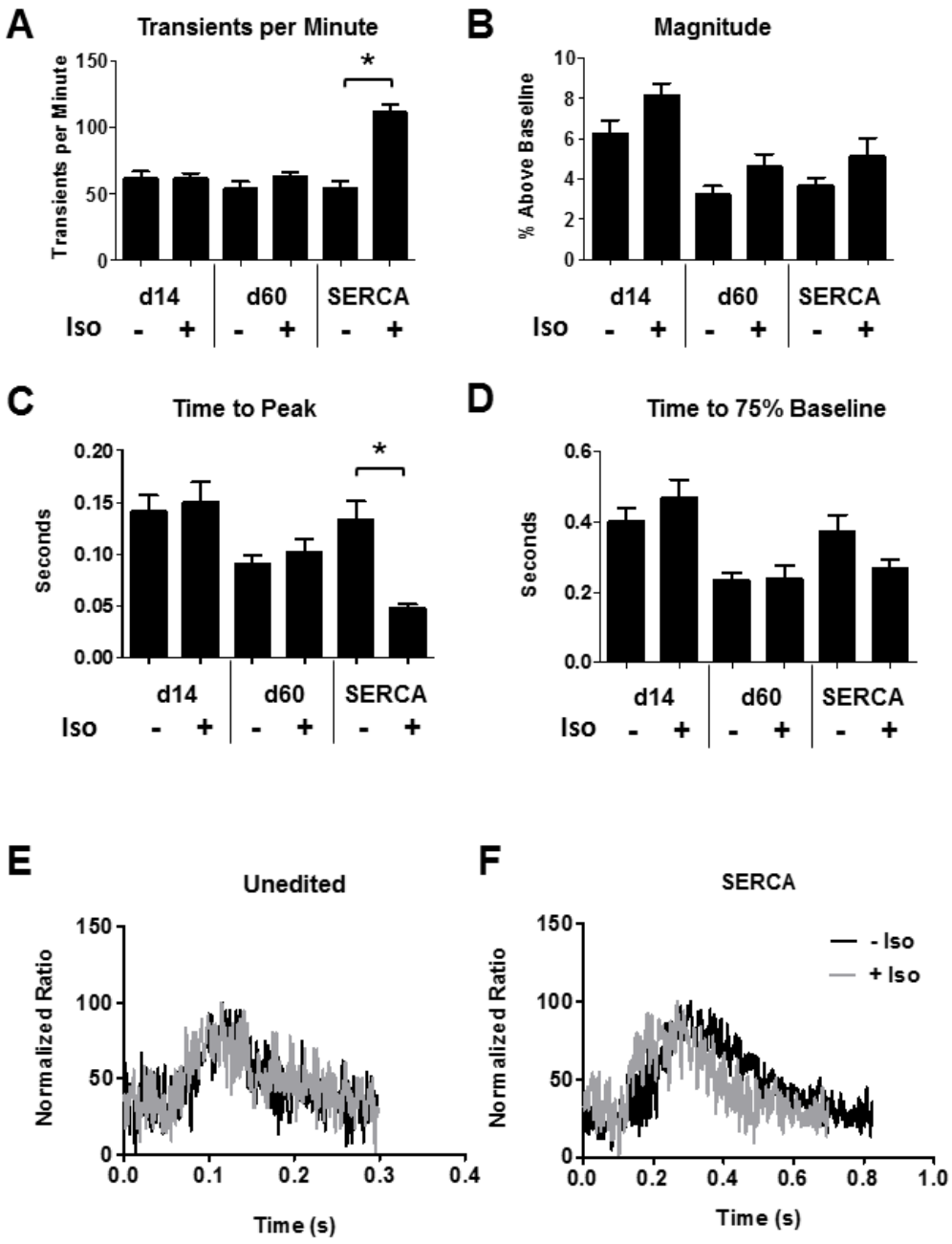


Figure 3.3. Calcium transients in hiPSC-CMs, unedited at day 14 and day 60 post-differentiation, and SERCA-overexpressing hiPSC-CMs at day 60 post-differentiation, at baseline and in response to isoproterenol. A, calcium

transients per minute (mean 61.22 ± 11.89, 61.5 ± 8.08, 53.67 ± 13.03, 62.91 ± 7.44, 54.23 ± 11.60, 111.5 ± 11.2, $p < 0.05$). B, magnitude of transient peak as a percent of baseline ratio (mean 6.26 ± 1.30, 8.15 ± 1.22, 3.20 ± 1.09, 4.61 ± 1.31, 3.47 ± 1.64, 5.11 ± 1.81). C, time to peak of transient from baseline (mean 0.14 ± 0.32, 0.15 ± 0.39, 0.09 ± 0.02, 0.10± 0.03, 0.13 ± 0.04, 0.05 ± 0.008, $p < 0.05$). D, time from transient peak to 75% baseline (mean 0.40 ± 0.08, 0.47 ± 0.10, 0.23 ± 0.05, 0.24 ± 0.07, 0.37 ± 0.10, 0.26 ± 0.04). E, representative traces in unedited cells before and after isoproterenol. F, representative traces in SERCA-overexpressing cells before and after isoproterenol.

