Cardiomyocyte Differentiation With Cyclic Mechanical Strain

by

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Abstract

The creation of cardiomyocytes from pluripotent stem cells has the potential to revolutionize the treatment of heart disease, the discovery and testing of drugs, and our understanding of human physiology. Thus far, differentiation protocols have mainly focused on recapitulating the biochemical signalling events of cardiac organogenesis, and the resulting cardiomyocytes exhibit a fetal-like phenotype. We postulated that cyclic mechanical strain can mimic the mechanical environment of the developing heart, and, when applied in conjunction to biochemical differentiation protocols, can increase the efficiency of differentiation and the maturity of the resulting cells. Using an induced pluripotent stem cell line derived from human fibroblasts, we derived spontaneously contracting cardiomyocytes via treatment with activin A and bone morphogenetic protein 4 (BMP4). We observed that differentiation of induced pluripotent stem cells to cardiomyocytes is sensitive to cyclic strain, with the application of 5% continuous cyclic strain at 1Hz having the effect of inhibiting spontaneous contractions and disrupting sarcomere formation.
Preface

This thesis is original, unpublished, independent work by the author, E.J. Zhao.
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Glossary

5'-UTR  5' untranslated region, a segment of DNA that is not used to code for amino acids, upstream of the actual amino acid sequence

αMHC  α myosin heavy chain

ATP  adenosine triphosphate

bFGF  basic fibroblast growth factor

βMHC  β myosin heavy chain

BMP  bone morphogenetic protein, a group of growth factors that play roles in the patterning of tissues during development

BMP4  bone morphogenetic protein 4

BSA  bovine serum albumin

CAT  chloramphenicol acetyltransferase

CER1  Cerberus

cTnT  cardiac troponin T

CMV  human cytomegalovirus

DAPI  4',6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to DNA

DC  direct current

DIC  differential interference contrast

DKK1  Dickkopf homologue 1

DMEM  Dulbecco’s Modified Eagle’s Medium

ECM  extracellular matrix
EDTA ethylenediaminetetraacetic acid, used as a chelating agent in tissue culture
EGF epidermal growth factor
EOMES eomesodermin, also known as T-box brain protein 2 (TBR2)
ESRRG estrogen-related receptor gamma
ETS2 homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)
EGFP enhanced green fluorescent protein
FBS fetal bovine serum, a common adjuvant to tissue culture media
FEA finite element analysis
FGF fibroblast growth factor
FGF2 fibroblast growth factor 2 (basic)
FITC fluoresceine isothiocyanate, a fluorescent molecule with emission and excitation wavelengths similar to EGFP
GATA a family of transcription factors named for their ability to bind the DNA sequence “GATA”
GATA4 GATA binding protein 4
GFP green fluorescent protein
GMT GATA4, MEF2c, and TBX5, the three factors used by Ieda et al. [34] to transdifferentiate fibroblasts to cardiomyocyte-like cells
HAND heart- and neural crest derivatives-expressed, a family of genes that regulate ventricular development
HAND1 heart- and neural crest derivatives-expressed protein 1
HAND2 heart- and neural crest derivatives-expressed protein 2
HCN4 potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4
hERG human ether-à-go-go-related gene, a gene that encodes a subunit of the ion channel whose inhibition can lead to long QT syndrome
hESC human embryonic stem cell
**HSVTK** herpes simplex virus thymidine kinase

**IGF-1** insulin-like growth factor 1

**iPSC** induced pluripotent stem cell

**IRES** internal ribosomal entry site

**JAK** Janus kinase, a family of proteins with the ability to phosphorylate and activate downstream proteins

**Kan** kanamycin resistance

**KLF4** Kruppel-like factor 4 (gut), one of the four Yamanaka factors used to induce pluripotency in somatic cells

**LEFTY1** left-right determination factor 1

**LIN28** Lin-28 homolog A

**MACSAT** magnetically actuated cellular strain assessment tool

**MCS** multiple cloning site

**MEF2** myocyte enhancer factor-2, a family of transcription factors known to control cellular differentiation

**MEF2C** myocyte enhancer factor 2C

**mESC** mouse embryonic stem cell

**MESP** mesoderm posterior, a family of transcription factors known to initiate the network of cardiac regulatory network of transcription factors

**MESP1** mesoderm posterior 1

**MESP2** mesoderm posterior 2

**MHC** myosin heavy chain

**MOSFET** metal-oxide-semiconductor field-effect transistor

**MYC** v-myc avian myelocytomatosis viral oncogene homolog, one of the four Yamanaka factors used to induce pluripotency in somatic cells (also known as c-Myc)

**MYOCD** myocardin, a nuclear protein known to play a role in cardiogenesis and differentiation of the smooth muscle cell lineage
**MYOD1**  myogenic differentiation 1, the master regulatory of skeletal muscle differentiation

**NANOG**  NANOG, a transcription factor expressed by pluripotent stem cells

**Neo**  neomycin resistance

**NK2**  A family of transcription factors involved in organogenesis

**NKX2-5**  NK2 homeobox 5, a transcription factor known to play a role in heart development

**OCT4**  octamer-binding transcription factor 4, a transcription factor expressed by pluripotent stem cells and one of the four Yamanaka factors used to induce pluripotency in somatic cells. Also known as OCT3, OCT3/4, and POU5F1 (POU class 5 homeobox 1)

**PBS**  phosphate-buffered saline

**PCR**  polymerase chain reaction

**PDGF**  platelet-derived growth factor

**PDMS**  polydimethylsiloxane

**PI**  propidium iodide, a fluorescent molecule

**PKA C-α**  protein kinase A, catalytic subunit α

**ROCK**  rho-associated, coiled-coil containing protein kinase

**SMARCD3**  SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily D, Member 3

**SOX2**  SRY (sex determining region Y)-box 2, one of the four Yamanaka factors used to induce pluripotency in somatic cells, known to regulate embryonic development maintain stem cells in the central nervous system

**SRF**  serum response factor

**SV40**  simian virus 40, a polyoma virus capable of infecting monkeys and humans

**RT-PCR**  reverse-transcriptase polymerase chain reaction

**T**  brachyury homolog (mouse)

**TBX**  T-box, a family of genes that possess the T-box DNA-binding domain

**TBX5**  T-box 5
TGF-β  transforming growth factor β
THY1  thymocyte differentiation antigen 1
ZFPM2  zinc finger protein, FOG Family Member 2
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Chapter 1

Introduction

1.1 Cardiac regeneration

Why can some animals regenerate entire lost limbs while humans cannot hope to regrow even a fingertip? Why do some human tissues – liver, for example – possess drastically higher regenerative capacity than others? What if we could heal heart damage by growing new, functional heart tissue? If we could better model genetic cardiomyopathies by growing a patient’s cardiomyocytes \textit{in vitro}, could we find new, more effective treatments? How much time and money could we save if we had phenotypically mature cardiomyocytes on which to test candidate drugs for cardiotoxicity?

These are the questions that drove me to complete the work presented herein.

1.2 Cardiac development

To attempt cardiac regeneration, we must first have a thorough understanding of cardiac development during embryogenesis and fetal development. Let us embark upon a survey of embryology, with emphasis on the pre-cardiac mesoderm [53, 71].

The fertilized egg, known as the zygote, is a single cell that undergoes mitotic division without cell growth to produce a ball of cells known as the morula. In a process known as compaction, the blastomeres form a spherical shell, known as the trophoblast, with a lump on the inside, known as the inner cell mass (Figure 1.1). The trophoblast then forms a single-layered epithelium and the sphere fills with fluid. The trophoblast eventually develops into the fetal portion of the placenta, while the inner cell mass gives rise to the embryo. The embryo at this stage is known as the blastocyst. A natural question emerges: how do the uniform clump of cells that comprise the morula know which to become, the trophoblast or the inner cell mass? Experimenter have shown that transcription factors play a role in the specification of the fate of blastomeres. In the uniform morula, all cells express octamer-binding transcription factor 4 (OCT4) and NANOG, and indeed the expression of these transcription factors is used as a marker of the pluripotency of stem cells. However, OCT4
Figure 1.1: A schematic of human zygote development from ovulation to the hatched blastocyst. A: Oocyte with polar body surrounded by zona pellucida (grey border). B: Two-cell stage. C: Four-cell stage. D: Eight-cell stage. E: Sixteen-cell stage (morula). F: Blastocyst with inner cell mass (pink) surrounded by trophoblast (blue).

and NANOG are downregulated in the cells that make up the trophoblast, while it is maintained in the inner cell mass.

In the next stage of development, the inner cell mass (also known as embryoblast) splits into 2 layers: the epiblast and the hypoblast (also known as primitive endoderm). Together these two layers comprise the bilaminar embryonic disc, which will later give rise to the germ layers of the embryo. Almost simultaneously, the amniotic cavity begins to form in the epiblast, lined with a layer of cells derived from the epiblast (Figure 1.2). Next, the two-layered embryonic disc gains a third layer in the process of gastrulation, giving rise to the three germ layers – endoderm, mesoderm, and ectoderm. Correct morphogenesis is achieved through tight spatiotemporal regulation of signalling molecules such as bone morphogenetic protein (BMP)s, fibroblast growth factor (FGF)s, and Wnt proteins. Researchers have used these signalling proteins to great effect in the *in vitro* differentiation
Figure 1.2: Soon after the blastocyst implants into the endometrium, the inner cell mass forms a circular, two-layered disc called the embryonic disc. The two types of cells that comprise the two layers are known as epiblasts and hypoblasts.

The morphological marker of the beginning of gastrulation is the appearance of the so-called “primitive streak,” which originates as a thickened ridge of cells in the epiblast (Figure 1.3). This ridge forms with one end (the caudal end) abutting the edge of the embryonic disc and the other end (the cranial end) extending towards the centre of the embryonic disc. The appearance of the primitive streak breaks the symmetry of the embryonic disc and establishes the embryo’s craniocaudal axis, its dorsal and ventral surfaces, and its right and left sides. The process of cardiomyogenesis from the mesoderm has been most thoroughly studied in the mouse, so we now move to the mouse model for a closer view of the signalling events that direct tissue specification. Mouse embryology uses the convention of prefixing the number of days since ovulation with “E” to indicate the age of the conceptus. Thus an oocyte at ovulation is at E0, and fertilization usually occurs at E0.5.

At E4.5, the mouse embryo has entered the late blastocyst stage, but the spatial arrangement of cell groups still resembles that of the human embryo. There is an outer layer of cells (the trophec-
Figure 1.3: Gastrulation, the specification of the three germ layers, begins with the formation of the primitive streak (shown as the divot in the green mesodermal cells in cross-section of the embryonic disc). Cells along the primitive streak migrate into the bilaminar disc to give rise to the mesoderm.

toderm) that surrounds an inner cell mass, comprised of the epiblast and the primitive ectoderm, as shown in Figure 1.4 [4]. After implantation, the mouse embryo undergoes a dramatic morphological folding to create the so-called “egg cylinder”. The cells of the primitive endoderm invaginate to form a double-walled cup, holding the cells of the epiblast at the bottom of the cup and the cells of the extraembryonic ectoderm near the top of the cup. This break of symmetry establishes the proximodistal axis: the opening of the cup points to the proximal end of the embryo, while the bottom of the cup is distal end (Figure 1.5). At E5.0, cells in the proximal epiblast begin producing NODAL, a signalling peptide that exerts its effects by activating transcriptional modulators (Figure 1.6) [4]. NODAL tells cells of the extraembryonic ectoderm nearest the epiblast to maintain expression of bone morphogenetic protein 4 (BMP4). This, in turn, signals to the proximal epiblast cells to produce Wnt3.

The anteroposterior symmetry is broken on E5.5, when what is now the anterior visceral endoderm begins expressing the Wnt inhibitor Dickkopf homologue 1 (DKK1) and the NODAL inhibitors left-right determination factor 1 (LEFTY1) and Cerberus (CER1). NODAL and Wnt signalling continues in the posterior epiblast [10]. About 6 hours later, Wnt signalling tells the cells of the posterior epiblast to produce the mesoendodermal markers brachyury (T) and eomesoderm-min (EOMES). Together, T and EOMES activate mesoderm posterior 1 (MESP1), which begins the cardiac specification protocol in earnest [8].

