Role of non-myocytes in engineering of highly functional pluripotent stem cell-derived cardiac tissues

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2013
ABSTRACT

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Abstract

Massive loss of cardiac tissue as a result of myocardial infarction can create a poorly-conducting substrate with impaired contractility, ultimately leading to heart failure and lethal arrhythmias. Recent advances in pluripotent stem cell research have provided investigators with potent sources of cardiogenic cells that may be transplanted into failing hearts to provide electrical and mechanical support. Experiments in both small and large animal models have shown that standard cell delivery techniques suffer from poor retention and engraftment of cells. In contrast, the transplantation of engineered cardiac tissues may provide improved cell retention at the injury site, creating a more localized paracrine effect and yielding more efficient structural and functional repair. However, tissue engineering methodologies to assemble cardiomyocytes or cardiac progenitors into aligned, 3-dimensional (3D) myocardial tissues capable of physiologically relevant electrical conduction and force generation are lacking. The objective of this thesis was thus to develop a methodology to generate highly functional engineered cardiac tissues starting from pluripotent stem cells.

To accomplish this goal, we first derived purified populations of cardiac myocytes from mouse embryonic stem cells (mESC-CMs) by antibiotic selection driven by an α-myosin heavy-chain promoter. Culture conditions that yielded robust mESC-CM electrical coupling and fast action potential propagation were optimized in
confluent cell monolayers. We then developed a microfabrication-based tissue engineering approach to create engineered cardiac tissues ("patches") with uniform 3D cell alignment. We found that, unlike in monolayers, mESC-CMs required a population of supporting cardiac fibroblasts to enable the formation of 3D engineered tissues. Detailed structural, electrical and mechanical characterization demonstrated that engineered cardiac patches consisted of dense, uniformly aligned, highly differentiated and electromechanically coupled mESC-CMs and supported rapid action potential conduction velocities between 22 – 25cm/s and contractile force amplitudes of up to 2mN.

Next, we sought to circumvent the use of primary cardiac fibroblasts by utilizing a single pluripotent stem cell-derived source, multipotent cardiovascular progenitors (CVPs) capable of differentiating into vascular smooth muscle and endothelial cells in addition to cardiomyocytes. CVPs were derived from mouse embryonic stem cells and induced pluripotent stem (iPS) cells by antibiotic selection driven by an Nkx2-5 enhancer element. Similar to mESC-CMs, CVPs formed highly differentiated cell monolayers with electrophysiological properties that improved with time in culture to levels achieved with pure mESC-CMs. However, unlike mESC-CMs, CVPs formed highly functional 3D engineered cardiac tissues without the addition of cardiac fibroblasts, enabling engineered cardiac tissues to be formed from a single, entirely stem cell-derived source.
Finally, we explored mechanisms of synergistic cardiac fibroblast/myocyte signaling in 3D engineered tissues by using cardiac fibroblasts of different developmental stages in the settings of direct 3D co-culture as well as in conditioned media studies. When co-cultured with fetal cardiac fibroblasts, mESC-CMs were capable of two-fold faster action potential propagation and 1.5-fold higher maximum contractile force generation than when co-cultured with adult cardiac fibroblasts. These functional improvements were associated with enhanced mESC-CM spreading and upregulation of important ion channel, coupling, and contractile proteins. Conditioned medium studies revealed that compared to adult fibroblasts, fetal cardiac fibroblasts secreted distinct paracrine factors that promoted mESC-CM spreading and spontaneous contractility in 3D engineered tissues and acted via the MEK-ERK pathway. Quantitative gene expression analysis revealed paracrine factor candidates that may mediate this action.

In summary, this thesis presents methods and underlying mechanisms for generation of highly functional cardiac tissues from pluripotent stem cell sources. These techniques and findings provide foundation for future engineering of human ES and iPS cell-based cardiac tissues for therapeutic and drug screening applications.
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1. Introduction

Myocardial infarction due to coronary artery occlusion results in significant loss of working cardiomyocytes and impaired cardiac output. Since resident cardiac stem cells and circulating progenitor cells are only able to contribute minimally to tissue regeneration, the human heart has limited capacity to regenerate itself after injury, often resulting in pathological remodeling and heart failure. Approximately 1.3 million Americans suffer from myocardial infarction every year, and approximately 5.7 million are afflicted with heart failure\(^1\), resulting in an estimated total cost to society of approximately $300 billion.

In general, the process of healing after an injury typically involves the infiltration of inflammatory cells to clear out dead tissue and to release cytokines that signal to fibroblasts and endothelial cells. Activated fibroblasts secrete granulation tissue, forming a rich environment for endothelial cells to form a new vascular bed. In tissues such as the liver, skin and skeletal muscle, resident progenitor cells differentiate into functional cells, completing the wound healing process. However, the heart often undergoes pathological wound healing because (1) it has a very limited resident progenitor pool, and (2) it is under a constant requirement to produce mechanical work. As a result, there is slippage of collagen and muscle fibers in the weakened segment of the heart, leading to dilation of the ventricle and further damage. The heart has various
short-term compensatory mechanisms to cope with the increased workload placed on the remaining tissue, such as increase in beta adrenergic stimulation and hypertrophy of muscle tissue, but these eventually lead to further pathological remodeling and death.

Nevertheless, there is encouraging evidence from non-mammalian vertebrate systems as well as in fetal and neonatal mammals that corrective cardiac wound healing can take place. Zebrafish and salamanders have been shown to regenerate significant portions of their hearts through the de-differentiation, proliferation and re-differentiation of existing cardiac cells. Fetal sheep and neonatal mice have recently been shown to be capable of recovering from myocardial infarction and resection of the ventricular apex. In general, it appears that systems such as immature hearts which contain a significant population of progenitor cells capable of replacing lost muscle tissue are capable of corrective cardiac wound healing.

Cell-based therapies seek to augment the endogenous wound healing process through the delivery of progenitor cells or differentiated cardiac myocytes. The advent of pluripotent stem cells, particularly induced pluripotent stem cells, has redoubled interest in this putative mode of therapy since they are: (1) highly proliferative, capable of generating therapeutically-relevant numbers of cells; (2) capable of differentiating to cardiac progenitor cells or differentiated cardiac myocytes through various stages of development; (3) amenable to genetic manipulation for basic studies. In addition, it has
become evident that the success of future cardiac cell therapies will be highly dependent on our ability to overcome the problem of low retention and survival of implanted cells\textsuperscript{2,3}. Potential approaches to address this issue include: (1) co-injecting cells with bioactive, in situ polymerizable hydrogels\textsuperscript{4}, (2) pre-conditioning cells with hypoxia or pro-survival factors\textsuperscript{5}, (3) genetic engineering of cells to enhance their angiogenic and/or anti-apoptotic action\textsuperscript{6-8}, and (4) the epicardial implantation of a pre-assembled tissue engineered patch\textsuperscript{9,10}. In particular, tissue patch implantation, although surgically more complex than cell or cell/hydrogel injections, may support long-term survival of delivered cells and exert a more efficient structural and functional cardiac tissue reconstruction at the infarct site\textsuperscript{11}.

Therefore, the objective of this thesis was to establish conditions under which highly functional engineered cardiac tissue patches with controllable structure could be formed from pluripotent stem cell-based sources. In particular, we focused on developing a reproducible, well-defined tissue engineering strategy that could be used to explore (1) the conditions under which terminally differentiated stem cell-derived cardiac myocytes could form functional tissues; (2) the capability of less mature, multipotential cardiovascular progenitor cells to differentiate towards a functional phenotype and form functional tissues; (3) the capability of fetal cardiac fibroblasts to mediate the formation of functional tissues as compared to adult cardiac fibroblasts. We
assessed electromechanical function by optical mapping of action potentials and calcium transients, sharp intracellular electrode recordings, as well as measurement of active contractile force. We also investigated the cellular composition, structural, gene and protein level changes to 2D monolayers and 3D engineered tissues by immunostaining, PCR and Western blot. Finally, we performed conditioned media studies to assess the effects of paracrine signaling on the evolution of function in engineered tissues.

Chapter 2 of this thesis describes relevant background on cardiac electrophysiology, cardiac development and wound healing, with an emphasis on the origin and phenotype of the different cardiac progenitor and supporting cell types. It also describes the current state of the art in cell-based therapies, including tissue engineering, and how insights from development and wound healing have inspired and informed the design of strategies in cell-based therapy.