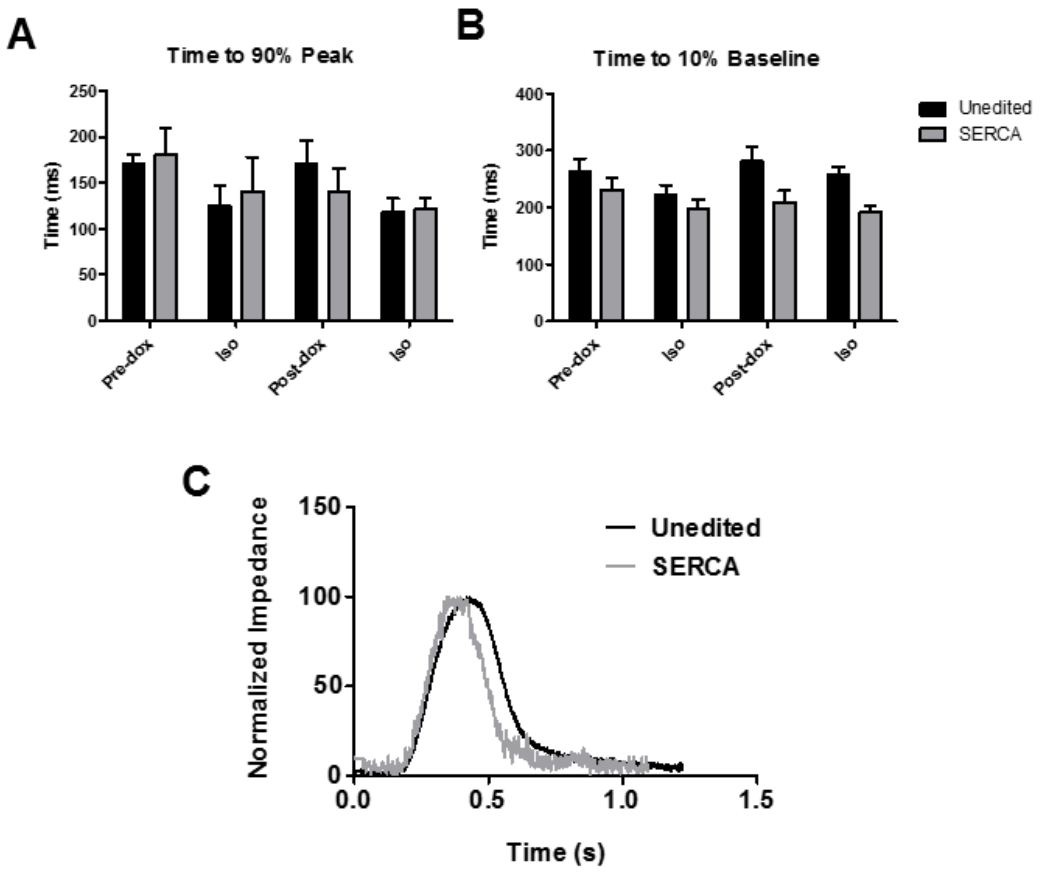


Figure 3.4. Impedance measurements in unedited and SERCA-overexpressing hiPSC-CMs, before and after treatment with doxycycline, with response to isoproterenol. A, time from 10% to 90% peak (mean in Unedited 170.2 ± 35.2, 124.7 ± 69.7, 170.5 ± 80.9, 118.3 ± 47.3, mean in SERCA 180.7 ± 124.7, 141.0 ± 159.0, 140.0 ± 110.2, 121.1 ± 52.7). B, time from 90% to 10% baseline (mean in Unedited 263.5 ± 69.5, 221.4 ± 55.7, 280.8 ± 84.8, 258.8 ± 37.6, mean in SERCA 231.2 ± 90.1, 197.9 ± 65.8, 209.1 ± 86.7, 191.9 ± 48.7). C, representative traces of impedance measurements of unedited and SERCA-overexpressing hiPSC-CMs after treatment with doxycycline, no isoproterenol.

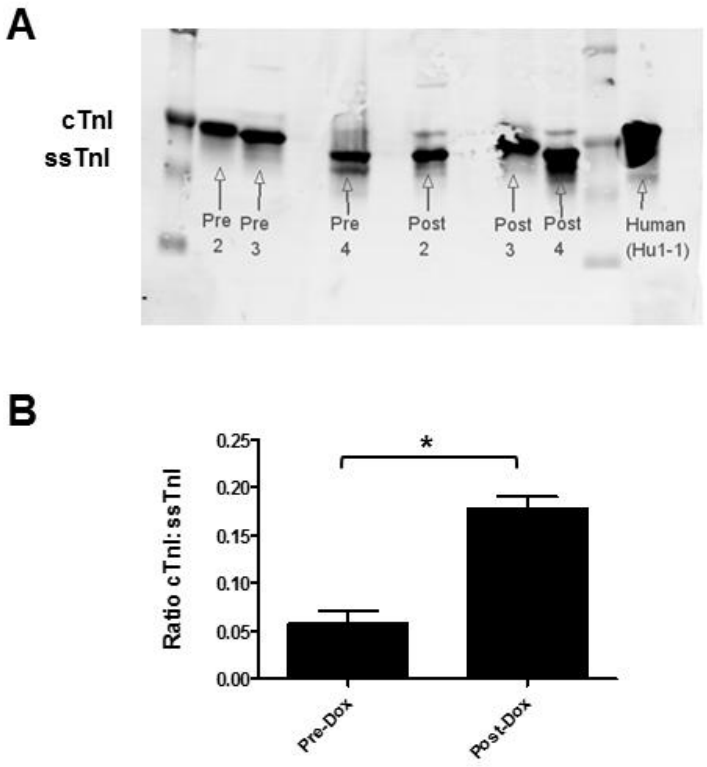


Figure 3.5. A, Western blot for cTnI and ssTnI in SERCA-edited hiPSC-CMs with and without doxycycline. B, quantification of Western blot (mean 0.04 ± 0.05, 0.16 ± 0.03, $p < 0.05$).