Figure 1.5: The primitive endoderm of the murine blastocyst invaginates to form a double-walled cup. This forms the so-called egg cylinder stage of the mouse embryo. A: morula. B: Early blastocyst stage with inner cell mass (pink) surrounded by trophoblast (blue). C: Late blastocyst stage with primitive endoderm (orange). D: Egg cylinder.
1.3 Gene regulatory networks for heart development

1.3.1 The “kernel” of cardiac regulatory genes

Studies of heart development in a wide range of organisms, from the phylogenetically ancient jellyfish to more modern mammals, has revealed the existence of an evolutionarily conserved network of transcription factors that control genes for muscle growth, patterning, and contractility [60]. The genes in this network are:

- NK2
- myocyte enhancer factor-2 (MEF2)
- GATA
- T-box (TBX)
- heart- and neural crest derivatives-expressed (HAND)
Work in *Drosophila* has revealed that, once established by upstream signals, this regulatory network is self-sustaining. Many other genes can be considered accessories to this core “kernel” of regulatory genes; some of these accessory genes have been used successfully to transdifferentiation somatic cells into cardiomyocytes, as described in Section 1.5.

As multicellular organisms increase in size, the need for a specialized vascular system to transport nutrients and wastes increases as well. The first structures that rose up for the task were mere peristaltic tubes lacking chambers and valves – they served merely to force fluid through the organism, and could not pump blood or fluids unidirectionally [6]. But as more complex organisms evolved, so their hearts became commensurately more sophisticated, with chambers dedicated to receiving and pumping blood, a conduction system ensuring synchronized contractions, valves and septa to ensure efficient unidirectional flow, and seamless connections to a closed vascular system [54, 72].

### 1.3.2 The gene-to-structure relationship

With increase in heart complexity, investigators have found a concordant increase in the number of paralogs for the genes of the cardiac regulatory network. The current theory states that gene duplication events during evolution increased the number of these regulatory transcription factors, increasing the possible combinations of gene expression and changing protein expression in different areas to create specialized structures [60]. Figure 1.7 shows the number of paralogs found in the cardiac tissue of representative animals, as well as their phylogenetic relationship.

The gene-to-structure relationship is most clearly demonstrated with the role of the *HAND* gene in ventricular development. There exists a one-to-one relationship between *HAND* genes and ventricles – amphibians and fish express only one *HAND* gene, and their hearts have only one ventricle. Zebrafish mutants without *HAND* expression do not form the ventricular chamber [87]. Mice and other mammals have two copies of *HAND*, with *HAND1* expressed in the derivatives of the primary heart field, and *HAND2* expressed in the derivatives of the secondary heart field [9]. Mice mutants without *HAND2* expression do not form a right ventricle [76], while mutant embryonic stem cells lacking *HAND1* cannot contribute to the left ventricle [65]. Silencing of both *HAND* genes by deleting *HAND2* and *NK2 homeobox 5 (NKX2-5)*, which regulates *HAND1* expression, results in a heart lacking ventricles altogether [9, 75].

### 1.3.3 Upstream control of the cardiac gene kernel

How does the kernel of cardiac regulatory genes establish itself in the developing organism? Work in model organisms has revealed that other genes were recruited to act as upstream initiators of the core kernel during evolution. For example, the *MESP* gene is required for the expression of *NK2* and *HAND* in cardiac progenitor cells of the tunicate *Ciona intestinalis*, a phylogenetically ancient chordate [18, 70]. In the mouse, *MESP1* and *mesoderm posterior 2 (MESP2)* are required for
The number of cardiac regulatory genes expressed in cardiac tissue of representative animals. Hearts with more structures and specialized tissues express a higher number of gene paralogs. From E. N. Olson. Gene regulatory networks in the evolution and development of the heart. *Science*, 313(5795):1922-1927, 2006. Reprinted with permission from AAAS.

specification of the cardiac mesoderm and coordinate the migration of multipotent cardiovascular progenitors [7].

### 1.4 Induced pluripotent stem cells

In 2006 and 2007, Takahashi and Yamanaka published the first reports of the creation of induced pluripotent stem cell (iPSC)s in mice and human cells [77, 78]. With the induction of the four transcription factors, \textit{OCT4}, \textit{SRY} (sex determining region Y)-box 2 (\textit{SOX2}), \textit{Kruppel-like factor 4} (\textit{gut}) (\textit{KLF4}), and \textit{v-my} avian myelocytomatosis viral oncogene homolog (\textit{MYC}), Takahashi and colleagues transformed human dermal fibroblasts into embryonic stem cell-like cells capable for forming cells of all three germ layers \textit{in vitro} and in teratomas. This seminal work catalyzed a flurry of efforts in both the differentiation of cardiomyocytes from iPSCs. Besides cardiac regeneration, researchers have been motivated by the need for a more accurate \textit{in vitro} model of the heart for the modelling of disease and the testing of candidate drugs.

The range of differentiation protocols share a common theme of attempting to recapitulate \textit{in vivo} cardiomyogenesis by recreating the signalling events of fetal development. The efficiency of these protocols is highly variable – the process is not robust and differences in the starting pluripotent cell population, the composition of the tissue culture medium, the concentration of growth
factors, the timing of growth factor application, and the morphology of the starting cell populations lead to different results [10]. Nonetheless, these methods can be classified into two main camps – the embryoid body method, in which pluripotent stem cells are grown in suspension and allowed to form spherical conglomerates of cells resembling the morula, and the monolayer method, in which pluripotent stem cells are grown to a high density.

The monolayer method is technically simpler and faster than the embryoid body method. It begins with 24 hours of treatment with Activin A, followed by 4 days of treatment with BMP4 without medium change. Both Activin A and BMP4 belong to the transforming growth factor β (TGF-β) superfamily of signalling proteins. Since the first report of this method was published by Laflamme et al. [45], others have made incremental improvements. Paige et al. [61] added Wnt3a during the first 24 hours, and DKK1 between days 5 and 11. Uosaki et al. [82] used a Matrigel overlay 24 hours before differentiation to enhance epithelial-mesenchymal transitions, used fibroblast growth factor 2 (basic) (FGF2) and removed insulin during days 1–5, and used DKK1 from day 5–7. Hudson et al. [33] saw improvements by adding the Wnt inhibitors IWR-1 and IWP-4 on day 3.

A major limitation to the in vivo application of the above techniques is the risk of generating teratomas via unspecific differentiation of iPSCs. Indeed, such risks have been verified in the mouse by Abad et al. [1]. Failure to engraft by the injected cells presents another challenge for clinical applications.

1.5 Transdifferentiation

In 1987, Davis et al. [20] reported the discovery of the master regulatory gene of skeletal muscle, myogenic differentiation 1 (MYOD1). With the expression of this single gene, fibroblasts and other cell types can be directly reprogrammed to adopt a skeletal muscle phenotype [15]. Researchers have since searched extensively for a similar master regulator of cardiac muscle differentiation, but the lack of success lead to a dwindling of efforts.

The recent results of iPSC reprogramming rekindled interest in direct reprogramming, or transdifferentiation, of somatic cells into cardiomyocytes. If successful, such a method would eliminate the risk of teratoma formation seen with the induction of pluripotency in vivo.

Using a strategy similar to Takahashi and Yamanaka [77], Ieda et al. [34] managed to reprogram murine cardiac and tail-tip fibroblasts into cells exhibiting the fetal cardiomyocyte phenotype. Starting from a pool of genes known to be highly expressed in embryonic cardiomyocytes and known to cause severe cardiac developmental defects when mutated, they identified GATA4, MEF2c, and TBX5 (GMT) as the minimum essential transcription factors for cardiac transdifferentiation. This report of successful transdifferentiation spawned a series of papers documenting improvements in reprogramming efficiency and the maturity of the resulting cardiomyocytes by adding various transcription factors, small molecules, and microRNAs.

In mice and murine cells, Song et al. [73] added HAND2 to the 3-factor cocktail (GMT) intro-
duced by Ieda et al., transdifferentiating non-myocyte cardiac cells in the mouse heart to beating myocytes and improving heart function following induced myocardial infarction. Protze et al. [62] took a more rigorous approach in selecting the set of transcription factors with which to induce reprogramming – instead of using the Takahashi group’s strategy of removing factors one at a time to find the minimum sufficient set, they tested all combinations of 3 factors from a pool of 10 candidates. They found that set of T-box 5 (TBX5), myocyte enhancer factor 2C (MEF2C), and myocardin (MYOCD) upregulated a broader set of cardiac genes than the GMT cocktail, as determined by quantitative PCR. Jayawardena et al. [40] also used a combinatorial approach to identify a set of microRNAs (miR-1, miR-133, miR-208, miR-499) sufficient to transdifferentiate a significant portion of cardiac fibroblasts into cardiac-like cells. Interestingly, they found that treatment with Janus kinase (JAK) inhibitor I increased reprogramming efficiency by ten-fold. Addis et al. [3] found that the combination of HAND2, NXX2-5, GATA binding protein 4 (GATA4), MEF2C, and TBX5 had a stronger reprogramming effect than GMT alone. Similarly, Christoforou et al. [17] found that MYOCD, serum response factor (SRF), MESP1 and SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily D, Member 3 (SMARCD3) enhance the inductive effects of GMT.

In human cell culture, Nam et al. [59] were the first to demonstrate the possibility of transdifferentiating fibroblasts to cardiomyocytes. They used a combination of transcription factors and microRNAs – GATA4, TBX5, HAND2, MYOCD, miR-1, and miR-133. This was followed closely by work from Wada et al. [83], who added MESP1 and MYOCD to the GMT cocktail. Fu et al. [27] added estrogen-related receptor gamma (ESRRG), MESP1, MYOCD, and zinc finger protein, FOG Family Member 2 (ZFPM2) to further enhance reprogramming.

Islas et al. [35] took an alternate approach to selecting the genes for cardiac transdifferentiation. Whereas previous groups used a combinatorial approach or one-at-a-time removal approach to find the optimal minimum subset of core cardiac regulatory genes to achieve reprogramming, Islas and colleagues tried genes that were known to be involved in upstream cardiac specification in model organisms (Section 1.3.3). They found that homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (ETS2) and MESP1 were sufficient activate the cardiac gene regulatory network and convert human dermal fibroblasts to cardiac-like myocytes.

Despite the extensive work in optimizing protocols, even the most effective reprogramming protocols struggle to attain efficiencies of 40% [74], and as yet unknown epigenetic states may preclude reprogramming, making some protocols difficult to reproduce [13]. In addition, all of the reprogramming protocols above are plagued by the same two problems: one, the cardiomyocyte-like cells generated are immature, resembling fetal cardiomyocytes in electrophysiology and calcium handling; two, the population of cells is heterogenous, with a variable mixture of atrial-, ventricular-, and nodal-type myocytes. Both problems must be overcome if we are to achieve a reliable population of cells with which to model cardiac disease, test candidate drugs, and achieve
cardiac regeneration *in vivo*. Since some of the cells will be nodal-type pacemakers, the problem of introducing arrhythmogenic ectopic pacemakers looms large.

Noticeably absent from the literature are works examining the effects of mechanical signalling on cardiomyocyte transdifferentiation and maturation. This is in spite of similar works in differentiation of pluripotent stem cells and knowledge of the role of tissue stiffness and cyclic mechanical loads on stem cell differentiation [23], the spontaneous beating of primary cardiomyocytes *in vitro* [24], the TGF-β and BMP signalling pathways [84] in ventricular myocytes, and the structural changes in atrial myocytes [21].

### 1.6 Defining characteristics of cardiomyocytes

The field of cardiac differentiation and reprogramming has not yet reached a consensus on where the distinction between differentiating cardiac progenitor (or somatic cell) and *de facto* cardiomyocyte should be drawn. The cells generated from differentiation and transdifferentiation experiments are heterogeneous and fall on a continuum of maturity from the starting cell population on one end and fully mature cardiomyocyte on the other. Typically, a large proportion of cells express baseline features (e.g., driving a cardiac-specific fluorescent reporter, such as α myosin heavy chain (αMHC)-enhanced green fluorescent protein (EGFP)), and ever-decreasing numbers of cells expressing more stringent criteria (e.g., calcium oscillations, action potentials, and spontaneous contraction).

Addis and Epstein [2] proposed that three criteria be met before a cell is to be considered successfully reprogrammed to a cardiomyocyte:

1. The cell should have a gene expression pattern (as determined by reverse-transcriptase polymerase chain reaction (RT-PCR), microarray, or transcriptome sequencing) that more closely matches those of cardiomyocytes than other cell types.

2. The cell should express structural proteins (e.g., cardiac troponin T (cTnT), αMHC, and α-actinin) and organize these proteins into sarcomeres

3. The cell should exhibit at least one functional attribute (e.g., action potentials, calcium oscillations, and spontaneous or induced beating)

### 1.7 Mechanical signalling in cell differentiation

#### 1.7.1 Mechanisms of mechanotransduction

How do mechanical forces outside the cell lead to changes in gene expression in the nucleus? Our current understanding of intracellular force transmission mechanisms involves a handful of actors, some of which are depicted in Figure 1.8. For example, integrins span the cell membrane, binding
to extracellular matrix (ECM) proteins (e.g., collagen, fibronectin) on the outside and intracellular proteins on the inside. Talin and vinculin act as adapter proteins to bind the intracellular domain of integrin to actin filaments of the cytoskeleton. Near the nucleus, the nesprin proteins connect the actin filaments to nuclear membrane proteins such as SUN1 and SUN2. The intranuclear domains of SUN1 and SUN2 interact with the nuclear envelope protein lamin, which can bind DNA and forms stable nuclear structures [37].