Chapter 3 describes general methods that are used throughout this thesis. It includes a detailed explanation of how different cell types such as primary cardiac fibroblasts, embryonic stem cell lines and induced pluripotent stem cell lines are derived or isolated, maintained and differentiated, and used to generate 2D monolayers or 3D engineered tissues. It also includes details of analytical methods used to assess tissue-level structure and function, such as immunohistology, immunoblotting, qRT-PCR,
optical mapping, measurement of active and passive tension, intracellular electrode recordings, and the monitoring of spontaneous beating and cell spreading.

Chapter 4 details the optimization of the methodology to fabricate high aspect-ratio elastomeric tissue molds capable of directing cell alignment in 3D. We further describe optimized methods for engineering of functional cardiac tissues starting from mouse embryonic stem cells. Described experiments reveal that pure populations of embryonic stem cell-derived cardiac myocytes are unable to form highly functional 3D engineered cardiac tissues by themselves, but can be stimulated to spread, align, and electrically couple with the inclusion of small numbers of cardiac fibroblasts. Optical mapping techniques are used to assess action potential propagation through the engineered 3D cardiac syncytium, and force measurements are used to evaluate contractile function.

Chapter 5 first describes methods for derivation as well as characterization of multipotent cardiovascular progenitors, purified from either differentiating embryonic stem cells or induced pluripotent stem cells, which can differentiate towards smooth muscle, endothelial and cardiac myocyte lineages. We show that unlike purified cardiac myocytes, cardiovascular progenitors can form highly functional engineered cardiac tissues without the addition of supporting cardiac fibroblasts. We also show through optical mapping, gene expression analysis and intracellular electrode recordings that
both 2D monolayers and 3D engineered tissues composed of cardiovascular progenitors are capable of attaining levels of electrophysiological maturity similar to late fetal or early neonatal myocardium.

Finally, in Chapter 6 we explore the impact of cardiac fibroblast developmental stage on the function of engineered cardiac tissues. We find that fetal cardiac fibroblasts enable the formation of more highly functional engineered tissues than adult cardiac fibroblasts, which is evidenced by increase in cell size, the upregulation of connexin-43 gap junctions, voltage-gated sodium channels, as well as sarcomeric proteins. We also find that conditioned media from fetal cardiac fibroblasts act via the MEK-ERK pathway to induce enhanced spontaneous beating and cardiac myocyte spreading in engineered cardiac tissues, which may underlie the observed enhancement of engineered tissue function.
2. Background

2.1 Cardiac Excitation, Conduction, and Contraction

2.1.1 Membrane excitability

Ion channels are specialized transmembrane proteins that admit the flow of ions into and out of the cell at a very high rate. While most cells possess ion channels that have housekeeping functions such as the maintenance of osmolarity, some cells have a unique complement of ion channels that render their membranes electrically “excitable”. These combinations of ion channels allow: (1) the buildup of large potential differences between the interior and the exterior of the cell (transmembrane potential); (2) large oscillations in transmembrane potential due to a triggering event (action potential), such as a local change in voltage, ion or chemical concentration. The transmembrane voltage of excitable cells thus behaves in a highly non-linear fashion, granting excitable cells the ability to sense, respond to, and transmit electrical signals. This forms the biological foundation for numerous physiological processes including neural transmission, muscle activation, sensory signaling, and hormonal activation. In the heart, the initiation and transmission of action potentials from cell to cell are critical for the coordination of complex spatiotemporal phenomena such as the cardiac contraction cycle.
2.1.2 The cardiac action potential

In humans, the adult ventricular action potential can be divided into five different phases (Figure 2.1). At rest (phase 4), most transmembrane ion channels are closed except for a subset of potassium ion-permeable kir2.x (e.g. Kir2.1 – 2.3) ion channels\textsuperscript{14}. Thus, the resting potential approximates the Nernst potential of potassium (\(~-80\text{mV}\)). When the cell experiences a local depolarization due to an external stimulus or an influx of ions from neighboring cells, it reaches a threshold voltage above which a large number of voltage-gated sodium channels (Nav1.5) are triggered, resulting in a rapid influx of sodium current (I\textsubscript{Na}) (phase 0). During this phase, membrane permeability is dominated by sodium channels, causing the transmembrane potential to rise quickly (upstroke) towards the Nernst potential of sodium (\(~+53\text{mV}\)). Near the peak of the upstroke, inactivation of sodium channels and activation of the transient outward potassium current (I\textsubscript{to}) creates a notch in the action potential (phase 1). The inward calcium current (I\textsubscript{Ca}) flowing through L-type calcium channels induces calcium release from intracellular stores (reviewed below) to generate and prolong the plateau phase of the action potential (phase 2). Finally, the combined activation of several different outward potassium currents (including I\textsubscript{Kr}, I\textsubscript{Ks}, and I\textsubscript{K1}) repolarizes the membrane potential (phase 3) back to its resting state (phase 4). Sodium-potassium ATPase
exchanges accumulated intracellular sodium ions for extracellular potassium ions in an ATP-dependent process to reset the resting ionic balance\textsuperscript{15}.

Although the main features of the cardiac action potential are similar in mice, differences in the expression of repolarizing potassium currents and different $I_{ca}$ kinetics result in a greatly abbreviated and triangular action potential profile, with almost no phase 2 plateau (Fig. 2.1).
Figure 2.1: Phases and currents of the ventricular action potential. (A) The human ventricular action potential (AP) can be divided into five distinct phases: rest (4), upstroke (0), notch (1), plateau (2), and repolarization (3). Activation profiles of the main ionic currents contributing to the action potential waveform are shown below. Inward currents are indicated as downward dark areas while outward currents are in the upward direction. (B) The murine ventricular action potential has different magnitude and activation kinetics of inward calcium and potassium currents, leading to a much more abbreviated, triangular action potential waveform. Adapted from Nerbonne et al, TCM vol. 14, No. 3, 2004
2.1.3 Propagation of the cardiac action potential

During the action potential, inward depolarizing current flows into neighboring cardiac cells via gap junctions. This flow initiates local depolarization of the membrane in neighboring cells, triggering the initiation and propagation of downstream action potentials. Normal intercellular electrical coupling permits the rapid spread of action potentials throughout cardiac tissue. During mouse heart development, connexin-45 is the first isoform to be expressed, and is then progressively downregulated in favor of first connexin-40, and finally connexin-43 (reviewed below). Connexin-43 is thus the main intercellular coupling protein responsible for propagation of action potentials in mature heart tissue.

Since gap junctions present zones of high intracellular resistance relative to the cytoplasm, action potential conduction in cardiac myofibers is discontinuous, and occurs more quickly within a cell than between cells. Tissues consisting of larger myocytes therefore tend to have fewer gap junctions per unit length, resulting in an increase in conduction velocity. Moreover, gap junctions between cardiomyocytes are located primarily at specialized end-end connections called intercalated discs. These properties, and in particular number of gap junctions per unit length of tissue, result in a distinctly anisotropic activation pattern as action potentials travel more quickly in the longitudinal (parallel to muscle fiber alignment) than in the transverse (orthogonal to
fiber alignment) direction\textsuperscript{19}. During disease states such as myocardial ischemia, gap junctional uncoupling\textsuperscript{20,21} causes a rise in effective intercellular resistance slowing the velocity of action potential propagation, which can result in conduction block and the formation of reentrant arrhythmias.

Conduction velocity has also been found to depend on the resistance of the extracellular domain\textsuperscript{22,23}. During ischemic episodes and myocardial infarction, poor blood flow leads to a rapid buildup of metabolic products and a rise in extracellular resistance, which can lead to a rapid decrease in conduction velocity\textsuperscript{22}. Simulations suggest that higher interstitial resistivity in regions of high source load mismatch can change activation pattern, leading to changes in the shape of the action potential wavefront\textsuperscript{23}. Last but not least, conduction velocity in cardiac tissue strongly depends on the availability of the voltage-gated sodium current\textsuperscript{24,25}.