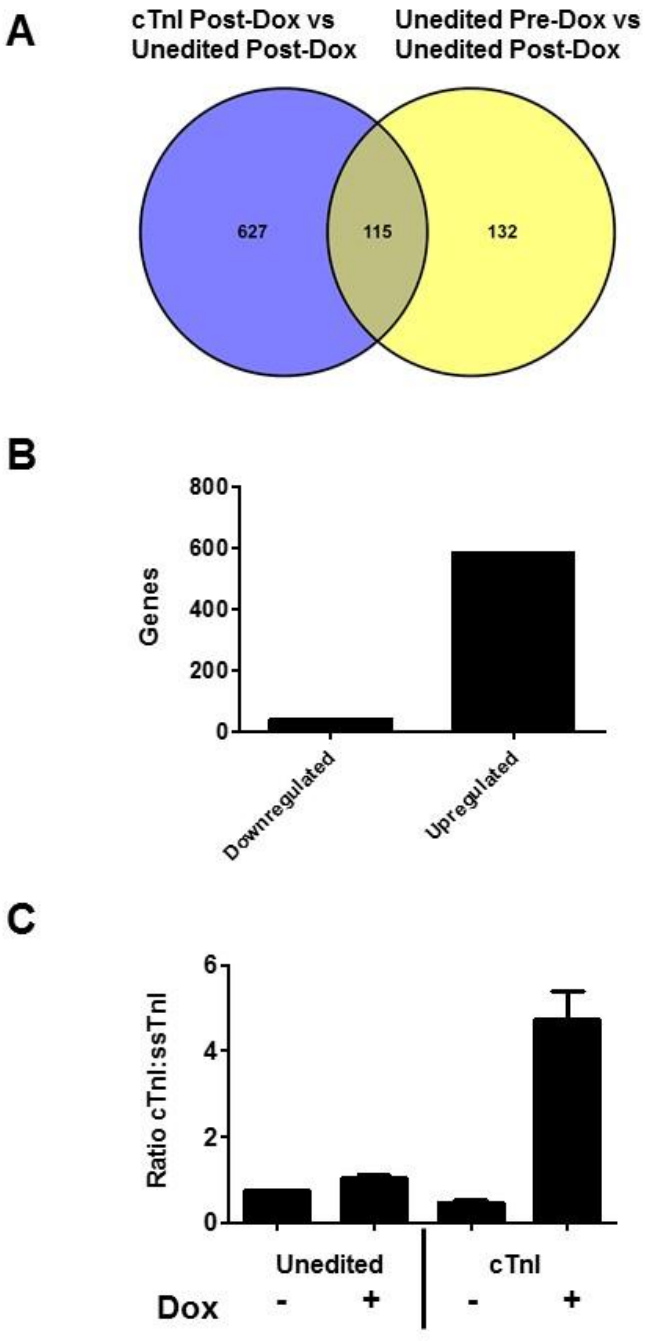
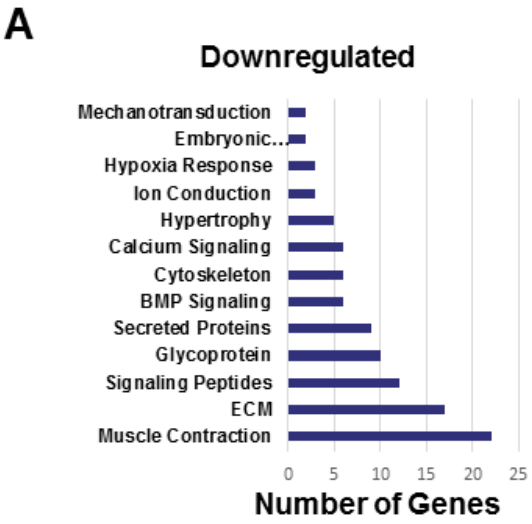
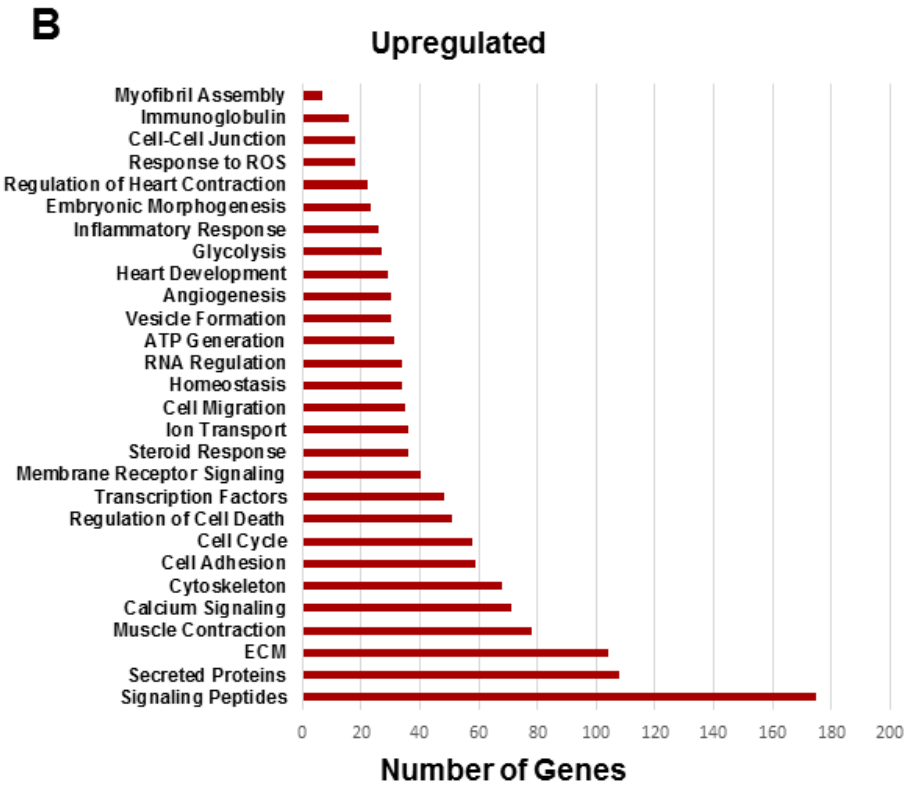