Researchers have long suspected that mechanical signalling plays an important role in cell behaviour [26], and indeed many working in the field of differentiation and transdifferentiation to cardiomyocytes have conjectured that the mechanical properties of the cardiac microenvironment may explain why in vivo experiments generally have better results than in vitro [63].

Engler et al. [23] further confirmed the importance of the mechanical properties of the extracellular environment by showing that mesenchymal stem cells can be directed to differentiate by varying only the substrate stiffness. When the non-muscle myosin IIs are blocked with blebbistatin, a selective inhibitor, the mesenchymal stem cells no longer respond to substrate stiffness. Macri-Pellizzeri et al. [48] reported similar findings with iPSCs.

### 1.7.2 Previous works involving mechanical strain

Researchers have long speculated that the cardiac microenvironment offered additional signalling cues to cells being differentiated or reprogrammed to cardiomyocytes [74]. This conjecture is based on the results of in vivo reprogramming and differentiation experiments, which generally show much higher rates of conversion to cardiomyocytes than similar experiments in vitro [63, 73]. Possible candidates for these so-called "non-soluble factors” that affect cell differentiation include tissue stiffness, extracellular matrix proteins, and mechanical strain.

Engler and Discher were pioneers in the exploration of the effects of mechanical signalling on stem cell differentiation. They found that mesenchymal stem cells can be directed to differentiate to neuronal, myoblastic, or osteoblastic lineages by varying only the stiffness of the substrate on which they grew [23]. It is thought that cells can sense the stiffness of the extracellular matrix by pulling on it and sensing the force required to deform the matrix with force transduction proteins.

Other workers have examined the effects of cyclic mechanical strain on stem cells and stem cell-derived cardiomyocytes. Horiuchi et al. [31] exposed mouse embryonic stem cell (mESC) to 10% at 0.17Hz (10 cycles per minute) and found that this stretching maintains expression of NANOG, a marker of pluripotency. Similarly, Saha et al. [68] and Saha et al. [67] stretched human embryonic stem cell (hESC) biaxially at 0.17Hz, 10% strain, and found that this inhibited the spontaneous differentiation of hESC and promoted their self-renewal.

Interestingly, varying the parameters of cyclic strain (frequency, magnitude, and duration) can also have pro-differentiation effects. Teramura et al. [79] stretched iPSCs at 0.2Hz, 15% strain, for 12 hours. This cyclic strain regimen decreased the expression of pluripotency markers NANOG,
Tulloch et al. [81] seeded hESC- and iPSC- derived cardiomyocytes into a three-dimensional collagen scaffold and subjected the scaffold to cyclic, uniaxial mechanical stress. This stretching treatment resulted in increased cardiomyocyte fibre alignment, myofibrillogenesis, and sarcomeric banding. The stretched cells showed greater hypertrophy and proliferation, and quantitative RT-PCR showed increased mRNA transcript levels of cardiac-specific genes. The stretching regimen began 16 days after the end of BMP4 treatment, after the start of spontaneous contractions. The scaffold was stretched at 1Hz, 5% strain, for 4 days.

1.8 Hypothesis

The aim of my thesis is to explore the phenomenological effects of cyclic strain on the efficiency of differentiation and transdifferentiation to cardiomyocytes. We hypothesize that cyclic mechanical strain, in conjunction with transgene expression and treatment with soluble factors, will increase differentiation and transdifferentiation efficiency, as measured by the number of cells activating a cardiac-specific fluorescent reporter (Section 2.2.2). We also hypothesize that cyclic mechanical strain can increase the maturity of generated cardiomyocytes, as measured by structural organization of sarcomeres and calcium handling abilities.

Chapter 2 specifies the details of my experimental procedures. In Chapter 3, I describe the design, fabrication, and characterization of a device used for the controlled application of strain to cultured cells. In Chapter 4, I report on the modification and testing of a cardiac-specific fluorescent promoter, and I present the results of differentiating cardiomyocytes from iPSCs. In Chapter 5, I discuss the implications of my results and speculate on possible directions for future works.
Chapter 2

Materials and methods

2.1 Cell culture

2.1.1 Cell adhesion coatings

Matrigel is a solubilized basement membrane protein mix extracted from the Engelbreth-Holm-Swarm mouse sarcoma. This tumour is rich in ECM proteins, including laminin, collagen IV, heparan sulfate proteoglycans, and entactin/nidogen. Growth Factor Reduced Matrigel coatings were used in the culture of iPSCs prior to differentiation. Growth Factor Reduced Matrigel has lower levels of epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and platelet-derived growth factor (PDGF). Matrigel solution was kept on ice to remain liquid and aliquots were frozen upon receipt. Coating of tissue culture wells was done by dissolving an aliquot of Matrigel in DMEM/F-12 medium (GE Healthcare Life Sciences Catalog #SH30023.FS), and immersing the well surface in the Matrigel solution at room temperature for 1 hour. Each 6-well plate was coated with 0.5mg of Matrigel.

Gelatin is a hydrolyzed mixture of collagens derived from animal connective tissue. It is often used to coat tissue culture surfaces to facilitate cell adhesion. Type B (lime-cured) gelatin powder (Fischer Scientific Catalog #G7-500) derived from bovine skin was dissolved in deionized and autoclaved water to make 0.1% (weight of solute in grams as a percentage of volume of solvent in millilitres) gelatin solution. For each well of a 6-well plate, 2mL of 0.1% gelatin solution was added. The tissue culture vessels were incubated with the gelatin solution at 37°C for 4 hours before use.

Fibronectin is another ECM protein commonly used to coat tissue culture surfaces to facilitate cell adhesion. Fibronectin also exists in a soluble form in blood plasma, which is the source of the fibronectin used in these experiments. Fibronectin was purified from human plasma by affinity chromatography using gelatin Sepharose 4B (GE Healthcare Life Sciences Catalog #17-0956-01).
Poly-L-lysine solution (0.01%, provider, catalog number) was used to pre-treat certain tissue culture wells prior to Matrigel coating in order to facilitate protein adhesion. The positively charged side chain of the amino acid polymer increases the binding of negatively charged proteins and cells. Coating was done inside a biosafety cabinet using sterile technique at room temperature with 1mL of poly-L-lysine solution per 6-well plate well. After 5 minutes, the coating solution was aspirated and the culture surface allowed to dry before introducing cells or additional coating.

2.1.2 BJ fibroblast

BJ fibroblasts were a gift from Dr. Christopher Maxwell of the Child and Family Research Institute and the University of British Columbia. They were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F-12 nutrient mix, 1:1 ratio, with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Medium was changed every 4-5 days. Passaging was done when the cells reached 90% confluence (i.e., coverage of the available growth substrate by the cells reached 90%). For dissociation, the cells were rinsed with phosphate-buffered saline (PBS) before incubation for 5-8 minutes with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.05% trypsin, GE Healthcare Catalog #SH30236.01).

2.1.3 Celprogen “primary cardiomyocytes”

Putative human cardiomyocytes derived from biopsy of adult cardiac tissue were purchased (Celprogen Catalog #36044-15). These cells were grown on 6-well plates with proprietary ECM adhesion coatings (Celprogen Catalog #E36044-15) and in proprietary medium with serum (Celprogen Catalog #M36044-15S). The cells were maintained at 37°C in an atmosphere of 5% carbon dioxide. Medium renewal was performed daily, and passaging was done at 80-90% confluence. Cells were harvested for experiments at 90% confluence.

2.1.4 Induced pluripotent stem cells

iPSC cells, iPS(IMR90)-4, from the WiCell Stem Cell Bank, were a gift from Sanam Shafaat Talab and Dr. Glen Tibbits of the Child and Family Research Institute and Simon Fraser University. The iPS cells were transformed from the IMR-90 fibroblast cell line, which was itself derived from the lungs of a 16-week female human fetus. The fibroblasts were passaged 18 times prior to reprogramming with OCT4, SOX2, NANOG, and Lin-28 homolog A (LIN28) [88]. Once transformed, the cells were passaged 41 times, the last 16 of which were cultured in mTeSR1 medium on a Matrigel coating. Once received, the iPSCs were cultured on Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix (Corning Product #356230).

The culture medium used was mTeSR™1 (Stemcell Technologies Catalog #05850), containing recombinant human TGF-β and recombinant human basic fibroblast growth factor (bFGF).
Complete mTeSR1 medium is made by adding the mTeSR1 5X Supplement to the mTeSR1 Basal Medium. The mTeSR1 5X supplement was thawed at room temperature or at 4°C. Aliquots of the complete mTeSR1 medium were frozen at -20°C until needed, at which point they were also thawed at 4°C. Once seeded, the medium was renewed daily until passing or initiation of differentiation protocol. The optimal time for passaging depends on seeding density and cell aggregate size at the time of seeding. Cell cultures were visually inspected and passaged when confluent islands of cells reached at least 2 mm in size. The culture medium was aspirated and the cells were washed with 1mL Versene® (EDTA) 0.02%, (Lonza Catalog #17-711E). The wash solution was aspirated and a fresh 1mL of Versene was used to treat the cells for 8 minutes. The Versene was then carefully aspirated without disturbing the cell layer. Next, 3mL of mTeSR1 was used to wash the cells off the culture surface. For maintenance passage, a 5mL pipet and Pipet-Aid® were used for gentle dissociation forces. The larger cell aggregates created this way showed faster recovery after replating. For seeding into wells prior to differentiation protocols, a manual pipettor was used to pipet up and down rigorously, resulting in almost fully dissociated cells that, once replated, grew into a more homogeneous monolayer. The split ratio varied from 1 to 12 for maintenance and expansion passaging, to 1 to 3 for differentiation experiments. Immediately after replating, the small-molecule rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor Y-27632 was added to the culture medium to a final concentration of 10µM. The use of ROCK inhibitor has been shown to increase viability of stem cells in vitro by reducing dissociation-induced apoptosis (also known as anoikis) [85].

Freezing the iPSCs was done by harvesting with Versene, as described above. After the 8-minute Versene treatment, 3mL of mFreSR®1 (Stemcell Technologies Catalog #05855) was used to wash the cells off the culture substrate. Multiple washes were done to ensure all cells were washed off. The cells from each 6-well plate were pooled in 10mL of mFreSR1, aliquoted into 10 cryovials, and put into an isopropanol freezing container to be frozen at -80°C. The vials were transferred to vapour phase nitrogen the next day.

2.1.5 Differentiation of iPSCs to cardiomyocytes

We used the monolayer method to differentiate iPSCs into cardiomyocytes [45]. iPSCs were thoroughly dissociated and seeded onto Matrigel-coated culture plates at a density of 100,000 cells/cm². After 3-4 days of daily medium renewal, the cells should reach 90% confluency. (If the cells do not grow as expected, extending this culture period generally does not result in greater confluence. Instead, non-adherent cells tend to proliferate and crowd out adherent cells, resulting in even lower confluence.) At this confluence, the mTeSR1 medium was replaced with RPMI 1640 basal medium with B-27 supplement (no insulin) (Life Technologies Catalog #A1895601). Recombinant human Activin A (R&D Systems Catalog #338-AC-010) was added to this medium to a final concentration of 100ng/mL. After 24 hours of Activin A treatment, the medium was refreshed and recombinant hu-
man BMP4 (R&D Systems Catalog #314-BP-010) was added to a final concentration of 10ng/mL. The cells were treated with BMP4 for 4 days without medium change so that secreted factors may accumulate and exert an autocrine or paracrine effect. After 4 days, the medium was changed to RPMI 1640 basal medium with B-27 supplement (complete) (Life Technologies Catalog #17504-044). This medium was renewed every 3 days. With traditional polystyrene cell culture substrates, spontaneous contractions can be observed 10 days after onset of Activin A treatment. With continued maintenance, the spontaneously beating collections of cells remodel and recruit more cells, creating larger areas of contraction.

2.2 Plasmids and transfection

2.2.1 Transfection

Transfection, the introduction of foreign DNA to a cell, was done using electroporation. Electroporation uses electrostatic fields to increase the membrane permeability of cells to DNA. To perform electroporation, we used the Nucleofector™ device from Lonza, with programs optimized for the cell types under experimentation. Program A-024 was used for fibroblasts, and G-009 was used for cardiomyocytes or cardiac-like cells. In lieu of Amaza proprietary transfection reagents, nucleofection was carried out in Opti-MEM® media (Life Technologies Catalog #31985-070). In most cases, nucleofection in Opti-MEM achieves comparable transfection efficiencies at much reduced costs [25].