\section*{2.1.4 Initiation of calcium transients and cardiac contraction}

In the mature heart, sarcolemmal invaginations known as t-tubules allow L-type calcium channels to exist in close proximity to ryanodine receptors of the sarcoplasmic reticulum (SR) in a structure known as a calcium release unit (Fig. 2.2). Ryanodine receptors serve as specialized, non-selective, high conductance channels to rapidly release calcium ions, which are stored in the sarcoplasmic reticulum at very high (millimolar) concentrations\textsuperscript{26}. During the cardiac action potential, inward calcium
current (I\textsubscript{Ca}) from L-type calcium channels triggers a release of calcium stores from the SR in a phenomenon known as calcium induced calcium release (CICR). CICR allows the relatively small influx of calcium ions from I\textsubscript{Ca} to be greatly amplified, resulting in a local increase in cytosolic calcium concentrations from about 100nM to about 1μM\textsuperscript{26}. These calcium ions then rapidly diffuse to the myocyte’s contractile apparatus, which consists of interlocking troponin/tropomyosin complexes, actin and myosin filaments (Fig2.3). Calcium ions bind to troponin, causing them to undergo a conformational change and thus allowing cross-bridges to form between actin and myosin, enabling ATP-dependent cellular contraction\textsuperscript{27}. The calcium ions are then rapidly recycled back into the SR by the sarco/endoplasmic reticulum calcium-ATPase (SERCA) or pumped out of the cell by the sodium/calcium exchanger\textsuperscript{28}. The sensitivity of the cardiac myocyte’s contractile apparatus to intracellular free calcium is modulated by numerous mechanisms, such as beta adrenergic stimulation\textsuperscript{29} and the intracellular calcium binding to regulatory molecules calsequestrin\textsuperscript{30}, calmodulin\textsuperscript{31} and phospholamban\textsuperscript{32}. 
Figure 2.2: Schematic representation of a calcium release unit. Depolarization of the cardiomyocyte occurs by Na⁺ influx through (i) voltage-gated sodium channels, leading to inflow of calcium into the cell via (ii) L-type Ca²⁺-channels and (iii) reverse mode Na⁺/Ca²⁺-exchanger. This induces calcium-induced-calcium release from (iv) the sarcoplasmic reticulum via ryanodine receptors (RyR) into the cytoplasm. Calcium subsequently diffuses and binds to contractile elements, initiating contraction. During diastole, calcium leaves the cytoplasm via sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), (v) the Na⁺/Ca²⁺-exchanger, and plasma membrane Ca²⁺-ATPase. Adapted from DM. Bers, Nature 2002
2.2 Cardiac development

2.2.1 Lineage specification in cardiac development

Upon conception, the fertilized zygote embeds itself into the uterine wall to form a blastocyst. The blastocyst consists of an inner cell mass (from which embryonic stem cells are derived) that is surrounded by the trophoectoderm. The polar trophoectoderm and the inner cell mass develop into the epiblast and a layer of visceral endoderm, as well as extra-embryonic tissues. Gastrulation commences with the formation of the primitive streak, a structure that establishes bilateral symmetry and marks the site of gastrulation.

Cardiac progenitor cells are derived from the epiblast and are located in two patches on both sides of the cranial portion of the primitive streak. During gastrulation in mice (E6.5–7.5), cardiac progenitors and other epiblast cells ingress through the cranial primitive streak and through an epithelial-mesenchymal transformation (EMT) give rise to the mesoderm and the endoderm. The remnants of the epiblast form the ectoderm, giving rise to the three primary germ layers. Cardiac progenitors are exposed to signaling factors such as retinoic acid and FGFs in the process, helping to specify cells of the primary and secondary heart fields in a structure called the cardiac crescent (E7.5). Cells from the primary heart field contribute to the ventricles, AV canal and both
atria, whereas cells of the secondary heart field contribute to the outflow tract and all heart structures except the left ventricle\textsuperscript{37}.

At this stage, cells in the mesoderm express the transcription factors Mesp1 and Mesp2, which are required for mesoderm migration and formation of the heart fields\textsuperscript{35,38}. In response to signaling factors such as FGFs, BMPs and Nodal from the anterior endoderm and neural tube, an area of reduced \textit{Wnt3a/Wnt8} expression and activity is created, inducing activation of Nkx2-5, GATA4, Tbx5 and other transcription factors\textsuperscript{37}. Nkx2-5 (Nkx2.5) is the earliest molecular marker of cardiac lineage cells in both the primary and secondary heart fields, and the expression of its associated gene has been used as a specific marker for cardiac progenitor cells in transgenic mice\textsuperscript{39,40}. Nkx2.5 cooperates with zinc finger transcription factors of the GATA family to activate cardiac gene expression\textsuperscript{35}. Cardiac progenitor cells in the secondary heart field also express the transcription factor Islet1 (\textit{Isl1}), which may be required for their proliferation, survival and migration\textsuperscript{36}. \textit{Isl1} gene expression has also been successfully used to mark cardiac progenitor cells in transgenic mice\textsuperscript{41,42}. After specification, cardiac progenitors converge along the ventral midline of the embryo to form a beating heart tube (E8.5), which consists of distinct myocardial and endocardial layers separated by a layer of extracellular matrix\textsuperscript{35,37}. 

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At this stage, heart tube cells express contractile proteins such as α-myosin heavy chain (Myh6), which are cardiac specific and critical for their function. There is also evidence that cardiac progenitors at this stage express chamber-specific markers such as the ventricular-specific myosin light chain (Mlc-2v), suggesting that their fate may be genetically programmed. The genes associated with these contractile proteins have successfully been used to mark cardiac myocytes in transgenic mice.\textsuperscript{43,44} Subsequent to this, rightward looping of the heart tube occurs, which forms the various cardiac compartments, correctly orients the ventricles and aligns the nascent chambers with the vasculature.\textsuperscript{35}

### 2.2.2 The origin and function of cardiac fibroblasts

During cardiac development, epicardium is formed by the outgrowth of proepicardial cells over the heart tube. Proepicardial cells undergo EMT to form the subepicardial mesenchyme, subsequently migrating into the myocardium, giving rise to the majority of cardiac fibroblasts.\textsuperscript{45-47} Mesoangioblasts (self-renewing mesenchymal-like progenitor cells associated with the walls of the large vessels) have also been shown to be capable of giving rise to a minor population of cardiac fibroblasts.\textsuperscript{48} During the neonatal and adult stages, bone marrow derived cells have also been shown to be capable of migrating to myocardium and giving rise to cardiac fibroblasts.\textsuperscript{49} Cardiac fibroblasts do not possess a unique cell-specific marker or subset of markers, and are instead generally recognized \textit{in vitro} by their relatively strong adhesiveness to tissue culture plastic, and ability to stain strongly for vimentin and prolyl hydroxylase.\textsuperscript{50,51} Cardiac fibroblasts
are present together with cardiac myocytes at least as early as E11.5 - 12.5, and increase in numbers throughout development. Cardiac fibroblasts are generally recognized as intensely vimentin+ and SMA− population of cells in the heart, and have been isolated using Thy1 and DDR2 as positive cell surface markers. In fresh adult mouse heart tissue digests, the percentage of isolated cells being DDR2+ cardiac fibroblasts has been reported to be 27 – 37%, whereas in fetal mouse hearts it has ranged from 8.3% at E12.5 to 28.7% at E16.5. Unlike other cell types in the heart, cardiac fibroblasts do not have a basement membrane, allowing them to be distinguished from other cardiac cells.

An interesting study by Ieda et al. has shown that embryonic cardiac fibroblasts induce a hyperplastic response in embryonic cardiomyocyte mediated by heparin-binding EGF-like growth factor and β1 integrin activation. In contrast, adult cardiac fibroblasts induce only a weak hyperplastic response, inducing embryonic cardiomyocytes to undergo hypertrophy instead. Several pro-hypertrophic cardiac fibroblast paracrine factors have been identified in the literature, including endothelin-1, cardiotrophin-1, interleukin-6 (IL-6), transforming growth factor-β1 (TGF-β1), insulin-like growth factor-1 and high molecular weight fibroblast growth factor 2 (FGF-2). Some of these factors, such as endothelin-1, are produced at high levels only in the presence of cardiomyocyte-secreted angiotensin II, and are known to play a role in the progression of pathological remodeling and heart disease. However, their significance in development is uncertain. Others, such as IL-6 and TGF-β, are known to
be developmentally significant. It is clear that the growth and maturation of the heart is governed by complex processes that regulate interplay between hyperplasia and hypertrophy of cardiomyocytes in a 3-dimensional milieu, suggesting that in vitro studies in stem cell-derived engineered cardiac tissues may yield significant insights into the development of functional myocardium.