Figure 3.6. A, Venn diagram showing genes with significantly different ($P < 0.05$) read counts in cTnI-overexpressing hiPSC-CMs versus unedited hiPSC-CMs

after treatment with doxycycline. 627 genes in the left-most region were used for the analysis. B, Of the 627 genes, 41 were downregulated and 586 were upregulated.


A
Downregulated
Mechanotransduction
Embryonic...
Hypoxia Response
Ion Conduction
Hypertrophy
Calcium Signaling
Cytoskeleton
BMP Signaling
Secreted Proteins
Glycoprotein
Signaling Peptides
ECM
Muscle Contraction
0 5 10 15 20 25
Number of Genes


B
Upregulated
Myofibril Assembly
Immunoglobulin
Cell-Cell Junction
Response to ROS
Regulation of Heart Contraction
Embryonic Morphogenesis
Inflammatory Response
Glycolysis
Heart Development
Angiogenesis
Vesicle Formation
ATP Generation
RNA Regulation
Homeostasis
Cell Migration
Ion Transport
Steroid Response
Membrane Receptor Signaling
Transcription Factors
Regulation of Cell Death
Cell Cycle
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Calcium Signaling
Muscle Contraction
ECM
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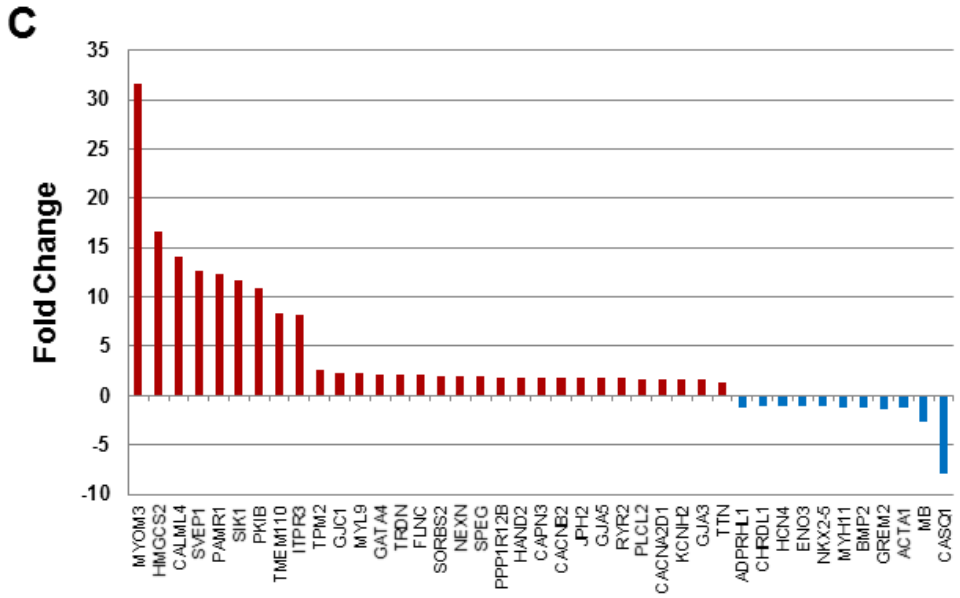

Figure 3.7. A, major pathways involved in downregulated genes. B, major pathways involved in upregulated genes. C, important genes that were upregulated or downregulated.

Chapter 4

General Discussion

Conclusions

The experiments in this thesis have focused heavily on the physiological function of human induced pluripotent stem cell-derived cardiac myocytes. Broadly, this thesis focuses on two major topics- first, the force-producing properties of hiPSC-CMs in their native state, that is, without marked genetic, pharmacologic, electrical, or mechanical modifications, utilizing the state of the cells as they are grown and differentiated according to the current commonly-used protocols[66]. Second, we focused on changes to physiological function of hiPSC-CMs that occur in response to a major, but conceptually simple, modification to the cell, which is the overexpression of a wild-type, developmentally important protein that has an immediate role in either the calcium handling or contractile properties of the myocyte.