2.2.2 Reporter for cardiac-specific myosin heavy chain

Myosin is a protein that generates force from the chemical potential energy stored in adenosine triphosphate (ATP). The myosin present in cardiac muscles is a large protein made up of two heavy subunits (the myosin heavy chains), two light subunits (the myosin light chains), and two regulatory subunits. The human genome encodes 17 different isoforms of myosin heavy chain (MHC)s, but only αMHC and β myosin heavy chain (βMHC) are present in cardiac muscle [55].

Researchers have taken advantage of the tissue specificity of αMHCs, as well as previous work cloning and characterizing its promoter, to create a reporter construct for cardiac-like cells. Specifically, the ~5000 base pairs upstream of the transcription start site of of αMHC was used as a promoter driving fluorescent reporters (i.e., EGFP and mCherry) [28]. When the cells enter a cardiac-like transcriptional state, it begins to transcribe αMHC mRNA, but this also activates the transcription of the fluorescent proteins, which enables the quantification of differentiation efficiency with flow cytometry. Previous workers have used lentiviruses to create pluripotent stem cells stably expressing bioengineered reporter constructs [44]. Despite the utility of these techniques, lentivirus transduction always carries the risk of random genomic integration and tumorigenesis.
We thus created a plasmid containing the same reporter construct that can be transiently expressed after introduction to cells via nucleoporation.

We began with the pEGFP-N1 plasmid backbone (Addgene plasmid #6085-1) and the αMHC reporter construct created by Kita-Matsuo et al. [44] (Addgene plasmid #21229). The αMHC promoter was cloned into the pEGFP-N1 multiple cloning site (MCS) with XhoI and AgeI. Then the human cytomegalovirus (CMV) promoter was excised with AseI and NheI, and a blank annealing oligo was used to close the plasmid. The resulting plasmid’s map is shown in Figure 2.1.

2.2.3 Vehicle for introducing ETS2 and MESP1

We created a bicistronic plasmid vector capable of mammalian expression carrying the genes ETS2 and MESP1. A map of the plasmid is shown in Figure 2.2.

The CMV immediate early promoter and enhancer is used to express transgenes in a variety of cell lines. Transcription factors bind to the enhancer region to facilitate transcription, while the promoter region contains DNA sequences for both transcription factors and RNA polymerase to bind and initiate transcription. The IRES allows 2 proteins to be translated from the same bicistronic mRNA [38, 39]. IRES originates from the 5’ untranslated region (5’-UTR) of the encephalomyocarditis virus, and folds into secondary hairpin structures that allows the eukaryotic ribosome to bind and initiate translation. The precise cellular mechanism for this ribosomal binding is still unclear. Polyadenylation is the addition of several adenine bases to the 3’ end of an mRNA molecule, which allows the mRNA to remain stable while being transported from the nucleus and translated. The SV40 polyadenylation signal is an RNA element that promotes efficient polyadenylation [86]. The f1 origin of replication is derived from the f1 phage. This enables a single-stranded copy of the plasmid to be made and packaged into a phage particle. The pIRES2 plasmid contains a neomycin/kanamycin-resistance cassette for bacterial and eukaryotic cell selection. The protein encoded by the cassette hails from the Tn5 transposon of E. coli, and confers resistance to both neomycin and kanamycin. Cells that have taken up the plasmid can thus be grown in media or on agar containing these antibiotics, allowing us to select for these cells. The expression of this gene is driven by an SV40 early promoter/enhancer sequence. The tail end of this cassette contains the polyadenylation sequence from the herpes simplex virus thymidine kinase (HSVTK) gene. Eukaryotic cells stably transfected with a plasmid containing this cassette can thus be selected for using the G418 antibiotic. The pUC origin of replication allows the plasmid to be replicated in E. coli. This origin of replication enables each cell to maintain a high copy number of plasmids (over 500).

ETS2 and MESP1 were purchased from DNASU (clone ID HsCD00002188 and HsCD00080018). These genes were amplified using polymerase chain reaction (PCR) with custom-designed primers that included flanking restriction enzyme sites (see Table 2.1). The PCR products were agarose gel-purified and ETS2 was then inserted upstream of the IRES. Downstream of IRES, the EGFP cassette was removed and replaced with MESP1.
Figure 2.1: Map of the αMHC reporter plasmid. The CMV promoter of the pEGFP-N1 plasmid was replaced with the αMHC promoter.

Table 2.1: PCR primers for the amplification and cloning of ETS2 and MESPI.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MESPI.FOR</td>
<td>5’-CTCGAGGAATTCAATGGCCCAGCCCCTGTGC-3’</td>
</tr>
<tr>
<td>MESPI.REV</td>
<td>5’-AATCCCGGGCTACAACCTGAATGATTTCGGAATC-3’</td>
</tr>
<tr>
<td>ETS2.FOR</td>
<td>5’-GAATTCCAGATCTCATCATGTAATGATTTCGGAATC-3’</td>
</tr>
<tr>
<td>EST2.REV</td>
<td>5’-GTGACGGATCCATGTTAGAAAGCTTCCC-3’</td>
</tr>
</tbody>
</table>
Figure 2.2: Map of the pIRES2 plasmid vector used to deliver ETS2 and MESP1 to the cells of interest. This plasmid features a CMV immediate early promoter and enhancer, an IRES sequence, an MCS upstream of the IRES sequence, a SV40 polyadenylation signal downstream of the EGFP gene, an f1 single-strand DNA origin, a Kan\textsuperscript{r} and Neo\textsuperscript{r} cassette, and a pUC origin of replication. Restriction sites are shown in bold with leader lines indicating their relative position on the plasmid.

2.3 Lentivirus production and transduction

Lentivirus remains a popular choice for the introduction of transgenes to non-dividing cells for stable expression. The technology was based on the human immunodeficiency virus and was developed in the late '90s by the Trono lab.

2.3.1 Lentivirus production

Lentiviruses carrying the αMHC reporter construct were constructed with the help of Oksana Nemirovsky. We used a second-generation lentiviral packaging system, consisting of the psPAX2
packaging plasmid, the pMD2.G envelop plasmid, and the transfer plasmid containing our gene of interest. The separation of the components of lentivirus (i.e., envelope proteins, packaging proteins) is a safety feature used to lower the risk of generating replication-competent viruses capable of infecting humans. The system also contains other variations (e.g., truncated 3’ long terminal repeat, deletion of auxiliary virulence genes) that increase safety [90, 91].

The three plasmids were co-transfected into HEK-293FT cells, a cell line derived from human embryonic kidney transformed with the SV40 large T antigen, in order to create lentiviruses carrying our gene of interest. Transfection was done using Lipofectamine 2000®. The large T antigen functions to increase DNA replication by suppressing tumour suppressor proteins and pushing the cell from G1 to S phase. This also increases virus genome replication, a desirable effect for lentiviral particle production.

After transfection, the culture medium containing viral particles was collected and filtered to remove cells. The virus-containing medium was then centrifuged to obtain a pellet of viral particles, the medium aspirated, and the viral particles resuspended in PBS.

2.3.2 Lentivirus transduction

BJ fibroblasts were cultured using methods described in Section 2.1.2. BJ fibroblasts were seeded into a 24-well tissue culture plate at a density of $4 \times 10^4$ cells per well and allowed to attach for 16 hours. The next day, the medium was changed to include the lentiviral particle suspension. The cells were washed twice with PBS before the addition of the transduction medium (regular tissue culture medium and lentiviral suspension solution). A range of viral suspension volumes were used ($2 \mu$L, $5 \mu$L, $10 \mu$L, and $15 \mu$L), with and without the transduction adjunct hexadimethrine bromide, to gauge transduction efficiency. Hexadimethrine bromide (Polybrene) is a cationic polymer that increases transduction efficiency by reducing the charge repulsion between the virus and the cell membrane. Some cell types are sensitive to polybrene, so transduction was done with and without the adjunct to gauge toxicity. Polybrene was added to a final concentration of 4$\mu$g/mL. After 24 hours, the transduction medium was aspirated and the cells washed three times with PBS. From this point, cell culture and passaging proceeded as described in Section 2.1.2.

2.4 Immunocytochemistry

iPSC-derived cardiomyocytes were fixed in 3.7% formaldehyde fixation buffer and permeabilized with a buffer solution containing 0.1% TX-100. Anti-α-actinin antibody (Sigma-Aldrich #A7811) was applied at a 1:800 dilution. Anti-cTnT antibody (Abcam #ab10214) was applied at a 1:600 dilution. Bound primary antibodies were visualized with goat anti-mouse IgG, DyLight 488 conjugate (Life Technologies Catalog #35503). Specimens were mounted using ProLong Gold with 4’,6-diamidino-2-phenylindole (DAPI) (Life Technologies Catalog #P-36931).
2.5 Western blots

Western blotting was conducted on Celprogen cardiomyocytes with anti-cTnT antibody (Abcam #ab10214) at a 1:2000 dilution. Mouse whole heart lysate and Jurkat cell lysate were used as positive and negative controls, respectively. PKA C-α, a housekeeping gene, was used as a loading control.

2.6 Flow cytometry

Flow cytometry employs lasers to count and sort cells. Flow cytometers can separate individual cells on the basis of presence of fluorescent chromophores. Cell counting was performed at the Child & Family Research Institute (CFRI) Flow Core facility using the FACSCanto instrument (BD Biosciences). Post-acquisition analysis was done using FlowJo (Tree Star).
Chapter 3

Cyclic strain device for tissue culture

3.1 Cyclic strain in the heart

Modelling the ventricles of the heart as a hollow ovoid, we can define an orthogonal cardiac coordinate system as depicted in Figure 3.1. The radial axis is normal to the outer surface of the epicardium; the longitudinal axis is perpendicular to the radial axis, pointing from the apex of the ventricle to the base; the circumferential axis is perpendicular to both the radial and longitudinal axes and points counterclockwise around the ring of the epicardium, when looking from apex to base. Moore et al. [52] characterized the strain evolution of the normal human left ventricle during systole using magnetic resonance imaging. They measured peak systolic strains of ∼45% in the radial axis, ∼20% in the circumferential axis, and ∼16% in the longitudinal axis.

The goal of strain devices for tissue culture is to controllably mimic some aspect of this strain in vitro.

3.2 Previous devices and their limitations

Many devices for probing cellular responses to cyclic strain have been previously described. They have in common the use of an elastomeric membrane as a growth surface for cells and the means by which cells are strained. The membrane may be functionalized, for example with ECM proteins, to facilitate cell adhesion and proliferation. The devices differ in the way the membrane is stretched.

Flexcell® International Corporation has long marketed a pneumatically-driven tissue strain setup, complete with proprietary control software (Figure 3.2). A vacuum pump generates the pulling force that strains the membrane, parts of which are stretched against loading posts to generate defined strains. Beyond the high cost associated with such systems, they are limited by the slow response times of the pneumatic system (max stimulation frequency of 5Hz) and their large size (difficult to integrate with tissue culture incubators).

Our lab has addressed some of these limitations with the magnetically actuated cellular strain
in order to conceptualize the strains generated and felt by cardiomyocytes, it helps to establish a cardiac coordinate system, as used in echocardiography. Such a system has a radial axis, a circumferential axis, and a longitudinal axis.

assessment tool (MACSAT), which uses an electromagnet to pull on permanent magnets clamped on both sides of the membrane (Figure 3.3) [42]. However, scaling up the device to strain multiple wells simultaneously proved difficult, as each electromagnet required a signal generator and current amplifier.

3.3 Design of the HexCycler

To address the above issues I designed the HexCycler, featuring 6 independently controlled direct current (DC) motors, each driving a Scotch yoke actuator, shown in Figure 3.4 that converts the rotation of the motor to linear motion of a piston head that pushes the membrane from below. The final design is the end result of many iterative prototypes, including rough functional prototypes machined from polycarbonate and iterations with 3D-printed components. The supporting frame of the HexCycler is made from waterjet-cut aluminum sheet metal (6061 aluminum alloy) so as to be corrosion-resistant and autoclavable (Figure 3.5). A BioFlex 6-well plate from Flexcell International
Figure 3.2: The Flexcell® FX-5000™ Tension System uses vacuum pressure to strain an elastomeric membrane on which cells are cultured. Image courtesy of Flexcell International Corporation.
Figure 3.3: The magnetically actuated cellular strain assessment tool (MACSAT) uses an electromagnet to exert force on permanent magnets attached to elastomeric membranes of the BioFlex tissue culture plate.
Figure 3.4: The motor assembly of the HexCycler features a Scotch yoke mechanism (also known as slotted link mechanism) that turns the rotational motion of the DC motor into linear motion.

Corporation is clamped to the top stage of the device, and the motor assembly below pushes a piston up and down to distend the membrane.