2.2.3 Structural and functional characteristics in cardiac development

During development, early embryonic cardiomyocytes are rounded in shape and express primarily connexin-45 and connexin-40 gap junctions, as well as mechanical adherens junctions and desmosomes in a uniform circumferential pattern. As development progresses, connexin-45 and connexin-40 gap junctions are progressively downregulated, and connexin-43 gap junctions are upregulated. Simultaneously, the unorganized myofibrils in early cardiomyocytes become organized to form distinct I, A, and Z bands in the later embryonic stages. At prenatal stages, the pattern of gap junction expression changes from uniform to punctate. Cells start to attain well-defined rod shape and by 3-4 weeks post birth, mechanical adherens junctions and desmosomes move to the longitudinal ends of the cardiomyocytes to form mature (staircase-shaped) intercalated discs. By 6-8 weeks post birth, gap junctions also migrate to intercalated disks to co-localize with mechanical junctions. T-tubules appear as minor sarcolemmal invaginations in the late embryonic stage and gradually grow.
deep into the cytoplasm to achieve mature appearance 3-4 weeks after birth. Occurrence of T-tubules is associated with the formation of couplons and the appearance of calcium sparks. As the T-tubules become fully mature, the L-type Ca\(^{2+}\) channels in T-tubules co-localize with Ryanodine receptors for more efficient excitation-contraction coupling.

Described structural maturation is paralleled by the functional changes in action potential shape, conduction, Ca\(^{2+}\) handling, and generation of contractile force. Specifically, depolarized resting potential and fast spontaneous activity present in all cardiomyocytes during early embryonic stage are gradually replaced with a quiescent cell phenotype in atrial and ventricular myocytes. With further development, cardiac resting potential hyperpolarizes, the slope of action potential upstroke, velocity of action potential propagation, and maximum firing rate increase, while action potential duration decreases (Table 2.1). These changes are caused by the gradual alterations in the expression and kinetics of membrane ion channels and pumps. Simultaneous with action potential changes, intracellular Ca\(^{2+}\) transient increases in amplitude and attains faster kinetics. Sarcolemmal Ca\(^{2+}\) inflow through L-type Ca\(^{2+}\) channels and Na\(^+-\)Ca\(^{2+}\) exchanger, as the main source of the Ca\(^{2+}\) transient in embryonic cardiomyocytes, becomes dominated by the release of Ca\(^{2+}\) from sarcoplasmic reticulum (SR) in postnatal
and adult myocytes \cite{24}. These cellular changes are paralleled at the organ level with an increase in the generated contractile stress (Table 2.1) and passive stiffness\cite{25}.
Table 2.1: Structural and functional changes during rodent heart development.

<table>
<thead>
<tr>
<th>Time</th>
<th>Early Embryonic (Tubular Heart)</th>
<th>Late Embryonic / Fetal (Looping/segmentation)</th>
<th>Neonatal</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>Round to polygonal cell shape, irregular myofilament arrangement 1</td>
<td>Nascent T-tubules are formed as minor sarcolemmal invaginations 12</td>
<td>Rod cell shape 2</td>
<td>Mature T-tubules form as deep membrane invaginations into cytoplasm 1</td>
</tr>
<tr>
<td></td>
<td>Adherens junctions and desmosomes are uniformly distributed in cell membrane 2</td>
<td>Myofilaments arrange into long, compact, and well-organized myofibrils 11</td>
<td>Adherens junctions and desmosomes start to move to cell ends and localize in intercalated discs 11</td>
<td>Co-localization of ryanodine receptors and Ca^{2+} channels 2</td>
</tr>
<tr>
<td></td>
<td>Formation of nascent T-discs 1</td>
<td>Adherens junctions and desmosomes localize to myofibril attachment sites 3 4</td>
<td>Development of I and A bands 5</td>
<td>Fully mature intercalated discs are formed 2</td>
</tr>
<tr>
<td></td>
<td>Cardiomyocytes are mainly mononucleated until after birth 6</td>
<td>Well-defined T-discs form 2</td>
<td>Cardiomyocytes are mainly bi-nucleated 6</td>
<td>Cardiomyocytes are mainly bi-nucleated 6</td>
</tr>
<tr>
<td>Function</td>
<td>Connexin-43 is weakly expressed in all cardiac compartments 2</td>
<td>Downregulation of connexin-45 8</td>
<td>Connexin-43 gap junction move to intercalated discs 11</td>
<td>Connexin-43 gap expression of connexin-43 gap junctions in cell membrane 1</td>
</tr>
<tr>
<td></td>
<td>Low conduction velocity (0.8 mm/s in left heart tube) 2</td>
<td>Initiation and increase of uniform connexin-40 and 43 expression in cell membrane 13</td>
<td>Punctate expression of connexin-43 gap junctions in cell membrane 1</td>
<td>High conduction velocity (50-45 cm/s) 13</td>
</tr>
<tr>
<td></td>
<td>Low (dV/dt)_{max} (88.2 V/s in E18.5 mouse ventricular cells) 10</td>
<td>Conduction velocity increases (7-21 cm/s in mouse ventricles) 14</td>
<td>Initiation of calcium spiking 2</td>
<td>(dV/dt)_{max} is maximum (221 V/s in adult mouse) 13</td>
</tr>
<tr>
<td></td>
<td>Long action potential duration (APD_{90} = 100-200 ms in E18.5 mouse) 11</td>
<td>Action potential duration shortens (APD_{90} = 74 ms in E18 mouse) 13 14</td>
<td>Conduction velocity increases (24-29 cm/s in mouse ventricles) (Valiyaveettil, 2001 #16)</td>
<td>Action potential duration is short (APD_{90} = 8 ms, APD_{90} = 20.7 ms in adult mouse) 12</td>
</tr>
<tr>
<td></td>
<td>Low contractile stress (0.56 mmHg/mm^2, E10.5 mouse cardiomyocytes) 11</td>
<td>Contractile stress increases (0.91-1.8 mmHg/mm^2, E18.5-19.5 mouse cardiomyocytes) 12</td>
<td>Action potential duration shortens (APD_{90} = 28 ms, APD_{90} = 42 ms in neonatal mouse) 17</td>
<td>Contractile stress is maximum (6-8 mmHg/mm^2, 6-8 wk adult mouse) 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Contractile stress increases (3.1 mmHg/mm^2, D7 mouse cardiomyocytes) 12</td>
<td></td>
</tr>
</tbody>
</table>
2.3 Cardiac pathology and wound healing

2.3.1 Myocardial infarction, wound healing and scar formation

Myocardial infarction (MI) occurs when a blood supply to a portion of heart tissue is interrupted, resulting in cardiac ischemia and the loss of significant amounts of cardiac tissue. The most common cause of MI is the occlusion of a coronary artery due to the rupture of an atherosclerotic plaque. Soon after the onset of MI, the heart goes through a wound healing process that can be divided into four phases, characterized by: (1) cardiac myocyte death; (2) inflammation; (3) formation of granulation tissue; (4) remodeling and scar tissue formation. This sequence of events can be found during wound healing in many organs, such as the skin, but cardiac wound healing has certain unique traits. In particular, activated myofibroblasts can be found in the infarct scar many years after MI, whereas in other tissues myofibroblasts are rapidly cleared.

Phase 1 – cardiac myocyte death: Although cardiac cell death can occur rapidly upon ischemia, it takes at least 6 hours before the first detectable symptoms of cardiac
myocyte death can be observed in the blood stream due to poor blood flow in the infarct area and slow diffusion rates. Classic hallmarks of MI include elevated blood levels of troponin-T and creatine kinase. Cardiac myocytes die through the twin processes of apoptosis and necrosis. Necrosis is commonly thought to be responsible for early cell death (6 – 8hrs post MI), whereas apoptosis occurs over a longer time course (4hrs – 4 days post MI) and is associated with the onset of a severe inflammatory response.

**Phase 2 – inflammation:** Neutrophils rapidly infiltrate the infarct site in response to the release of cytokines such as TNF-α, IL-6 and IL-8 to begin the removal of dead cardiac myocytes through phagocytosis. Neutrophils also release further chemoattractants to facilitate the infiltration of lymphocytes and macrophages.

**Phase 3 – formation of granulation tissue:** Granulation tissue is a rich mixture of fibrinogen, fibronectin and collagen that is formed from blood plasma components and fibroblast-secreted materials. As early as 12 – 24hrs post-MI, early fibrin-rich granulation tissue begins to invade the infarct site and forms a loose collection of pre-scar tissue, rich in inflammatory cells, myofibroblasts and new blood vessels. Granulation tissue persists for 2-3 weeks post-MI.