The first major project utilized traction force microscopy (TFM), a technique that has been in use for several years now to examine contractile properties of various cell types including fibroblasts[145], keratocytes[146], osteoblasts[147], and neonatal rat ventricular myocytes[148]. TFM allows us to optically determine the amount of force a cell produces against a substrate of known elastic modulus. We have implemented TFM here to determine the peak force of single isolated hiPSC-CMs, paced at a physiologic frequency of 1 Hz,

against physiologic substrate stiffness of between 2.5 to 9.3 kPa[244], normalized to cell area, based on the finding that peak force positively correlates with cell size.

In this part of the project, the emphasis was on determining the contractile function of hiPSC-CMs that had been differentiated according to the most commonly used protocol by current labs[66], a protocol that is fairly straightforward and simple, and that efficiently produces high numbers of cardiac myocytes. This was important to us, because we had felt that while some researchers had characterized aspects of their function[79,112], a fundamental understanding of force development, which is the central physiologic function underlying the very existence of myocytes, was lacking. Force output in hiPSC-CMs should be established before extrapolating results of more complex experiments, such as disease modeling by introduction of genetic mutations, to human physiology. Furthermore, we wished for other researchers to be able to use the results of our experiments to guide their own work.

These experiments provided us with evidence of physiology that was mostly in line with what we would expect, with some important caveats. First, that force production correlates with cell size- this is unsurprising, given the structure-function of a cardiac myocyte. However, unlike adult cardiac myocytes, we did not find force to correlate with cell width. We found that force production increased as hiPSC-CMs mature in culture. This relationship was evident to a

point where they plateau no matter whether they are continued to be kept in culture, and force normalized to cell area never reaches that of a NRVMs.

We found that hiPSC-CMs produce less force on stiffer substrates, which is in line with what some groups have found with hiPSC-CMs[161], but different than others[160]. A critical finding of this project was that the extracellular calcium concentration is crucial for cardiac myocyte development, with hiPSC-CMs that are grown in physiologic levels of extracellular calcium producing more force than those grown in RPMI + B27, which is the accepted growth media for hiPSC-CMs[66].

The second major project involved overexpression of critical cardiac proteins in hiPSC-CMs to modulate physiology and maturation. We chose two proteins that play important roles in the physiology and development of cardiac myocytes, and that would be likely to have significant effects on hiPSC-CM function. SERCA2a, responsible for removal of calcium from the cytoplasm during diastole, and for loading the SR with calcium[104], has effects on calcium handling, cell contractility, adrenergic responsivity, and cardiac myocyte development[188]. Next, we targeted cTnI, which is the adult isoform of troponin I and part of the thin filament. It is exclusively expressed in adult cardiac myocytes, and is partially responsible for the sarcomere's ability to accelerate contractility kinetics in response to adrenergic signaling[140].

The driving idea behind this part of the project was that we could use these proteins to modulate both the immediate physiology of the cell, as well as

the developmental status of the cell. With both genetically-engineered cell lines, those that had been edited to contain an inducible exogenous SERCA2a gene, and those that had been edited to contain an inducible exogenous cTnI gene, we found both cases to be true. Using a fluorescent calcium reporter, we found evidence that overexpression of SERCA led to enhanced calcium transient activity in the face of adrenergic stimulation (isoproterenol). Furthermore, these hiPSC-CMs had increased spontaneous transient rate and faster time to peak after isoproterenol challenge. Using an impedance measuring system, we were able to track contractility of syncytia of cells at baseline and in response to isoproterenol, and found that although both edited and unedited cells had faster contraction in response to isoproterenol, overexpression of SERCA led to shortened contraction times at baseline, as well as further response to isoproterenol.

Western blot of SERCA overexpressing cells showed increased expression of key markers associated with a switch to maturity, including cTnI, indicating cell-wide changes in gene expression involving genes not directly involved in calcium handling. Similarly, RNAseq analysis of cTnI overexpressing cells showed global changes in gene expression in edited cells treated with doxycycline compared to unedited cells treated with doxycycline for a month. Changes included upregulation of genes important to the maturation and development of cardiac myocytes, including cardiac myosin heavy chain, and downregulation of genes involved in earlier stages of cardiac myocyte

development, such as Gremlin, which is important for atrial myocyte differentiation but not ventricular[270].

This series of experiments, progressing from more descriptive analysis of baseline cellular function to advanced genomic engineering, depict hiPSC-CMs as an innovative and powerful research tool. Here, with intact physiologic and genomic function that in many ways resemble mature cardiac myocytes, we find evidence that hiPSC-CMs are able to produce force and contract with vigor, which is the ultimate purpose of a cardiac myocyte. Additionally, these hiPSC-CMs are able to appropriately respond to adrenergic stimulation, as seen by increased chronotropy and accelerated kinetics.

Alongside the similarities, these experiments reveal some significant insights that we believe should be taken into consideration by researchers in future experiments. For example, force production never quite reaches the same level as an NRVMs. Thus, any experiments involving mutations that decrease contractility should take this into account. In terms of gene and protein expression, we here confirm previous findings by us and other groups[112] that immature isoforms of certain proteins, such as troponin I, are predominantly expressed versus mature isoforms. Finally, the understanding that the standard differentiation and growth media used for hiPSC-CMs, RPMI + B27 supplement, has an extremely low calcium concentration, and that this significantly impacts the development of hiPSC-CMs compared to media with physiologic calcium

levels. This finding should be an important technical consideration for future experiments.