The magnitude of membrane strain is controlled by raising and lowering the upper stage of the HexCycler. Figure 3.12 shows the range of estimated strains achievable with the HexCycler and BioFlex membrane for various max membrane displacements. Since the Scotch yoke mechanism creates sinusoidal linear motion, the impingement of the piston on the membrane will be a "half-wave rectified" form of this sinusoid, as shown in Figure 3.6. The maximum amplitude of oscillation can be varied by changing the disc of the Scotch yoke mechanism.

3.3.1 DC motor control with the Arduino

The open-source electronics platform of the Arduino massively simplified the control of the DC motors of the HexCycler. Coupled with the Adafruit motor shield, this off-the-shelf solution is an affordable and easy-to-use alternative to specialized proprietary systems. The heart of the Adafruit motor shield is the TB6612 metal-oxide-semiconductor field-effect transistor (MOSFET) driver, which uses the Arduino 8-bit digital output to control the large currents required to power the motors. This gives the HexCycler 256 discrete speed settings for a given motor.
Figure 3.5: The HexCycler couples 6 independently-driven DC motors to achieve high parallelism. Its frame is made from waterjet-cut 6061 aluminum alloy, making it sterilizable and corrosion-resistant. The 6-well BioFlex plate is shown in magenta.

Figure 3.7 shows the frequency response of the HexCycler under simulated experimental conditions. The line of best fit, which determines the digital input-to-frequency response relationship, is

\[ f = 0.0259d - 0.4522 [\text{Hz}] \]  

(3.1)

where \( f \) is the frequency of oscillation and \( d \) is the digital input, an integer between 0 and 255. The \( R^2 \) value for this linear regression is 0.9995.

3.4 Characterizing strain with finite-element analysis

The radial symmetry of the tissue culture well and piston creates 2 main strain axes – radial and circumferential (Figure 3.8). With an upward deformation from the impinging piston, the membrane experiences positive strain (i.e., elongates) in the radial axis, somewhat smaller positive strain in the circumferential axis, and negative strain in the z axis.

To better quantify this strain, I modelled the elastomeric membrane of the BioFlex 6-well plate
Figure 3.6: Depending on the height of the upper stage of the HexCycler, the sinusoidally oscillating piston may only come into contact with the membrane for part of its cycle. This figure models a hypothetical setup with 5mm of piston travel that impinges on the membrane. The resulting motion of the membrane in direct contact of the piston is shown in blue.

using COMSOL Multiphysics, a finite element analysis (FEA) software package. The geometric dimensions of the well are measured from the BioFlex plate. I exploit the radial symmetry of the culture well to simplify the model. Figure 3.9 shows the cross-section of the membrane along a radial strip, as well as the meshing of the model (i.e., the division of the model volume into small elements on which to apply the constitutive equations). The centre of the culture well is $r = 0$, and the outside edge is at $r = 17.5$, giving the membrane a diameter of 35mm. The thickness of the membrane is 0.5mm. The green section of Figure 3.9 denotes the section of the membrane that is pushed by the HexCycler’s piston.

The model is given a fixed constraint on the outer edge, and a prescribed displacement is applied to the bottom of the membrane with $r \leq 3$ to simulate the piston pushing up on the membrane (Figure 3.9). This method of modelling avoids the complexity of modelling the interface between the piston and membrane, but has the drawback of not accurately capturing strain over the areas of the membrane that overlay the piston, since it fails to account for membrane stretching and sliding in this area.
Figure 3.7: Empirical determination of the HexCycler’s frequency response under operating conditions. The frequency was measured using the time required for 30 oscillations. The line of best fit, with equation $f = 0.0259d - 0.4522$, has $R^2 = 0.9995$.

Figure 3.8: The radial symmetry of the BioFlex tissue culture membrane gives rise to three orthogonal axes: radial, circumferential, and $z$ (axial).
Figure 3.9: A radial cross-section of the BioFlex tissue culture membrane. The left-most edge of the rectangle is the axis of radial symmetry, around which the rectangle is revolved to generate a thin circular membrane. The green section indicates the area that directly contacts the HexCycler’s piston. The orange boundary is given a prescribed displacement, representing the pushing action of the HexCycler’s piston. The red boundary is a fixed constraint, representing the clamping of the BioFlex membrane to the edge of the tissue culture well. The meshing of the membrane is also shown.

3.4.1 The Ogden model for hyperelastic materials

Since the potential deformations of the membrane are large relative to the dimensions of the membrane, a hyperelastic material model is needed to capture the nonlinear stress-strain behaviour. Hyperelastic materials are elastic materials that possess a strain-energy function. Kim et al. [43] demonstrated that the second-order Ogden model best matched empirically measured stress-strain relationships of various polydimethylsiloxane (PDMS) polymer mixtures.

The Ogden model for incompressible materials begins with a strain energy density function $W$:

$$W(\lambda_1, \lambda_2) = \sum_{p=1}^{N} \frac{\mu_p}{\alpha_p} \left( \lambda_1^{\alpha_p} + \lambda_2^{\alpha_p} - \lambda_1^{-\alpha_p} - \lambda_2^{-\alpha_p} - 3 \right) \text{[MPa]}$$  \hspace{1cm} (3.2)

Here, $N$ is the order of the model, $\lambda_i$ are the principal stretches, and $\alpha_p$ and $\mu_p$ are experimentally derived parameters. The Ogden parameters determined by Kim et al. [43] are listed in Table 3.1.
Table 3.1: Empirically derived values of Ogden hyperelastic model parameters [43].

<table>
<thead>
<tr>
<th>Ogden Parameter</th>
<th>Value</th>
</tr>
</thead>
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<tr>
<td>$\alpha_1$</td>
<td>63.4885 MPa</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>0.041103 MPa</td>
</tr>
<tr>
<td>$\mu_1$</td>
<td>$6.371 \times 10^{-10}$</td>
</tr>
<tr>
<td>$\mu_2$</td>
<td>3.81166</td>
</tr>
</tbody>
</table>

The BioFlex plate uses a “polyorganosiloxane” elastomer as its flexible membrane [5]. The material properties of this elastomer are taken from empirically derived values of a PDMS mixture that best approximates its Young’s modulus. Most sources estimate the Poisson’s ratio of PDMS to be $\sim 0.5$, which I approximate in my simulations with 0.49999 in order to avoid the singularity in the calculation of the bulk modulus [57].

3.4.2 Model results

Figure 3.10 shows the magnitude of the three principal strains when the membrane is stretched to 5mm above resting position. The large peak at $r = 3$mm is due to the sharp discontinuity between the loaded section of the model (i.e., the section given a prescribed displacement) and the rest of the membrane. Strain values near this area do not reflect reality, since the model does not account for membrane sliding over the piston. Figure 3.11 shows the directions of these principal strains throughout the radial cross-section of the membrane. As expected, we see positive stretch along the radial axis and compression in the z-axis. The second principal strain, corresponding to the circumferential axis for most of the membrane, is perpendicular to the plane of the diagram and not shown. However, in the region with $r < 3$mm, the model erroneously flips the second and first principal strains.

Figure 3.12 shows the range of achievable strains with membrane displacements of 4mm to 10mm. The simulation suggests that, for moderate membrane displacements, there will be a plateau region (4mm $< r < 16$mm) in which the strain is relatively homogeneous.

3.5 Validating the effects of the HexCycler’s strain on cultured fibroblasts

In order to test that the HexCycler is capable of exerting biologically relevant strains to cultured cells, I recreated one of the known effects of cyclic strain on cultured fibroblasts – these spindle-shaped cells will align themselves such that their long axes are perpendicular to the axis of tensile strain.

Figure 3.13 shows differential interference contrast (DIC) images of BJ fibroblasts before and after the application of cyclic strain. The cells begin oriented randomly, but after 24 hours of
Figure 3.10: Principal strains as a function of distance away from the centre of the culture well.

5% cyclic strain at 1Hz, they become aligned to a common orientation. Figure 3.14 shows the distribution of fibroblast orientations before and after stretch, along with unstretched controls. The range of orientations clearly narrows in the cells receiving the strain treatment. The orientation angle is defined to be the angle of the long axis of the cell, relative to the horizontal of the image. The average orientation of strained cells is subtracted from each orientation value to normalize the orientation across several images to zero degrees.
Figure 3.11: Vectors denoting the directions of principal strains. The first principal strain is an elongation along the radial axis, while the third principal strain is a contraction along the z-axis. The biologically relevant strain (i.e., the strain the cultured cells feel) is the first principal strain.
Figure 3.12: First principal strain across the BioFlex tissue culture membrane for piston displacements of 4mm to 10mm.

Figure 3.13: DIC microscopy images of fibroblasts before (left) and after (right) 24 hrs of 5% cyclic strain at 1Hz. The cells reorient themselves such that their long axis is perpendicular to the axis of radial strain (white arrow).
Figure 3.14: Scatter box plot of fibroblast orientations, measured as the inclination from the horizontal of the longest cell axis. Cells with no obvious longest axis were excluded from the analysis. Mean and standard deviations are indicated with red lines. Unstrained negative controls show no significant change in cell orientations, but strained cells align to a common orientation (normalized to 0 degrees) after 24 hours of exposure to 5% cyclic strain.
Chapter 4

Results

4.1 Validating the αMHC reporter

To verify that the αMHC reporter is capable of driving expression of green fluorescent protein (GFP), we tested its response to thyroid hormone. Previous work characterizing the αMHC gene had shown the existence of thyroid response elements in its 5’ upstream region [80]. It is thought that these thyroid response elements mediate the hormonal regulation of MHC isoform switching during development [49]. The thyroid hormones triiodothyronine (T3) and its prohormone, tetraiodothyronine (T4), have been used in vivo and in vitro to stimulate the expression of αMHC. More specifically, Gustafson et al. [30] used T3 to drive the expression of a transfected fusion gene (the αMHC promoter fused to chloramphenicol acetyltransferase (CAT)) in cultures of rat cardiomyocytes. The same group has shown that treatment of rat cardiomyocytes in vitro with T3 up-regulates αMHC and down-regulates βMHC [29]. Lompré et al. [46] showed that injection of T4 into rats has similar effects, increasing the transcription of αMHC mRNA and decreasing the transcription of βMHC.

4.1.1 Thyroid hormone fails to activate αMHC reporter transduced by lentivirus

We tested the ability of T3 and T4 to induce expression of αMHC. BJ fibroblasts were stably transduced with the αMHC reporter construct described in Section 2.2.2 (see Section 2.3 for detailed methods of lentivirus production and transduction).

Figure 4.1 and Figure 4.2 show the results of treatment of T3 and T4, respectively. The concentrations of T3 and T4 were based on reported values in the literature. Cells transduced with EGFP act as positive controls for both flow cytometry and the lentiviral transduction process. The emission from intracellular EGFP chromophores is captured using both the fluorescein isothiocyanate (FITC) and propidium iodide (PI) channels, which have bandpass filters around their emission spectrum peaks of 519nm and 617nm, respectively. This allows for 2-dimensional plots of emission intensity that better separate positive and negative populations. The emission profile of
Figure 4.1: Flow cytometric analysis of BJ fibroblasts stably transduced with the αMHC reporter. Treatment with various concentrations of T₃, the active form of thyroid hormone, does not result in increased expression of EGFP. FITC-A: fluorescein isothiocyanate channel emission intensity, arbitrary units. PI-A: propidium iodide channel emission intensity, arbitrary units. Despite achieving a viral transduction rate of ~72% and ~43% of the BJ fibroblasts (EGFP control group), negligible αMHC reporter driven fluorescence was observed from either the T₃ or T₄ treated groups. The transduced αMHC reporter is not inducible by thyroid hormone in the BJ fibroblast.

4.1.2 Thyroid hormone fails to activate αMHC reporter transfected using nucleoporation

We also examined the response of the the αMHC reporter plasmid to thyroid hormone. Figure 4.3 shows the result of treatment of transfected BJ fibroblasts with T₄. Cells were treated for 4 days be-
Figure 4.2: Flow cytometric analysis of BJ fibroblasts stably transduced with the αMHC reporter. Treatment with various concentrations of T₄, the circulating prohormone of thyroid hormone, does not result in increased expression of EGFP.

fore nucleoporation, then replated and treated for 2 more days before harvesting for flow cytometry.

Despite achieving a nucleofection rate of 30–40% of the BJ fibroblasts (EGFP control group), negligible αMHC reporter driven fluorescence was observed from the T₄ treated group. The transfected αMHC reporter is not inducible by T₄ in the BJ fibroblast. The lack of thyroid hormone inducibility in these constructs may be due to the difference in transcription factor profiles of BJ fibroblasts compared to primary cardiomyocytes or differentiated cardiomyocytes. Tsika et al. [80] speculated that the transcription factors required for T₃ inducibility are tissue-specific.