**Phase 4 – ventricular remodeling and scar tissue formation:** As myofibroblasts secrete and cross-link increasing quantities of collagen type I, the scar tissue increases in tensile strength and stabilizes. The scar tissue decreases in cellularity through apoptosis of...
resident cells, although myofibroblasts have been observed to persist in cardiac scar tissue\textsuperscript{27}. With the need to maintain a baseline ejection fraction, the remaining viable heart tissue is placed under a heavier mechanical load, and undergoes a process of dilation and hypertrophy that continues until the distending forces are counterbalanced by the tensile strength of the collagen scar\textsuperscript{82}.

2.3.2 Cardiac wound healing in immature mammalian hearts

In contrast to the well-established cardiac wound healing process observed in adults, fetal and neonatal mammals have a much greater regenerative potential, and have reported to recover from severe cardiac insults\textsuperscript{83,84}. Post-MI wound healing in fetal sheep subjected to coronary ligation was associated with a much smaller inflammatory response as compared to adult sheep, proliferation of cardiac myocytes and lack of scar formation\textsuperscript{83}. In neonatal mouse hearts, a similar regenerative response has recently been reported. Cardiac wound caused by resection of the ventricular apex was healed by formation of a blood clot, a robust inflammatory response, resorption of the clot, and population of the wound with proliferating cardiac myocytes\textsuperscript{84}. In both of these cases, the hearts of the afflicted animals were able to recover completely with no significant decrease in cardiac function, and no persistent scar was formed. Although reduced inflammation and higher proliferative capacity are clearly important facets of the improved healing response in immature hearts, one should not discount the possible
contribution of fetal cardiac fibroblasts to the wound healing response given the central role of fibroblasts in scar formation.

2.4 Cell-based therapies for heart disease

Since myocardial infarction (MI) in humans typically results in the loss of over a billion cardiac myocytes, the question of cell source has always been one of critical importance to cell-based therapies. Conventional wisdom suggests three criteria that a potential cell source must fulfill: (1) donor cells must be available in therapeutically-relevant quantities close to the number that was lost in injury; (2) donor cells must ideally be immunologically compatible with the host and must integrate into the host organ environment; (3) donor cells must be capable of replacing the functionality of the lost tissue. It is controversial whether any cell source to date has satisfied all three of these criteria.

2.4.1 Autologous cells

Autologous cell sources are typically obtained through biopsies, and in some cases may be expanded in vitro to obtain a therapeutically-relevant number of cells. They are immunologically compatible with the host, but most of these cell types lack significant potential to differentiate into functional cardiomyocytes. Cell injection into the infarct border zone has been attempted both in animal models as well as clinical trials with a variety of autologous cells, including skeletal myoblasts\textsuperscript{85,87}, bone marrow
derived stem\textsuperscript{88-93} and progenitor cells\textsuperscript{94}, circulating endothelial progenitor cells\textsuperscript{92,93,95,96},
adipose derived stem cells\textsuperscript{97,98}, amniotic stem cells\textsuperscript{99} and cardiac stem cells\textsuperscript{100,101}. The
observed benefits from these treatments have been modest and often transient in nature,
being attributed mainly to paracrine effects rather than to contact-mediated actions of
implanted cells.

Skeletal myoblasts that can be readily obtained via biopsy and expanded \textit{in vitro}
have been the first autologous contractile cell source used in clinics\textsuperscript{102}. Although these
cells do not electrically couple to cardiac myocytes\textsuperscript{103}, they have been found to exert
therapeutic action via secretion of paracrine factors including stromal derived factor 1
(SDF-1), hepatocyte growth factor (HGF), and vascular endothelial growth factor
(VEGF)\textsuperscript{104-106}. In addition to skeletal myoblasts, multipotent adult stem cells from bone
marrow, peripheral blood, and the heart have been used in several clinical trials for
treatment of post-infarction disease\textsuperscript{107,108}. Their therapeutic benefits have also been
attributed to paracrine action of implanted cells at the site of infarction. While most
adult-derived stem cells including bone marrow\textsuperscript{-}, adipose\textsuperscript{-}, or cardiac-derived stem
cells\textsuperscript{88-90} can express cardiac markers under specific conditions \textit{in vitro}, their potential to
yield functional cardiomyocytes capable of generating action potentials and active
contractions is significantly limited\textsuperscript{109-112}. As such, these cells do not represent a viable
cell source for functional cardiac tissue engineering.
2.4.2 Pluripotent stem cells

Unlike adult cells, pluripotent stem cells are able to differentiate into any cell type in the body (except extra-embryonic placenta) and are capable of robust proliferation. Pluripotent stem cells are commonly studied in the form of embryonic stem (ES) cells and induced pluripotent (iPS) cells, although other forms of stem cells (such as spermatogonial stem cells, parthenogenetic stem cells and somatic cell nuclear transfer stem cells) exist which share many of the properties of ES cells and iPS cells. ES cells are derived from the inner cell mass of the pre-implantation blastocyst, and can be propagated \textit{in vitro} in their undifferentiated state on feeder layers of embryonic fibroblasts using either leukemia inhibitory factor and bone morphogenetic protein 4 (mouse ES cells), or basic fibroblast growth factor (human ES cells). In contrast, iPS cells are derived by the overexpression of 3 – 4 transcription factors in adult somatic cells\textsuperscript{113-115}. This treatment results in epigenetic alterations in a small number of cells that allow them to revert to a pluripotent state. iPS cells are similar to ES cells and are propagated and differentiated using similar protocols.

Unlike many uni- or multi-potent cell types derived from adult sources, pluripotent stem cells exhibit significant levels of spontaneous differentiation towards functional, contractile cardiomyocytes. “Cardiac-like” cells derived from adult sources typically have poor morphology and sarcomeric organization, and lack the ability to
contract or fire sustained action potentials\textsuperscript{11,116-118}. In contrast, ES and iPS cells have been repeatedly shown to differentiate to functional cardiomyocytes\textsuperscript{5,119-130}. Robust differentiation protocols exist, ranging from embryoid body formation to the more recent guided differentiation systems, which can be scaled up to reproducibly generate therapeutically-relevant numbers of cardiomyocytes\textsuperscript{5,125,131,132}. Pluripotent stem cell derived cardiomyocytes have been well-characterized, and typically exhibit normal morphology, well-organized sarcomeres, and electro-mechanical properties that range from embryonic to neonatal levels of functionality\textsuperscript{119,124,127,129,130}.

ES cell-derived cardiac myocytes were recently shown to be capable of repopulating and integrating with injured host myocardium, mechanically reinforcing the heart and suppressing the incidence of arrhythmia. In the study, Shiba et al\textsuperscript{133} derived therapeutically-relevant quantities of cardiac myocytes ($1 \times 10^8$ cells, 63% $\beta$-MHC$^+$) from human ES cells, which were then injected into cryoinjured guinea pig hearts. The injected cells were observed to: (1) partially remuscularize 8.4% of the scar area; (2) partially rescue cardiac fractional shortening from 20% to 25%; (3) greatly reduce the rate of sustained spontaneous and induced tachycardia; (4) electrically couple with host myocardium as evidenced through use of the genetically encoded calcium indicator GCamP3. This study unequivocally showed that pluripotent stem cells are able
to do what few other cell types can: generate new, functional, therapeutically useful myocardial tissue.

Of note is that iPS cells derived from the host’s own skin cells have generally been assumed to be immunologically compatible with the host, but that notion has recently been challenged\(^\text{134}\) and remains controversial. Nevertheless, recent clinical trials with human ES cell derivatives have shown that immunological complications can be minimized either by working in immune-privileged parts of the body such as the intraocular space\(^\text{135}\), or through temporary low-dose immune-suppression\(^\text{136}\). These findings are highly encouraging for future applications, and firmly establish pluripotent stem cells as the premier cell source for regenerative medicine.