The most important takeaway from these experiments, though, is the power of hiPSC-CMs as a genetically malleable system for human cardiac muscle research. The immaturity of hiPSC-CMs, and the fact that, only several weeks previously, they were derived from pluripotent cells, is a boon to the field in that their genomes are amenable to editing by genome editing technologies, such as TALENs, as shown here. Furthermore, because we have access to and control over the developmental environment of the cells the entire way from pluripotency to terminally differentiated cardiac myocyte, there are seemingly endless possible ways of manipulating hiPSC-CMs in order to study factors that are important to cardiac development.

Genome editing provides a precise and permanent way of introducing genetic modifications to the cell. Genome editing can be used to introduce mutations, or correct mutations in an already-mutated cell line. It can be used to knock out a gene that may be developmentally important, but that may result in embryonic lethality, limiting its ability to be studied in vivo. It can also be used to overexpress a gene of interest that may have significant impacts on myocyte function. Here, we have used genome editing to permanently introduce an inducible gene expression system. Our data demonstrate that genome editing can be used effectively and efficiently to implement hiPSC-CMs as a tool for

studying physiologically relevant genes and their role in human cardiac muscle function and development.

Clinical Implications

hiPSC-CMs have several potential roles in clinical medicine. First, hiPSC-CMs can be used as a model of disease. Second, hiPSC-CMs can be used for drug discovery. Third, hiPSC-CMs can be used as a transplantable therapy. As a model system, as mentioned earlier, there have been attempts to utilize hiPSC-CMs for drug testing and drug discovery. For example, they have been proposed as an alternative to other cell types overexpressing the hERG channel. There have been some encouraging results, including validation of several previously-validated drugs[91]. hiPSC-CMs are an attractive tool for drug discovery because, unlike other cell types, they express not only hERG, but other ion channels that may be potentially arrhythmogenic[90]. Furthermore, hiPSC-CMs have action potentials, which are a sum of currents through several ion channels, rather than a single channel, and which can be measured using electrical recording.

However, hiPSC-CMs can have considerable variability of action potential waveform, presumably from differential contribution of ion channels[253] and from reduced currents compared to adult cardiac myocytes, such as the inwardly rectifying I_{K1} current, and spontaneous activity due to unopposed funny current I_f[271]. To improve hiPSC-CMs as a model of drug discovery, it may be beneficial to utilize genome engineering to overexpress channels that are poorly expressed,

such as IK1, or to use siRNAs to reduce If. Also, because intracellular calcium concentrations[75] have effects on membrane currents, such as by influencing the NCX, or by signaling through calmodulin, overexpression of SERCA2a may have important effects on the action potential, and may result in an action potential that more closely resembles that of an adult cardiac myocyte.

As a model of cardiac disease, hiPSC-CMs have shown the potential to be used to study mechanisms of disease, as well as potential therapeutic treatments. Here, several examples have already been mentioned of researchers that have used hiPSC-CMs derived from patients with various cardiovascular diseases, such as long QT[75], Timothy syndrome[81], and catecholaminergic polymorphic ventricular tachycardia[82]. While most of these studies have so far served to corroborate findings found in mouse models of the disease or in human patients, some of these studies have discovered additional findings that may be relevant to disease mechanisms. Thus, the CPVT study was able to show that dantrolene, shown to have beneficial effects in mouse model of CPVT[272], also has positive effects on the phenotype of the derived hiPSC-CMs.

Our genome editing and gene induction data highlights the potential of using hiPSC-CMs as a model of disease. While it can be convenient to derive pluripotent cells from patients that are known to have a disease-causing mutation, it is not possible to do so for every disease. It is also quite difficult to separate the effects of the mutation from the effects of potentially confounding factors, such as SNPs present in other genes, although these biases may be

mitigated by comparing cells from patients with a disease against cells from their siblings. However, because hiPSC-CMs can be modified according to the desires of the researcher, mutations that are desirable to study, but may not be found in patients, could be introduced precisely into the genome of the pluripotent stem cell. Furthermore, entire genes could be transiently upregulated or downregulated at any point in the development of the cell, such as at the beginning of differentiation, in order to model congenital diseases and abnormalities, or farther along in the differentiation process, in order to model diseases in more mature cardiac cells.

However, as has been repeatedly stated above, our data shows that hiPSC-CMs are still quite immature regardless of how long the cells have been in culture, and this affects the quality of data the comes out of physiologic studies. Studies on the effects of mutated genes in the context of their effects on contractility and calcium handling give us only hypothetical scenarios of how those mutations might affect an adult cell. To counteract this, it may be useful to upregulate a gene associated with maturity, such as SERCA2a or cTnI, and then examine the effects of the mutation or disease-causing gene. In this way, results from those experiments will be more relevant to adult cardiac disease, especially in the study of congestive heart failure, which is one of the most critical health problems facing the world today[2].

Finally, hiPSC-CMs may prove to be an important and powerful therapeutic agent in heart failure, ischemic heart disease, and cardiac infarction.