4.1.3 The αMHC reporter is activated by iPSC-derived cardiomyocytes

Spontaneously beating cardiomyocytes were derived from iPSCs using the techniques described in Section 2.1.5. On day 31 after the beginning of the differentiation protocol, the cells were trypsinized, transfected via nucleoporation with the αMHC reporter, and replated in tissue culture wells coated with gelatin and fibronectin. Three days after replating, the cells were trypsinized
Figure 4.3: Flow cytometric analysis of BJ fibroblasts transiently transfected with the αMHC reporter. Treatment with various concentrations of T₄, the circulating prohormone of thyroid hormone, does not result in increased expression of EGFP.

Figure 4.4 shows the expression of EGFP as measured by flow cytometry. Plotting the cells along the dimensions of forward scatter (FSC, proportional to cell size) and side scatter (SSC, proportional to cell granularity) reveals two distinct populations – low side scatter and high side scatter. The high side scatter population exhibits low transfectability (panel B), as only ~7% of EGFP-transfected cells were fluorescent. The low side scatter population exhibits high transfectability (panel C), with ~48% of EGFP-transfected cells fluorescing. The difference in transfectability may be partially due to the nucleofection program used, which was optimized for transfecting cardiomyocytes (see Section 2.2.1). The high side scatter population showed negligible αMHC reporter driven fluorescence, while a small proportion of low side scatter cells, ~4%, were definitively positive expressers of αMHC-driven EGFP. The high side scatter population’s low activation of the αMHC, as well as its low transfectability using the nucleofection program optimized for
cardiomyocytes, suggests that it is likely a fibroblast population. Assuming \( \sim 48\% \) of the low side scatter cells were successfully transfected with the \( \alpha \)MHC reporter, then one may estimate that \( \sim 8.8\% \) of this population (i.e., 4.23% divided by 48.5%) is positive for \( \alpha \)MHC as an indication of a cardiomyocyte-like cell type.

### 4.2 Primary cardiomyocytes lose core phenotypes \textit{in vitro}

We wanted to test the \( \alpha \)MHC promoter in a known cardiomyocyte to establish a true positive control. We purchased a cardiomyocyte cell line (Celprogen Catalog #36044-15) and transfected the \( \alpha \)MHC reporter using nucleofection. The results of this test are shown in Figure 4.5.

Though these cells showed a small amount of \( \alpha \)MHC-driven fluorescence, it was not the definitive result we expected for a supposed cardiomyocyte cell line. Given the transfectability rates of \( \sim 30\% \) and \( \sim 50\% \) for the two populations (demarcated by forward and side scatter profile), these cells only expressed \( \alpha \)MHC-driven fluorescence at rates of 3.6% and 5.0%. To verify the identity of the cell line we tested for the expression of cardiac-specific proteins. The protein of interest was cTnT (also known as cardiac troponin T2, or TNNT2), a core part of the contractile apparatus of cardiomyocytes that also contains troponin I, troponin C, and tropomyosin. Together, these proteins regulate myosin and its ability to generate force by ratcheting against actin.

Figure 4.6 shows the lack of expression of this protein in Celprogen cardiomyocytes. It is typical of cultures of primary human cardiomyocytes to rapidly lose cardiac phenotypes \textit{in vitro}, and these cells, though marketed as possessing cardiac-specific proteins, are no exception. Both early (p2) and late passage (p6) cells fail to express cTnT. To rule out overgrowth by rapidly proliferating fibroblasts, we cultured the same cells in medium containing cytosine arabinoside to suppress the growth of rapidly dividing cells. However, these cells cultured with this drug also failed to express cTnT. The lack of this core element of the cardiac contractile apparatus indicates that these cells are not reliable models of human cardiomyocytes.

### 4.3 Cyclic strain affects iPSC differentiation to cardiomyocytes

Inspired by the high efficiencies of \textit{in vivo} transdifferentiation, we asked if we can increase \textit{in vitro} cardiac differentiation efficiency by recreating some of the mechanical aspects of the cardiac microenvironment. To this end, we modified the monolayer differentiation method of Laflamme et al. [45], adding cyclic mechanical strain to explore whether this could speed up the differentiation process, convert more of the starting cell population to cardiomyocytes, generate more mature cardiomyocytes with more functional characteristics, or generate a more uniform population of cardiomyocytes.
Figure 4.4: Flow cytometric analysis of iPSC-derived cardiomyocytes transiently transfected with the αMHC reporter and EGFP as a positive control. A: Forward scatter (FSC) vs side scatter (SSC) separates the cells into two distinct populations – high and low side scatter. B: Cells with high side scatter do not exhibit good transfectability and do not activate the αMHC reporter. C: Cells with low side scatter have high transfectability. A small proportion of cells definitively express the αMHC-promoter driven EGFP.
Celprogen cardiomyocytes were nucleofected with the plasmid αMHC reporter to test promoter-driven EGFP activity. Untransfected cells were used as negative controls, while cells transfected with EGFP were used as positive controls. A: Forward and side scatter profile of the cells reveal two distinct populations, dubbed “singles” and “doubles”. B: The “singles” population was less transfectable, as shown by ~29% of cells expressing EGFP, and did not show αMHC-driven fluorescence to a significant degree. C: The “doubles” population was more transfectable, as shown by ~50% of cell expressing EGFP, but did not show any more αMHC-driven fluorescence.
Figure 4.6: Western blot assay for presence of cTnT in Celprogen human cardiomyocytes. Jurkat: a human T lymphocyte cell line used as a negative control. p6: Celprogen cardiomyocytes at passage 6. p2 (Ara-C): Celprogen cardiomyocytes at passage 2, maintained in medium containing cytosine arabinoside to suppress fibroblast growth. p2: Celprogen cardiomyocytes at passage 2. Mouse heart lysate was used as a positive control. PKA C-α, a housekeeping gene, was used as a loading control.

4.3.1 Cardiomyocyte differentiation is sensitive to cyclic strain parameters

Figure 4.7 shows the modified cardiac differentiation protocol. The iPSCs are cultured to 90% confluence in a well of the BioFlex plate in anticipation of the application of cyclic strain. Straining of the cells begins after the 4 days of BMP4 treatment (i.e., the end of the biochemical differentiation). The cyclic strain was applied continuously at 1Hz, with a peak piston displacement of 5mm, translating to an average strain of 5% in the plateau region of the membrane (Figure 3.12). A non-strained control well in the BioFlex plate was used, as well as a control well seeded on traditional tissue culture polystyrene.

Figure 4.8 shows a representative spontaneously beating cluster in the non-strained control well of the BioFlex plate. Whereas the non-strained control and the polystyrene control exhibited spontaneous beating 10 days after the start of Activin A treatment, the strained well never exhibited spontaneous beating while strain was applied. However, after cyclic strain was stopped, 19 days after the start of Activin A treatment, spontaneous beating was observed. This suggests that the strain exerted by the HexCycler was in a physiologically appropriate range to interfere with excitation-contraction coupling. Cyclic strain also had an effect on the formation of the cardiomyocyte contractile appara-
**Figure 4.7:** A schematic of the differentiation protocol used to generate cardiomyocytes from iPSCs. Cells are seeded on Matrigel on Day 0 and reach 90% confluence by Day 4. Activin A (100ng/mL) is applied for 24 hours, followed by 4 days of BMP4 treatment (10ng/mL) without medium change. After BMP4 treatment, 5% cyclic strain is applied continuously at 1Hz using the HexCycler.

**Figure 4.8:** A: A spontaneously beating cluster of cardiomyocytes. The yellow line indicates the region used for kymograph analysis. B: Kymograph analysis gives a beat frequency of 0.35Hz.

tus, as shown by immunocytochemistry. **Figure 4.9** shows staining of iPSC-derived cardiomyocytes. Cell nuclei are stained blue by DAPI, which binds to regions of DNA rich in adenine and thymine. The proteins of interest are stained green. Those nuclei not surrounded by green belong to non-cardiomyocytes, most likely cells that have differentiated to a cardiac fibroblast lineage. Both the unstrained and strained cells stained positively for cTnT, but the strained cells exhibited punctate staining while the unstrained cells had more filamentous staining. Sarcomeric striations revealed by α-actinin staining were common in the unstrained specimen, but much rarer in the unstrained specimen. This suggests that the strain regimen hindered the maturation of these cardiomyocytes.
Figure 4.9: Cyclic strain affects the formation of sarcomeric striations. Anti-α-actinin antibody was used to stain for sarcomeric striations. Striations are common in the unstrained specimen (inset) but much rarer in the strained specimen. DAPI stains cell nuclei blue, while green indicates either cTnT or α-actinin.
Chapter 5

Discussion and conclusion

5.1 Reporters of cardiac-like myocytes

Based on previous work characterizing the thyroid response elements present in the αMHC promoter [80], we expected thyroid hormone to be able to activate the αMHC reporter, even in non-cardiac cells. We wanted to activate the αMHC-EGFP fluorescence reporter with thyroid hormone to establish a baseline reading as a positive control. However, we determined that the αMHC reporter is not inducible by thyroid hormone in non-cardiac cells. This suggests that, in addition to thyroid hormone, other unknown cardiac-specific transcription factors are required to mediate the response of the αMHC promoter to thyroid hormone.

We delivered the αMHC reporter into BJ fibroblasts using both lentiviral transduction and transfection using nucleoporation. The lentiviral transduction system co-opts the gene delivery mechanisms of the human immunodeficiency virus to create stably transduced cells. Both gene transfer techniques are capable of generating cells that stably express the transgene, though lentiviral transduction can achieve higher efficiency than nucleofection [11]. The strong electric fields used to permeabilize cell membranes in nucleofection also result in a significant amount of cell lysis, and not all permeabilized cells successfully take up plasmid DNA. Both these factors contribute to the lower transfection efficiency of nucleofection compared to lentiviral transduction.

When transfected, the αMHC fluorescence reporter is not significantly activated by iPSC-derived cardiomyocytes, with only 8.8% of cells fluorescing. Previous reports have used lentiviral transduction to stably integrate this reporter into pluripotent stem cells prior to differentiation to cardiac lineages [27, 35, 44]. For example, Fu et al. [27] report that 18.1% ± 11.2% of human fibroblasts transduced with cardiac-transdifferentiating factors activated a similar αMHC fluorescence reporter. Future work with this reporter may require such lentiviral methods.

Laflamme et al. [45] were able to achieve over 50% βMHC-positive cells using the same monolayer differentiation protocol used in our experiments, but they began with human embryonic stem
cells. This is likely an over-estimate of the differentiation efficiency, as it was determined by visual inspection of fixed and stained specimens that obscures the underlying support layer of fibroblast-like cells. βMHC is the isoform of MHC predominantly expressed in the human ventricle, while αMHC is predominantly expressed in the atrium. Thus the low activation of our αMHC reporter may be a reflection that the cardiac differentiation protocol generates predominantly ventricular myocytes [64]. Much of the difference in differentiation efficiency could also be accounted for by epigenetic variability between starting cells. Kattman et al. [41] demonstrated that human embryonic stem cells lines have different cardiac differentiation capacities, and it has been speculated that iPSCs have similar if not greater variability [10]. iPSC cell line variability may be due to the cells used to create the cell line [50], as well as the passage history [61].

That said, activation of the reporter only represents a single aspect of the cardiac phenotype. A more rigorous profiling of gene expression, and functional testing of electrophysiology and calcium handling characteristics will be required to fully assess the maturity of differentiated cells.

5.2 The effects of cyclic strain on cardiomyocyte differentiation and maturation

Organogenesis is a delicately choreographed event that is sensitive to changes in timing. We showed that the application of ~5% cyclic strain at 1Hz at the end of biochemical differentiation in the monolayer method of cardiac differentiation inhibits spontaneous contractions and disrupts the formation of sarcomeres. It remains to be seen whether altering the timing, frequency, and/or magnitude of strain may speed up or improve differentiation. The HexCycler, with its 6 independently driven actuators, is well-positioned to facilitate the optimization of these parameters.

The presence of spontaneously contracting clusters of cardiomyocytes indicates the presence of pacemaker cells (also known as nodal cells, for the sinoatrial node). These pacemaker cells entrain the nearby myocytes to contract in synchrony. Though not definitively shown with immunofluorescence staining, the presence of these multicellular synchronously beating islands implies the successful formation of intercalated discs between cardiomyocyte. These cell interfaces consist of adherens junctions, desmosomes, and gap junctions and enable the cells to form a electrochemical syncytium.

Immunocytochemistry and bright-field microscopy reveals that the cardiomyocytes are supported by a layer of non-myocytes, likely cells that have differentiated to the cardiac fibroblast lineage. These cells, along with the pacemaker cells that drive the spontaneously beating cell clusters, are indicative of the heterogeneity of the cell population. Each tissue culture well likely contains cardiac fibroblasts, nodal cells, atrial myocytes, and ventricular myocytes.