### 2.5 Tissue engineering therapies for heart disease

Traditionally, tissue engineering approaches involve \textit{in vitro} generation of structural and functional substitutes of native tissue by use of one or more of the following: living cells, natural or synthetic materials (scaffolds), soluble or bound growth factors, and tissue culture bioreactors\(^\text{137}\). Their expected benefits over direct cell injection include superior retention of cells at the injury site and superior control over tissue structure and function. During the past decade, different methodologies for \textit{in vitro} engineering of cardiac muscle tissue have been developed by our group\(^\text{138-141}\) as well as others\(^\text{9,10}\) using primary cardiomyocytes isolated from neonatal rat ventricles. Initial
efforts concentrated on seeding primary neonatal rat ventricular myocytes into porous polymeric scaffolds composed of poly-lactic-co-glycolic acid (PLGA) using rotating vessels or orbital shaking\textsuperscript{138-143}. Attached cardiomyocytes remained viable and interconnected to form functional syncytia capable of conducting action potentials\textsuperscript{138,139,141}. Although directional cues to align seeded cardiomyocytes in 3D could be introduced through methods such as sucrose leaching\textsuperscript{141}, the high mechanical load imposed by the rigidity inherent to these polymeric matrices made macroscopic contractions impossible.

Compared to polymer scaffolds, naturally-derived hydrogels (e.g., collagen, fibrin, matrigel) have several properties that make them well-suited for the engineering of functional anisotropic cardiac tissues, including: (1) spatially-uniform cell seeding, (2) significant cell-mediated compaction that eventually yields high cell density, (3) abundant cell surface receptor binding sites that facilitate cell spreading and survival, and (4) high mechanical compliance that permits macroscopic tissue contractions and allows the application of tensile forces to align cells in 3D\textsuperscript{51,144-147}.

Previously, tissue engineered patches composed of skeletal myoblasts have been shown in animal studies to exert increased functional benefit over cell injections\textsuperscript{104-106}. Tissue engineered skeletal myoblast sheets, pioneered by Okano’s group, have also been implanted in a limited number of patients with post-infarction dilated cardiomyopathy.
The outcomes of these studies are awaiting future assessment\textsuperscript{148,149}. Tissue patches generated from bone marrow, peripheral blood and cardiac stem cells have been applied in animal studies where they have been shown to exert increased functional benefits compared to standard cell injections\textsuperscript{11,150}. A small clinical trial on 10 patients has also demonstrated the feasibility and safety of implanting collagen-based scaffolds seeded with bone marrow cells\textsuperscript{151}.

\textbf{2.5.1 The supporting role of non-myocyte cells in cardiac tissue engineering}

Multiple studies have shown that the inclusion of stromal cells in engineered tissue constructs enhances neonatal rat cardiomyocyte survival, spreading, and functional integration in host tissue. Narmonova et al. showed\textsuperscript{152} that co-culture of neonatal rat cardiomyocytes and endothelial cells from the heart and lung within a hydrogel composed of synthetic, self-assembling amphiphilic peptides resulted in the formation of capillary-like networks of endothelial cells and promoted spreading and reorganization of neonatal cardiomyocytes along the capillary structures, yielding synchronized contractions and enhanced expression of gap junction protein connexin-43. In contrast, cardiomyocytes cultured alone aggregated into sparse clusters and underwent significant apoptosis and necrosis. Naito et al. showed\textsuperscript{153} that collagen gels containing cardiomyocyte-enriched heart cell populations derived by enzymatic digestion and differential preplating of neonatal rat ventricular cells developed inferior
contractile performance compared to cardiac population derived without differential preplating (0.3mN vs. 0.7mN). The non-cardiac cells in this mixed population were found to consist primarily of cardiac fibroblasts, as well as some endothelial and smooth muscle cells that were observed to form capillary-like structures.

Similarly, stromal cells also exert a beneficial effect on ES cell-derived cardiomyocytes. Caspi et al. showed\textsuperscript{154,155} that human ES cell-derived cardiomyocyte proliferation was enhanced with the addition human umbilical vein endothelial cells (HUVECs) and mouse embryonic fibroblast-derived mural cells in engineered cardiac tissue constructs. Stevens et al. showed\textsuperscript{156} that when HUVECs and embryonic fibroblasts were included in scaffold-free aggregated human ES cell-derived cardiac myocyte patches, the supporting cells formed microvascular structures that anastomosed with host coronary circulation, allowing enhanced delivery of blood to engrafted cells. Recently, Tulloch et al. found that the addition of HUVECs to human ES cell-derived cardiac myocytes in engineered tissue constructs increased the proliferative capacity of cardiac myocytes, but did not enhance hypertrophy\textsuperscript{146}. Furthermore, recent studies by Pfannkuche et al. and Xi et al. indicated\textsuperscript{157,158} that mouse embryonic fibroblasts supported the integration of stem cell-derived cardiomyocytes onto either collagen sponges or avital myocardial tissue slices, possibly implying that fibroblasts enhance the expression of cell-adhesion receptors on cardiomyocytes.
Taken together, these studies suggest that endothelial cells enhance the proliferation of ES cell derived cardiac myocytes and reduce cell death in engineered tissue constructs, whereas embryonic fibroblasts stabilize nascent vascular structures and may enhance the integration and spreading of cardiomyocytes within the scaffold. Notably, the embryonic fibroblasts used in these experiments were not of cardiac origin, since the preparation of primary mouse embryonic fibroblasts involves the removal of internal organs such as the liver and heart before enzymatic dissociation\textsuperscript{157,158}. Since fibroblasts are known to be a transcriptionally diverse cell type even within the same organ\textsuperscript{159}, it is likely that the non-cardiac fibroblast-cardiomyocyte interactions reported in engineered tissues thus far may not accurately reflect the signaling that occurs between cardiac fibroblasts and cardiomyocytes \textit{in vivo}.
3. Methods

3.1 Cell isolation

3.1.1 Isolation of adult murine cardiac fibroblasts

Timed pregnant (E13.5) CD-1 mice were anesthetized by isofluorane treatment and euthanized by resection of both cardiac ventricles. The ventricular tissue was immediately washed in PBS to remove residual blood, and then finely minced to slurry (< 1mm³). The slurry was collected in a 50ml conical tube, and subjected to 4x 20 min serial digestions at 37°C with a solution containing 0.5mg/ml collagenase type II and 0.5mg/ml trypsin in 1x ADS buffer¹⁶⁰ (Table 3.1) which had been adjusted to pH of 7.4 using 1N sodium hydroxide or hydrochloric acid, and to osmolarity of 285-300 mOsm using sucrose. At the end of each digestion, liberated cells were aspirated as a supernatant together with the enzyme solution and placed on ice in a fresh 15ml conical tube with an equal volume of serum-containing medium (Table 3.2). Liberated cell fractions were collected and pelleted by centrifugation at 500xg for 5 min, resuspended in fresh medium and allowed to attach to a gelatin-coated tissue culture flask overnight. Cells were expanded on gelatin without passaging for four days in serum-containing medium (Table 3.2) and then frozen for storage.
3.1.2 Isolation of fetal murine cardiac fibroblasts

Fetal murine cardiac fibroblasts from E13.5 day embryos were isolated in collaboration with Dr. Donghui Zhang. Following euthanasia, uteri from the mother mice were removed to allow isolation of embryos, numbering approximately 10 per mouse. By using a dissection microscope and a pair of fine-point Dumont forceps (Fine Science Tools Inc.), the hearts were removed from each embryo and then rapidly digested by 4x 3 min serial digestions using a solution of 2mg/ml collagenase type II (Worthington Biochemicals) in ADS buffer with mechanical disruption. Liberated cells were pelleted at 500xg, resuspended in fresh medium, and allowed to attach to gelatin-coated tissue culture flasks. Cells were grown for without passaging for four days in serum-containing medium (Table 3.2) and then frozen for storage.