It has been proposed that hiPSC-CMs injected into a diseased myocardium may provide mechanical support, electrical support, metabolic support, or paracrine signaling support to the native cardiomyocytes[30]. The desired maturity of the transplanted cells may depend on the desired outcome. For example, an immature hiPSC-CM may be more likely to promote cardiac regeneration in the native tissue due to secretion of developmentally important growth factors or secretion of immature extracellular matrix proteins. On the other hand, a more mature cardiac myocyte may be more likely to provide structural support through increased force development, or increased electrophysiological support through mature calcium handling or action potentials. Furthermore, our modified cells show a greatly enhanced response to isoproterenol. This, along with mature calcium handling and ionic currents, may make mature transplanted cells less prone to arrhythmia, and may make them less prone to causing re-entrant circuit arrhythmias in the native heart[64].

Future Directions

This project, while a substantial effort, barely scratches the surface of possibilities of what we can learn from hiPSC-CMs. Utilizing traction force microscopy, we have shown that it is possible to precisely measure contractility by measuring the peak force production of a single, isolated hiPSC-CM. We have begun to investigate factors that affect force production, such as length of differentiation, substrate stiffness, cell geometry, and extracellular calcium

concentration. This is a powerful tool, and has the potential to be used to explore additional force-modulating factors. These could be divided into elements that are present in the embryo, factors that are present in the adult heart, external factors such as drugs and chemical modulators, and individual genes, along with their isoforms and splice variants.

As hiPSC-CMs most closely resemble embryonic or immature cardiac myocytes, factors that affect an embryonic cardiac myocyte's ability to produce force may be examined using traction force microscopy. For example, it has been shown that the stiffness of the extracellular environment that the cell senses affects things such as development of action potentials[273], and likely has an impact on the amount of developed force produced by a myocyte. Factors that affect hypertrophy, such as insulin and IGF[274], as well as other maternal and fetal hormones, may affect contractility, as may growth factors that affect cardiac patterning and growth[55]. Electrical stimulation has been shown to have effects on hiPSC-CM development[275] as well. All of the above treatments are easily implementable in hiPSC-CM culture and may be valuable sources of improved maturity in terms of contractility and force.

In the adult heart, many of the same factors that affect the embryonic heart are also present, except that in this case, alterations in these environmental factors may cause improved function, as in an exercised heart, or decreased function, as in a failing heart. Extracellular stiffness "felt" by the myocytes has already been noted to have a key role on myocyte function, as failing hearts are

significantly stiffer than non-failing hearts[244]. Electrical abnormalities, as arrhythmias, are strongly linked to heart failure, although it is currently hard to say whether arrhythmias have as much effect on contractility as cardiac myocyte derangement has on arrhythmogenesis[276]; hiPSC-CMs may serve to elucidate this. Hormones, such as insulin[277] or cortisol[278], have been shown to have crucial effects on cardiac function, and may be easily introduced into hiPSC-CM culture to probe their effects.

As mentioned above, a particularly promising medical application of hiPSC-CMs is in drug discovery and testing. There are numerous drugs either available clinically or tested experimentally that have been shown to modulate cardiac myocyte contractility. Drugs such as digoxin, which increases intracellular calcium[279]; levosimendan, which increases calcium sensitivity[280]; and omecamtiv mecarbil, which activates myosin[281], are all considered either full or partial positive inotropes, and might be expected to increase force production. These applications highlight hiPSC-CMs as a potential template for drug discovery. Conversely, negative inotropes, such as milrinone, which is a PDE3 inhibitor[283], would be expected to have negative effects on force production.

We have shown that genetic engineering and overexpression of physiologically important genes such as SERCA2a and cTnI have significant effects on contractility and gene expression. An important next step is to determine whether cells that overexpress either of these genes also have altered force development. Genome engineering and gene transfection can be used to

over- or under-express a wide variety of genes. These might fall into the category of sarcomeric proteins that have direct effects on force development, such as myosin heavy chain and myosin light chain isoforms[283-284] or myosin binding protein C[285], or proteins that modify the function of sarcomeric proteins, including the adrenergic signaling pathway, including adrenergic receptors and protein kinase A[286]. Other potentially force-modifying genes include those involved in hypertrophy, such as Akt[194], or genes involved in ion handling, such as the sodium potassium ATPase or dihydropyridine receptor[287].

Additionally, TFM can and should be modified to keep up with current technological developments in the field. For example, the assay used in its current form in this thesis suffers from the classical imaging trade-off between resolution and image capture speed- because the PIV algorithm relies on high-resolution images of small 0.2 μm fluorescent beads with a high signal-to-noise ratio, images were captured at a rate of 3-4 frames per second. Because the time to peak and time to relaxation of an isolated contracting myocyte are on the order of hundreds of milliseconds[267], the absolute peak contraction may be missed by the assay. Utilization of a faster microscope would allow peak to be captured precisely, and may allow contractility kinetics to be explored by the assay as well, assuming images are captured at constant intervals. High resolution microscopy is improving to the point where this may be possible in the near future[288]. Other improvements may come in the form of improved microcontact printing or hydrogel formation that may reduce the amount of labor involved in each assay,

or may improve survivability of hiPSC-CMs during the process of dissociation and replating onto hydrogels.