Though the inhibition of spontaneous contraction coincided with lack of sarcomeres in our experiments, the lack of spontaneous beating may counterintuitively indicate greater maturity – it has been shown that primary myocytes from embryonic hearts beat spontaneously in vitro [22], while
those isolated from adult hearts do not beat without stimulation [2, 58]. This is attributed to the
greater expression of certain ion channels as cardiomyocytes mature, which alters the electrophysi-
ology and thus the excitation-contraction threshold of cells [19, 69].

5.3 Potential applications of in vitro cardiomyocytes and future
directions

A systematic review of the global burden of disease revealed that cardiomyopathy and myocarditis
killed an estimated 443,300 people in 2013, an increase of 51% over the number of deaths in 1990.
The same study puts the number of deaths attributable to all heart disease, including rheumatic heart
disease, ischaemic heart disease, hypertensive heart disease, atrial fibrillation, and endocarditis, at
over 10.1 million [56]. The uses of a reliable source of in vitro cardiomyocytes to understand and
treat these diseases are numerous. I discuss some of these applications below.

5.3.1 Using iPSC-derived cardiomyocytes to model cardiomyopathies

Great strides have been made in using iPSCs from patients with known cardiomyopathies to model
disease and gain insights into possible treatments. For example, Itzhaki et al. [36] used these tech-
niques to study dermal fibroblasts from a patient with long QT syndrome to generate iPSCs, then
differentiated these iPSCs to cardiomyocytes. (Long QT syndrome is named for the extension of the
time interval between the Q and T waveforms of the electrocardiogram.) With this high-fidelity in
vitro model of patient-specific disease, they were then able to test existing and novel pharmacologi-
cal agents to see which had positive effects on the disease phenotype. Perhaps even more exciting is
the prospect of modelling cardiac diseases without a known genetic component, and the modelling
of the response of cardiac tissues to infectious agents.

5.3.2 Mature cardiomyocytes for drug toxicity testing

In the 1990s, several drugs were withdrawn from the market after it was revealed that they were
responsible for multiple unexpected cardiac deaths [14]. The pharmaceutical industry later learned
that these drugs adversely affected ion channels that altered the electrophysiology of cardiomy-
ocytes, which prolonged the QT interval and put the patient at risk of fatal arrhythmias, much like
patients with hereditary long QT syndrome. In response to these deaths and the regulations that
followed, the pharmaceutical industry has spent hundreds of millions of dollars testing candidate
drugs for cardiotoxicity. Lacking a reliable in vitro model, the current standard of testing involves
a “thorough QT study” in which healthy human volunteers are exposed to the drug to see if it pro-
longs their QT interval. This testing procedure is arduous, expensive, and worst of all, puts healthy
volunteers at risk.

iPSC-derived and transdifferentiated cardiomyocytes have the potential to dramatically increase
the fidelity of these tests while decreasing costs. While such *in vivo* models of the heart may not be able to recreate a full electrocardiogram, one may use patch-clamp techniques to measure the effect of drugs on a cardiomyocyte’s ion channel conductances. It is known, for example, that inhibition of the human ether-à-go-go-related gene (hERG) potassium ion channel can lead to long QT syndrome [14]. However, the accuracy of these models depends on the ability to generate mature cardiomyocytes, as many of the ion channels targeted by cardiotoxic drugs are only expressed in adult cells.

### 5.3.3 Cellular therapy for heart failure

Overcoming the poor regenerative potential of cardiomyocytes could revolutionize the treatment of heart failure. Robey et al. [66] estimated that 1 billion new cardiomyocytes are needed after myocardial infarction to restore function. The source of these cells could be cardiomyocytes generated from directed differentiation of iPSCs or cardiomyocytes transdifferentiated from native cardiac fibroblasts. Several groups showed that *in situ* transdifferentiation of cardiac fibroblasts to cardiomyocytes can modestly improve cardiac function in mice after induced myocardial infarction [40, 63, 73]. Such *in situ* techniques do not have to contend with the problem of cell engraftment, but those protocols that involve reprogramming somatic cells to iPSCs followed by directed differentiation to cardiomyocytes run the risk of generating teratomas [1].

Alternatively, cardiomyocytes may be produced *in vitro* and implanted to a failing heart to restore function. Such a technique may use also the patient’s own cells as starting materials to avoid immunological rejection. Much work remains to be done to ensure that the transplanted cells engraft to the existing myocardium, and that homogeneous populations of cells are transplanted to match their targeted repair site and avoid ectopic pacemakers that may be arrhythmogenic.

### 5.3.4 Tissue engineering

Tissue engineering is defined as the growth of three-dimensional tissues *in vitro*, with the ultimate goal of creating a fully functional organ by directing the proliferation, differentiation, and remodelling of cells. The current state of the art of directed cardiac differentiation remains far removed from realizing this goal, though engineered cardiac constructs (loops of cardiac tissue) have been employed in animal models to improve cardiac function after infarction [89]. Fine control over the results of cardiac differentiation, in terms of the mixture of cell types and maturity, will be needed for the success of such a construct in humans.

### 5.3.5 Future directions

Problems of iPSC variability leading to poor reproducibility of differentiation have plagued our experiments, as well as those of our collaborators. The mTeSR1 medium used in the culture of our
iPSCs contain bovine serum albumin, which is known to exhibit considerable variability from lot to lot [51]. Indeed, the creators of the mTeSR1 medium acknowledge this variability and emphasize rigorous quality control [47]. More recently, Chen et al. [12] developed a chemically defined, albumin-free medium called E8 that has been shown to able to sustain iPSC pluripotency in culture. Use of E8, in conjunction with a recombinant truncated version of vitronectin (an ECM glycoprotein) as an alternative to Matrigel, may reduce some of the variability seen in our experiments.

The applications in Section 5.3 could all benefit from a reliable method to separate and quantify the cardiac cell populations (atrial myocytes, ventricular myocytes, nodal cells, and cardiac fibroblasts). Such a method would also be crucial for the optimization of differentiation protocols, to determine the effects of novel stimuli. Such quantification and separation of cell populations may be done with flow cytometry, employing a panel of cell-type–specific antibodies. For example, αMHC and βMHC may be used as a rough marker of atrial and ventricular myocyte populations, respectively [64], while potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) may be used as a marker of nodal cells [16], and thymocyte differentiation antigen 1 (THY1) may be used as a marker of cardiac fibroblasts [32]. Such a high-throughput quantitative method could be complemented with more detailed imaging and functional measures, such as immunofluorescence imaging, calcium flux imaging and patch-clamping. The presence of calcium-handling proteins, such as the ryanodine receptor, calsequestrin, and phospholamban, can be verified with RT-PCR and immunofluorescence imaging. The use of cell-type specific markers can also verify whether the high side-scatter population of Figure 4.4 is of a fibroblast, endothelial, or some other lineage.

We have observed improvements in cardiomyocyte beating synchrony and increases in the size of spontaneously beating clusters over the period of several weeks in culture. We postulate that this may be due to the electromechanical influence of more mature cardiomyocytes on less mature cells. As the more mature cardiomyocytes contract, they pull on adjacent cells and depolarize their membranes (if the cells have formed electrochemical couplings via gap junctions). This may be enough to tip the immature cell towards a cardiac lineage. The combination of electrical signalling and mechanical stretch presents another dimension for bioengineers to recreate the cardiac microenvironment. More specifically, the tissue culture well can be outfitted with electrodes to create an electrochemical cell. The experimenter can then bias the two electrodes to create an electric field and force the cells to depolarize.

In both cyclic mechanical stretch and entrained electrical depolarization, the nascent cardiomyocyte will no doubt be sensitive to parameters of frequency and magnitude. The cell can be thought of as a simple harmonic oscillator with a time-varying natural frequency. If we perturb the system at the correct frequency, with a gentle enough magnitude not to obliterate delicate internal structures and balances, the cell may respond by developing the phenotypes of a mature cardiomyocyte. The aim of future work will be to find these natural frequencies.
5.4 Conclusion

The field of cardiac engineering has advanced considerably over the last decade, and exciting advances in bioreactor design are imminent. The problems to be solved at this juncture of engineering and cell biology will no doubt be of interest to interdisciplinary scholars of the future.
Bibliography


Appendix A

Design and fabrication

Computer aided design (CAD) was done with NX (Siemens). Prototypes were fabricated in the UBC Department of Mechanical Engineering Student Machine Shop. An iterative design and prototyping process was followed, with rapid prototyping facilitated by the use of the Asiga Pico 3D printer and the OMAX® MAXIEM abrasive waterjet cutter. The NX modelling module was used to create parts for 3D printing. Files were exported to a stereolithography format (STL) compatible with the Asiga Pico 3D printer. The NX sheet metal module was used to create parts for waterjet cutting. Sheet metal flat pattern files were exported to a CAD format compatible with the Intelli-MAX Layout software (DXF). The DXF files were used to create tool paths for the waterjet cutter in OMAX Layout. Cut quality was set to 3 (medium) and tabs were created after using the auto-path tool to hold work pieces in place. The tool paths were saved as ORD files and transferred to the OMAX MAXIEM waterjet cutter. Device components were waterjet cut out of gauge 14 and gauge 6 aluminum sheet metal (6061-T6). Bending of the sheet metal was done with a box-and-pan brake. The toggle clamp of the HexCycler was purchased from McMaster-Carr (Catalog #5128A39).

Open-source CAD files for the design of the HexCycler are available at [https://github.com/paradeofwolves/HexCycler](https://github.com/paradeofwolves/HexCycler). Drawings for the components of the HexCycler are included in Section A.1.

A.1 Drawings of HexCycler components
Bend Radius = 3.00
Bend Angle = 90.00
Bend Direction = down

HexCycler Top Plate

DATE: 2015-05-15
DRAWN BY: Eric Zhao
MATERIAL: 6061-T6
MATERIAL THICKNESS: 1.63

SCALE: 1:2
FILENAME: top_plate
SHEET REV: A

ALL DIMENSIONS IN MM
Bend Radius = 3.00
Bend Angle = 90.00
Bend Direction = up

Bend Radius = 3.00
Bend Angle = 90.00
Bend Direction = down

a place of mind

BioFlex Bracket

DATE 2015-05-15
DRAWN BY Eric Zhao
MATERIAL 6061-T6
MATERIAL THICKNESS 1.63

SCALE 2:1
FILENAME flexcell_holder_flange
SHEET REV A

Scale 1:1
ALL DIMENSIONS IN MM
Motor Holder Bottom

Bend Radius = 3.00
Bend Angle = 90.00
Bend Direction = up

Scale 1:1

Title Block:
- Date: 2015-05-15
- Drawn By: Eric Zhao
- Material: 6061-T6
- Material Thickness: 1.63
- Scale: 2:1
- All Dimensions in mm
Bend Radius = 3.00
Bend Angle = 90.00
Bend Direction = down
a place of mind

Motor Disc

DATE: 2015-05-15
DRAWN BY: Eric Zhao
MATERIAL: 6061-T6
MATERIAL THICKNESS: 4.06

SIZE: A4
FILENAME: yoke_disc
SCALE: 5:1
SHEET REV: A

ALL DIMENSIONS IN MM
Appendix B

Tissue culture protocols

B.1 iPSC maintenance and differentiation protocols

We used a protocol for culturing pluripotent stem cells that does not rely on a supporting layer of non-pluripotent feeder cells. The lack of a feeder layer necessitates a layer of ECM proteins to facilitate the attachment of the iPSCs. We used a thin coating of Matrigel as this protein coating. Matrigel is frozen below -20°C, liquid at 4°C, and gels rapidly at room temperature. Thus Matrigel should be kept frozen until ready for aliquoting and should be kept on ice during aliquoting.

B.1.1 Aliquoting Matrigel

1. One day before aliquoting, place 4 centrifuge racks and 1 sterile container of 1.5mL tubes in the -80°C freezer.

2. Calculate the volume of Matrigel needed per tube. The recommended coating density is 0.5mg per 6 well plate, so aliquots of 0.25mg, 0.5mg, and 1mg should be made. Matrigel concentration is measured in mg/mL and varies by lot.

3. Based on the volumes calculated above, place unopened box(es) of the appropriate sized pipette tips in the -20°C freezer.

4. Place the bottle of Matrigel in a covered container filled with ice in a 4°C refrigerator overnight. Make sure that the neck of the bottle is not submerged in ice.

5. Fill two small containers with ice and place both in the sterile biosafety cabinet. One container will hold the Matrigel bottle, while the other will hold the box of pipette tips.

6. Open the metal seal on the Matrigel bottle using the sharp end of a scoopula. Carefully remove the rubber cover of the bottle and place the bottle on ice.

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7. Retrieve one centrifuge tube rack from the freezer, place in the biosafety cabinet, and fill with microtubes.