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<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
<th>Vendor / Item number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue culture water</td>
<td>1 Liter</td>
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</tr>
<tr>
<td>NaCl</td>
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<tr>
<td>HEPES</td>
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<tr>
<td>NaH₂PO₄</td>
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<tr>
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<tr>
<td>KCl</td>
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<tr>
<td>MgSO₄•7H₂O</td>
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Table 3.1: 10x ADS buffer

<table>
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<th>Reagent</th>
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<td>Millipore / SLM-220-B</td>
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<td>Fetal bovine serum</td>
<td>75ml</td>
<td>Atlanta Biologicals / E1030</td>
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<tr>
<td>MEM NEAA</td>
<td>5ml</td>
<td>Stemcell Technologies / 07600</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>5ml</td>
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<tr>
<td>L-glutamine</td>
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<td>Sigma / G7513</td>
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<tr>
<td>ES-qualified 2-mercaptoethanol</td>
<td>0.5ml</td>
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</tr>
<tr>
<td>Gentamicin</td>
<td>0.5ml</td>
<td>Gibco / 15750-060</td>
</tr>
<tr>
<td>Leukemia inhibitory factor</td>
<td>0.5ml</td>
<td>Millipore / E6G1106</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>50mg</td>
<td>Sigma / A4403</td>
</tr>
<tr>
<td>(for ES and iPScell expansion)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(for ES and iPScell differentiation)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2: Serum-containing medium
3.1.3 Isolation of neonatal rat cardiac fibroblasts

Neonatal rat cardiac fibroblasts were obtained by enzymatic digestion of ventricles from 2-day old Sprague-Dawley rats (Charles River). Ventricles were minced to slurry and incubated in 0.7mg/ml trypsin (US Biologicals) in Hank’s Balanced Salt Solution (HBSS; Gibco) for 16 hours at 4°C with shaking at 70RPM. After neutralization with serum-containing medium, the tissue fragments were subjected to 4 – 5x 2-minute serial digestion with 1mg/ml collagenase type 2 (Worthington Biochemicals) in HBSS at 37°C with shaking at 90RPM. Supernatant containing liberated cells was quenched in an equal volume of serum-containing medium and placed on ice. Finally, cells were centrifuged and resuspended in warm serum-containing medium and subjected to 2x 1-hour pre-plating steps on tissue culture plastic. Attached cells comprised an almost pure population of cardiac fibroblasts, and were used for tissue engineering experiments after single passage.

3.2 Fibroblast purification by MACS and FACS

3.2.1 Magnetic Activated Cell Sorting (MACS)

Isolated fetal murine cardiac cells (consisting of a mix of cardiac myocytes and non-myocytes) (3.1.2) cultured in gelatin-coated tissue culture flasks were detached by treatment with 0.05% Trypsin-EDTA (Invitrogen), pelleted at 500xg, and resuspended in 170μl of a solution consisting of 20% FBS in DPBS + 10U/ml DNase I (Worthington
Biochemicals; “MACS buffer”). The cells were incubated with 30μl CD31 beads (Miltenyi Biotec) for half an hour at 4°C, washed twice and passed through a magnetic column (Miltenyi Biotec) to deplete endothelial cells. The flow-through was then pelleted, resuspended in MACS buffer and incubated with CD90.2 beads (Miltenyi Biotec), thus enabling cardiac fibroblasts to be retained once the cells were passed through a second magnetic column. Cardiac fibroblasts were then recovered by washing the column with MACS buffer, and were used directly in co-culture and paracrine experiments.

3.2.2 Fluorescent Activated Cell Sorting (FACS) analysis

Cell fractions were resuspended in MACS buffer (3.2.1) and incubated with CD90.2-PE and CD31-APC antibodies (BD Biosciences) for half an hour at 4°C. They were then washed twice in MACS buffer and sorted using a BD CANTO-II Analyzer. Viable cells were gated based on forward and side scattering, and determined to be positive or negative for the respective cell surface markers by using unstained cells as a negative control. Positive controls used were EL-4 neuroblastoma and MS-1 endothelial cell lines for CD90.2 and CD31 respectively.
3.3 Microfabrication and soft lithography

3.3.1 Fabrication of SU-8 silicon templates

10 cm diameter silicon wafers (Wafer World Inc.) were first cleaned for 15 minutes in a 1:3 mixture of hydrogen peroxide and concentrated sulfuric acid (“Piranha Etch”) at 80°C. The wafers were then washed in deionized water, dried with an air gun and dehydrated on a hotplate at 200°C for 15 minutes. Upon cooling, a 250μm thick layer of SU-8 100 photoresist (Microchem) was spun-coated onto the silicon wafer following the manufacturer’s protocol. The SU-8 coated wafer was then soft-baked at 95°C for 2 hours, after which the hotplate was switched off and the wafer allowed to cool to room temperature. Using this procedure, 5 additional 250μm-thick layers were sequentially added to a final thickness of 1500μm. The final soft-bake cycle was allowed to proceed at 95°C for additional 12 hours to promote evaporation of residual solvent from the thick SU-8 layer.

Photomasks were designed using Postscript and printed onto mylar transparencies (Advanced Reproductions). Photomasks were placed in contact with the soft-baked SU-8 layer while the areas outside the photomask boundaries were covered with aluminum foil to reduce the total surface area of SU-8 exposed to UV light, thereby reducing stress produced in the silicon wafer upon SU-8 crosslinking. The SU-8 coated silicon wafers were then exposed to UV light (365nm) at 12 mW cm⁻² in a series of twelve
1-minute exposures, each followed by a 2-minute rest interval so as to minimize heating of the photoresist layer. The exposed wafers were then post-exposure baked at 40°C for 48 hours to allow complete cross-linking of the exposed areas, followed by cooling to room temperature. Finally, the exposed wafers were developed overnight in polypropylene glycol monomethyl ether acetate (PGMEA) to dissolve unexposed SU-8, rinsed in isopropyl alcohol and dried using an air-gun.

3.3.2 Double-casting of PDMS tissue molds

SU-8 silicon templates were silanized overnight by placing them in a vacuum dessicator with approximately 200μl of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1 trichlorosilane (henceforth referred to as ‘silane’). Sylgard 184 PDMS (Dow Corning) base was carefully poured over the SU-8 silicon template and allowed to cure overnight at 65°C, forming a negative impression of the SU-8 template (PDMS negative). The PDMS negative was detached from the SU-8 template, silanized in a vacuum chamber overnight with 100-200μl silane, and used as a template to create positive PDMS tissue molds, which were then used for the casting and culture of tissue patches.

3.4 Derivation, maintenance, and differentiation of embryonic stem cell and induced pluripotent stem cell lines

3.4.1 Maintenance of mouse embryonic stem cells (ES cells)

Undifferentiated ES cells were routinely maintained on mitomycin-inactivated primary mouse embryonic fibroblasts (PMEFs; Millipore) in Embryomax high glucose
DMEM (Millipore) containing 15% FBS (Atlanta Biologicals) and Leukemia Inhibitory Factor (LIF; Millipore) (Table 3.2). When colonies were ~200μm in diameter, cells were trypsinized with 0.05% trypsin-EDTA (Gibco) and passed onto fresh mitomycin-inactivated feeder cells. One – 3 days prior to differentiation, ES cells were trypsinized and subjected to feeder layer subtraction by pre-plating on tissue culture plastic for one hour. Loosely-adherent ES cells were then washed off, and passaged at a ratio of 1:3 – 1:10 onto gelatin coated plates in medium containing LIF.

3.4.2 Derivation of induced pluripotent stem cells (iPS cells)

iPS cells were derived by epigenetically reprogramming cultures of primary mouse embryonic fibroblasts (PMEFs). We utilized a lentivirally-delivered, polycistronic, doxycycline-inducible expression vector allowing the controlled over-expression of the murine transcription factors Pou5f1 (Oct4), Sox2, Klf4, and Myc. We readily detected clusters of cells with an increased proliferation rate and a higher nuclear-to-cytoplasmic size ratio as compared to non-transduced PMEFs within two to three days following induction of transgene expression through the addition of doxycycline in culture medium containing serum (Table 3.2) and 2mM valproic acid (Sigma). Over the course of 14 days in culture, the clusters of proliferating PMEFs undergoing reprogramming grew larger, and within 14 days post initiation of transcription factor over-expression we detected compact, multi-layered cell colonies
with refractile borders closely resembling those of undifferentiated mouse ES cells. Individual cell colonies were picked and expanded on mitotically-inactivated PMEF feeder monolayers without doxycycline. Candidate cell colonies were then assessed based on their ability to maintain characteristic undifferentiated morphology as well as through positive immunostaining for pluripotency markers Pou5f1, Fut4 and Nanog. iPS cells were maintained in an undifferentiated state during routine culture in the same way as ES cells.

### 3.4.3 Differentiation of ES and iPS cells – mass suspension culture

On the day of differentiation (Day 0), purified ES or iPS cells were aggregated into embryoid bodies by suspending 4 million ES cells in 25ml ascorbic acid-containing media without LIF (Table 3.2) inside 15 cm diameter dishes (Falcon) coated with poly(2-hydroxyethyl methacrylate) (Sigma). Under these conditions, ES and iPS cells spontaneously aggregate into clusters (embryoid bodies). After 48 hours, media was changed by allowing embryoid bodies to settle out of suspension and carefully aspirating the supernatant. Subsequently, media was changed daily. On differentiation Day 8, 5 μg/ml of puromycin was applied for 5 days, and embryoid bodies were dissociated into single cells by serial digestion and used for further experiments.