In the second part of the thesis, we have demonstrated the enormous potential of hiPSCs and hiPSC-CMs to be modified and manipulated in order to modulate maturity and manage physiologic function. By introducing two genes, cTnI and SERCA2a, using an inducible promoter, we have shown proof-of-principle of real-time modulation of physiology. Knowing this, one can envision and almost unlimited number of experiments using genome editing to modulate function and maturity in hiPSC-CMs. Furthermore, having a permanently-inserted genetic system would allow us to examine the effects of gene induction alongside other techniques that may induce maturity, such as electrical stimulation or cyclical stretch, with the hope that they would work in synchrony to produce adult-like cardiac myocytes.

In this thesis, we used an inducible promoter, which has the benefit of allowing us to turn on in a time-dependent manner and when we believe it will have the most impact. For our purposes, this was at 42 days after the start of differentiation, a time point where the cells have reached some level of maturity compared to earlier time points, as based on traction force microscopy, but where the cells are still far from behaving like mature cardiac myocytes. However, it could be particularly useful to induce maturity genes at various time points, such as early in development, later in culture, or even before the differentiation process began, while the cells were undifferentiated pluripotent

stem cells. Furthermore, removing induction by removing doxycycline leading to a decrease in expression of the exogenous gene, would inform whether the effects are transient, lasting only as long as the protein is present, or whether it leads to permanent changes in the cell, indicating a more terminally mature cell type.

Tissue-specific promoters or developmental stage-specific promoters would be useful to study the effects of a gene without having to continuously expose the cell to doxycycline, which may potentially have unexpected effects on the cell, and which would be difficult to utilize in cells that were transplanted into a patient. Cardiac specific promoters, though, could be driven by a promoter such as the β-MHC promoter, which is present in both immature and mature cardiac myocytes[185]. The promoter could be used to drive additional genes associated with maturity, such as myosin light chain 2v[172], or it could be used to drive expression of siRNAs that inhibit translation of genes associated with immaturity, such as ANP, skeletal actin, or GLUT[178]. Alternatively, immature transcription factors and promoters could be used to induce expression of a more mature gene; for example, the transcription factor Nkx2.5, which is highly expressed in developing embryonic cardiac myocytes[289], could be used to drive expression of cTnI or α-MHC. One could envision a series of genes, each slightly more mature than the previous, and driven by a slightly less-mature promoter, leading to a chain reaction of gene expression that closely recapitulates cardiac development in the embryo. This might allow for testing that important

checkpoints along the path of differentiation from pluripotent cell to adult cardiomyocyte are met.

Physiologic function should be rigorously assessed after each modification, utilizing TFM or similar assays, in order to ensure that results from each experiment are directly comparable to each other. We have outlined several assays, including calcium handling, traction force microscopy, and impedance measurements for contractility, that are reliable and rigorous, and that test three essential functions of a myocyte: calcium, force, and kinetics. Other assays, including patch clamp and extracellular field potential, should be considered. Additionally, assays that measure the force of a syncytium of cells, rather than a single cell, should be used, as cells may behave differently in a syncytium than they do as isolated cells[235].

While we have used RNA sequencing, which is a powerful method of examining the complete set of genes expressed by the cell, to probe the developmental state and maturity of our cells, at the very least, important markers of maturity should be looked for by Western blot. Immunofluorescence or electron microscopy could also be used to study the structure of the cells. Immunofluorescence can provide indications of cell size and hypertrophy, as well as myofibril number and organization[290]. Electron microscopy can provide information about the ultrastructure of sarcoplasmic reticulum and T-tubules[291], mitochondria[292], and sarcomeres, including sarcomere size and shear angle[293].

Finally, mutations and genetic causes of disease should be studied and can be highly informative regarding human disease, once it is understood what role the wild-type protein plays in the physiology of an hiPSC-CM. For example, once we understand how the composition of the troponin complex in hiPSC-CMs affects the cell's contractility and adrenergic response, then it is appropriate to perform experiments using mutations in troponin T or troponin C. It may be that it is necessary to overexpress cTnI to a sufficient level before introducing mutations into other troponins, in order to be able to extrapolate results of those experiments to diseases in the adult or even childhood heart. It may turn out that modification of a wild-type protein, including overexpression of a mature protein, has no effect on the physiology of the cell. In this case, it would be reasonable to draw conclusions from experiments using mutations or modifications of that protein that could be relevant to human disease or modeling.

Taken together, this thesis demonstrates the vast significance of stem cells and stem cell-derived cardiac myocytes in the future of cardiovascular medicine. We have demonstrated that, while stem cell-derived cardiac myocytes represent an immature state, they have enormous potential for both scientific discovery and for therapeutic use in cardiovascular disease. We have shown that physiologically-relevant assays are available to measure important functional parameters, including force development, which are sensitive to physiologic stimuli including substrate stiffness and extracellular calcium concentration. A major advantage of hiPSC-CMs over mouse models is that they express human

proteins, which can be examined in the context of a human cell. A major advantage of hiPSC-CMs over primary human cardiac myocytes, though, is the incredible genetic malleability of the cells. Here we have shown precise genetic control over the cells as they develop from pluripotent stem cells to a terminally differentiated cell type. We have been able to examine the role of wild-type, physiologically important proteins on both the short-term function of the cell, as well as the long-term gene expression profiles. Human stem cell-derived cardiac myocytes are therefore a powerful tool for the future of cardiovascular health.

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