8. Aliquot Matrigel into each tube according to the volumes calculated above. Switch pipette tips every 5 tubes to keep the Matrigel cold and ensure sterility.

9. When the centrifuge tube rack is filled, label and transfer tubes to the -20°C freezer and retrieve a new rack. Work quickly to prevent the Matrigel from congealing.

B.1.2 Plating Matrigel

Matrigel cannot be thawed and refrozen, so each aliquot must be used at one time. However, excess Matrigel may be plated and used within 7 days.

1. Place a sterile 15mL conical tube, one P1000 pipetman, and a cold, sterile bottle of DMEM/F-12 media into the sterile biosafety cabinet.

2. Remove one aliquot of Matrigel from the freezer and add 1mL of DMEM/F-12 to the tube.

3. Gently pipette up and down to thaw and dissolve the Matrigel. Transfer to 15mL conical tube.

4. Add additional DMEM/F-12 to bring the total volume to 1mL for each well to be coated. Plate 1mL per well.

5. Tilt the well in orthogonal directions to fully cover the surface and allow to set for 1 hour at room temperature in the biosafety cabinet.

6. Wells may be used after the 1-hour coating, or stored for later use. Wells may be stored by adding 1mL of DMEM/F-12 to each well and placing in a 37°C incubator, or wrapping in parafilm and storing in the 4°C refrigerator. Stored wells must be used within 7 days.

7. If using plates that have been stored at 4°C, warm to room temperature in the biosafety cabinet for 1 hour before introducing cells.

B.1.3 Preparing complete mTeSR1 medium

The mTeSR1 medium is shipped in two components: basal medium and 5X supplement. The complete medium is made by adding the 5X supplement to the basal medium. The 5X supplement contains recombinant proteins that degrade rapidly at high temperatures and with repeated freeze-thaw cycles. For this reason, the supplement and the complete medium are kept frozen until use, and should not be warmed in a 37°C water bath. Avoid rough agitation of the medium or supplement that may form bubbles. The complete medium may be stored in the 4°C refrigerator for up to 2 weeks, or frozen at -20°C for up to 6 months.
1. Thaw the 5X supplement overnight in the 4°C refrigerator and mix thoroughly by gently inverting.

2. In a biosafety cabinet, add 100mL of the 5X supplement to 400mL of the basal medium. Mix thoroughly by gently inverting.

3. Aliquot the complete medium into sterile 50mL conical tubes and store in the -20°C freezer.

4. When needed, thaw an aliquot of the complete medium at room temperature or overnight at 4°C.

5. Unused medium can be stored at 4°C for up to 2 weeks, but should not be refrozen.

B.1.4 Preparing ROCK inhibitor stock solution

We used the small-molecule ROCK inhibitor Y-27632 (Figure B.1) to decrease anoikis and increase cell viability after dissociation.

1. Spin down the vial of Y-27632 powder at 10,000 rpm for 1 minute.

2. Place a P1000 pipetman in the biosafety cabinet.

3. Dissolve the Y-27632 powder in 624µL of PBS to create a 5mmol L⁻¹ stock solution.

4. Aliquot the stock solution to 3 microtubes of 208µL each.

5. Store the aliquots at -20°C. The solution is stable for up to 6 months.

B.1.5 Thawing iPSCs

Wear eye protection when handling vials that may have been submerged in liquid nitrogen, as these vials may explode when warmed.
1. Place a sterile 15mL conical tube, one 6-well plate coated with Matrigel, one P1000 pipetman, an aliquot of ROCK inhibitor, and mTeSR1 medium warmed to room temperature in the biosafety cabinet.

2. Add 1.5mL of mTeSR1 medium to each well to be plated with cells.

3. Replace the aspiration Pasteur pipette with a new sterile pipette.

4. Remove the vial of iPSCs from the liquid nitrogen dewar and roll it between your gloved hands for 15 seconds to remove frost.

5. Immerse the vial in a 37°C water bath without submerging the cap, swirling gently.

6. When only an ice crystal remains, remove the vial from the bath.

7. Ensure the cap is tight and immerse the vial into a 95% ethanol bath to sterilize the outside of the tube.

8. Air-dry the tube in the biosafety cabinet for 30 seconds.

9. Transfer the cells to the sterile 15mL conical tube using the P1000 pipetman.

10. Add 9mL of complete mTeSR1 medium drop-wise to the cells, gently rocking the tube while doing so to mix the cells and reduce osmotic shock.

11. Centrifuge the cells at 200 x g for 5 minutes.

12. Aspirate and discard the supernatant.

13. Using a 5mL pipette, re-suspend the cell pellet in 0.5mL mTeSR1 medium for every well that will receive cells.

14. Gently pipette cells up and down in the tube 3 times.

15. Slowly add 0.5mL of the cell suspension drop-wise into each well.

16. Add 4µL of 5mmol L⁻¹ ROCK inhibitor stock solution to each well to reach a final concentration of 10µmol L⁻¹.

17. Label the plate with the cell line, the passage number from the vial, the date, and your initials.

18. Place the plate in the incubator and gently rock the plate in orthogonal directions to evenly distribute the cells. Avoid circular motions to prevent the cells from pooling in the centre of the well.
Figure B.2: The appearance of iPSC colonies 1 day after plating (left) and immediately prior to passaging (right).

B.1.6 Feeding iPSCs

iPSC medium must be renewed daily until the cells are ready for freezing, passaging, or experimentation.

1. Observe the pluripotent stem cells using a microscope. If they require passaging, follow the procedures in Section B.1.7.

2. Warm a tube of mTeSR1 medium to room temperature in the biosafety cabinet for 15 minutes.

3. Replace the aspiration Pasteur pipette with a new sterile pipette.

4. Aspirate the spent medium with the sterile Pasteur pipette and a P200 pipette tip. Use a different pipette tip for each well to reduce the risk of contamination.

5. Add 2mL of fresh mTeSR1 medium to each well. Pipette against the side wall of the culture well to reduce fluid shear forces on the cells.

6. Return the plate to the 37°C incubator.

B.1.7 Passaging iPSCs

iPSCs are ready for passaging when contiguous colonies of cells reach a size of greater than 1mm in its widest dimension, as shown in Figure B.2. Passaging is also required when cells show visible morphological changes indicative of differentiation. We use EDTA (Versene, 0.02%) for passaging, which is a gentle method that gives clusters of cell aggregate instead of fully dissociated cells. The passage ratio can vary between 1:8 and 1:20, depending on the density of cell colonies after plating.

1. Warm a tube of mTeSR1 medium to room temperature in the biosafety cabinet for 15 minutes.
2. Place a P10 pipetman, EDTA solution, and an aliquot of ROCK inhibitor in the biosafety cabinet. A P200 pipetman and 15mL sterile conical tube may also be needed if the split ratio is high.

3. Replace the aspiration Pasteur pipette with a new sterile pipette.

4. Aspirate the Matrigel plating medium from each well to be seeded with new cells.

5. Add 2mL of mTeSR1 medium to each well.

6. Label the new plate with the cell line name, the new passage number, the date, split ratio, and your initials.

7. If harvesting from more than one well, stagger treatment with EDTA to avoid overexposure.

8. Aspirate the spent medium with the sterile Pasteur pipette and a P200 pipette tip. Use a different pipette tip for each well to reduce the risk of contamination.

9. Rinse each well with 1mL room temperature EDTA and aspirate.

10. Treat each well with 1mL room temperature EDTA for 7–9 minutes.

11. Aspirate the EDTA carefully, without disturbing the attached cell layer. If cells become free-floating, collect and spin down, remove EDTA, and resuspend with mTeSR1.

12. Using 3mL of mTeSR1 medium per well, hold a 5mL pipette perpendicular to the plate and gently wash the cells off the plate. Repeat if necessary, but do not use more than 3mL of medium per well to avoid contamination. Pipette gently to avoid bubbles, and avoid touching the cells with the tip of the pipette.

13. If harvesting from more than one well, use the same volume of medium to remove cells.

14. If the split ratio is high and the volume of cell suspension is small, pool the cell suspension in a sterile conical plate and use a pipetman for the next step. Otherwise, use a 5mL pipette.

15. Add the appropriate volume of cell suspension drop-wise to each well of the new plate.

16. Add ROCK inhibitor to each well to reach a final concentration of 10µmol L⁻¹.

17. Place the plate in the incubator and gently rock the plate in orthogonal directions to evenly distribute the cells. Avoid circular motions to prevent the cells from pooling in the centre of the well.

18. While cells are attaching, limit opening and closing of the incubator. If access is required, open and close the door gently.
B.1.8 Freezing iPSCs

1. Obtain a room-temperature isopropanol freezing container; the isopropanol must be replaced every 5 uses.

2. In the biosafety cabinet, label cryovials with the cell line, passage number (1 more than the number on the plate), freeze date, and your initials. Use a pen or labels that resist liquid nitrogen and ethanol.

3. Thaw the mFreSR1 cryopreservation medium in the biosafety cabinet.

4. Place a P1000 pipetman, 50mL sterile conical tube, and EDTA solution in the biosafety cabinet.

5. Replace the aspiration Pasteur pipette with a new sterile pipette.

6. If freezing more than one plate, stagger EDTA treatment to avoid overexposure.

7. Aspirate the spent medium with the sterile Pasteur pipette and a P200 pipette tip. Use a different pipette tip for each well to reduce the risk of contamination.

8. Rinse each well with 1mL room temperature EDTA and aspirate.

9. Treat each well with 1mL room temperature EDTA for 7–9 minutes.

10. Aspirate the EDTA carefully, without disturbing the attached cell layer.

11. Gently wash cells off using 3mL of mFreSR1 medium for each plate, transferring the medium from well to well.

12. Pool the cell suspension in the 50mL sterile conical tube.

13. Repeat harvest for any remaining plates and pool all the cells to create a uniform lot.

14. Add mFreSR1 medium to reach desired freezing density. One 6-well plate can be frozen into 10 cryovials, using a total of 10mL of mFreSR1 medium.

15. Use a 5mL pipette to gently mix the cell suspension.

16. Using the same pipette, aliquot 1mL of cell suspension to each prepared cryovial. Mix the pooled cells every 5 vials to maintain even distribution.

17. Place the cryovials into the isopropanol freezing container and place the container in the -80°C freezer overnight.

18. The next day, transfer the cryovials to liquid nitrogen storage.
B.1.9 Preparing Activin A and BMP4 stock solutions

Recombinant human Activin A and BMP4 were purchased from R&D Systems and are shipped in lyophilized powder form.

1. Create 5mL of sterile HCl solution and 5mL of sterile HCl solution containing 0.1% bovine serum albumin (BSA). Sterilize the solutions using a 0.2µm filter.

2. Dissolve 10µg of lyophilized Activin A in 100µL of sterile 4mmol L⁻¹ HCl solution to create a 100µg mL⁻¹ stock solution.

3. Make 10µL aliquots of the Activin A stock solution. This solution is stable for up to 3 months at -20°C.

4. Dissolve 10µg of lyophilized BMP4 in 200µL of sterile 4mmol L⁻¹ HCl solution containing 0.1% BSA to create a 50µg mL⁻¹ stock solution.

5. Make 5µL aliquots of the BMP4 stock solution. This solution is stable for up to 3 months at -20°C.

6. Dilute the 50µg mL⁻¹ stock BMP4 solution using sterile 4mmol L⁻¹ HCl solution containing 0.1% BSA to create a 5µg mL⁻¹ working solution.

B.1.10 Preparation of B-27–supplemented media

The differentiation of iPSCs is conducted in RMPI-1640 medium containing B-27 complete supplement, and B-27 supplement without insulin. Section 2.1.5 has details of when each supplement is required. B-27 is shipped frozen at a 50X concentration and should be protected from light.

1. Thaw B-27 supplements at 4°C.

2. Make 400µL and 100µL aliquots of B-27.

3. Label the microtubes with the type of supplement (B-27 with or without insulin), the volume, and the date.

4. When needed, thaw an aliquot of B-27 in the biosafety cabinet. The 100µL aliquot makes 5mL of complete medium, while the 400µL aliquot makes 20mL of complete medium.

5. B-27 supplement should not be freeze-thawed more than twice.

6. Cover a sterile conical tube with aluminum foil and label with the medium and supplement name, the date, and your initials. Place the tube in the biosafety cabinet.

7. Pipette 5mL or 20mL of RPMI-1640 into the covered conical tube.
8. Using a P1000 or P200 pipetman, transfer the B-27 supplement to the RPMI-1640 medium.

9. Gently invert the tube to mix, taking care not to form bubbles.

10. Complete RPMI-1640 medium with B-27 supplements is stable at 4°C for up to 7 days.

11. Prior to addition to cells, the complete RPMI-1640 medium with B-27 supplements should be warmed to room temperature, not in a 37°C water bath.