For digestion procedure, 20ml of 0.5mg/ml Trypsin was prepared by dissolving 10mg of dessicated Trypsin powder (Invitrogen) in calcium and magnesium-free HBSS
(Gibco), supplemented with DNase I (Worthington) to a final concentration of 10U/ml and sterile filtered (‘Solution 1’). At the same time, fresh 0.25% Trypsin-EDTA (Invitrogen) was defrosted and allowed to warm up to room temperature (‘Solution 2’). Mass suspension culture embryoid bodies were pipetted into a sterile 100ml glass bottle, allowed to settle and washed twice with DPBS to remove traces of serum. 8ml of Solution 1 was then added and used to digest the embryoid bodies at 37°C with 80RPM shaking for 20 minutes. The embryoid bodies were then allowed to settle out, and the supernatant was transferred to a 15ml conical tube, quenched with an equal volume of serum-containing medium (Table 3.3), and placed on ice. The digestion process was then repeated a second time with 8ml of fresh Solution 1. For the third digestion, the same procedure was carried out with Solution 2 used in place of Solution 1. At the end the 20 minute digest with Solution 2, an equal volume of serum-containing medium was added directly to the embryoid bodies + enzyme mixture and triturated with a 10ml pipet by pipetting the mixture up and down 10 times. It is then often best to use a 40μm cell strainer to separate the (small) undigested embryoid bodies from the released cells rather than waiting for them to settle out. The undigested embryoid bodies can then be washed once with DPBS and subjected to a fourth digestion step with Solution 2 (identical to the third digest) if deemed necessary. At the end of the 3-4 step serial digestion process, liberated cells from each stage should be counted. In our experience,
the first two digests typically contain a great deal of debris and very few cells, and can be discarded. Most cells are liberated during the third digest and a smaller number during the fourth digest.

Table 3.3: Composition of engineered tissue hydrogel

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
<th>Vendor / Item number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen solution (10mg/ml in DPBS)</td>
<td>2mg/ml</td>
<td>Sigma / F8630</td>
</tr>
<tr>
<td>2X DMEM</td>
<td>1X</td>
<td>Gibco / 31600-034</td>
</tr>
<tr>
<td>Matrigel</td>
<td>10%</td>
<td>BD / 354234</td>
</tr>
<tr>
<td>Thrombin</td>
<td>0.8U/ml</td>
<td>Sigma / T6634</td>
</tr>
</tbody>
</table>

3.4.4 Differentiation of ES and iPS cells – hanging droplets

Purified ES or iPS cells were resuspended at a density of 5x10^4 cells/ml in ascorbic acid-containing media without LIF (Table 3.2) and deposited as 20μl droplets on the inner surface of 15cm diameter tissue culture dish lids. Tissue culture dishes containing 25ml of PBS or tissue culture water were covered with the lids containing the deposited droplets, and incubated at 37°C. After 48 hours, the hanging droplets were washed into fresh tissue culture dishes previously coated with poly(2-hydroxyethyl methacrylate) and maintained as suspension cultures.
3.4.5 Derivation of mouse ES cell Myh6-puro cell line

D3 mouse ES cells (ATCC CRL-11632) were co-transfected by electroporation (Amaxa, A023) with two vectors. The first vector conferred constitutive expression of G418 resistance under SV40 promoter and puromycin N-acetyl-transferase (Invivogen, pORF39-PAC) expression under a 5.5Kb mouse Myh6 promoter (a kind gift from Dr. Jeffrey Robbins\textsuperscript{161}). The second vector conferred red fluorescence protein (pDsRed2-1, Clontech) expression under the same Myh6 promoter. Transfected ES cells were plated on G418 resistant, mitomycin-inactivated mouse embryonic fibroblasts (Millipore) and selected with G418 (300μg/ml) for 7-10 days followed by collection of 50 clonal colonies. These clones were passaged and induced to differentiate by formation of hanging droplets. The clones that expressed highest intensity of dsRed2-1 in the spontaneously contracting cardiac myocytes upon puromycin addition were chosen and used for further experiments.

3.4.6 Derivation of mouse ES and iPS cell Nkx2.5-puro cell lines

Similar to the derivation of Myh6-puro lines, Nkx2.5-puro cell lines were obtained by stable transfection of mouse ES or iPS cells with three vectors. The first vector conferred expression of puromycin N-acetyl-transferase driven by a 2.3kb Nkx2-5 enhancer element (a kind gift from Dr. Eric Olson \textsuperscript{162}) in series with the mouse Hsp68 minimal promoter. The second vector conferred expression of dsRed2-1 (Clontech)
driven by a 5.5kb Myh6 promoter. The third vector conferred the expression of hygromycin phosphotransferase driven by a mouse polII promoter, which allowed the selection of clonal colonies on hygromycin-resistant feeder layers using hygromycin.

3.5 Preparation of cardiac monolayers and engineered tissues

3.5.1 Engineered tissues made of mouse ES cell-derived cardiac myocytes and cardiac fibroblasts

For experiments involving adult and fetal murine cardiac fibroblasts, cells were defrosted one day before preparation of engineered tissues and plated onto gelatin-coated tissue culture flasks. MACS was then performed to separate cardiac fibroblasts from fetal cardiac cells on the day of the experiment. Adult cardiac fibroblasts were essentially pure (typically 85-90% CD90+/CD31- as shown in Chapter 6) and required no MACS purification. For experiments involving neonatal rat cardiac fibroblasts, cells were never frozen and were used at passage 1. Neonatal rat cardiac fibroblasts were also essentially pure\textsuperscript{163,164} and required no MACS purification.

Pure populations of ES-derived cardiac myocytes were derived by mass suspension culture, puromycin selection and enzymatic dissociation of differentiating Myh6-puro ES cell line embryoid bodies. Cardiac fibroblasts were mixed with cardiac myocytes in a 1:3 (adult and fetal murine cardiac fibroblasts) or 3:100, 6:100 or 12:100 (neonatal rat cardiac fibroblasts) ratio, suspended in a mixture of 2mg/ml fibrinogen + 10% Matrigel (Table 3.3), pipetted into microfabricated PDMS molds, and left in
incubator for 45 min to gel. The final cardiac myocyte cell density was 6.25 million cells/ml. The resulting engineered tissues were cultured in 12-well plates in serum-free N2B27 media (Table 3.4) on a rocker (~0.5Hz) at 37°C to facilitate transport of nutrients and oxygen into the engineered tissues.

**Table 3.4: Serum-free N2B27 medium**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
<th>Vendor / item number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F-12</td>
<td>225ml</td>
<td>Gibco / 11330-032</td>
</tr>
<tr>
<td>Neurobasal medium</td>
<td>225ml</td>
<td>Gibco / 21103-049</td>
</tr>
<tr>
<td>N2 supplement</td>
<td>5ml</td>
<td>Gibco / 17502-048</td>
</tr>
<tr>
<td>B27 supplement</td>
<td>10ml</td>
<td>Gibco / 12587-010</td>
</tr>
<tr>
<td>BSA fraction V</td>
<td>34ml</td>
<td>Gibco / 15260-037</td>
</tr>
<tr>
<td>MEM NEAA</td>
<td>5ml</td>
<td>Stemcell Technologies / 07600</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>5ml</td>
<td>Stemcell Technologies / 07000</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>5ml</td>
<td>Sigma / G7513</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5ml</td>
<td>Gibco / 15750-060</td>
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<tr>
<td>G-6-Aminocaproic acid</td>
<td>0.5g</td>
<td>Sigma / A2504</td>
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</tbody>
</table>

### 3.5.2 Preparation of cardiac monolayers

21mm diameter coverslips were punched out of Aclar™ sheets then sterilized in 70% ethanol for 10 minutes and dried with a vacuum aspirator. Coverslips were then coated with fibronectin for 1 hour by placing them in contact with 100μl droplets of 30μg/ml fibronectin solution (Sigma) spotted on sterile tissue culture plastic. Coverslips were carefully placed fibronectin-side-up in 12-well plates (Falcon), washed twice and immersed in DPBS. Enzymatically-dissociated cardiac myocytes were seeded at a
density of 0.8 million per coverslip and left to attach in 2ml of serum-containing medium (Table 3.2) overnight. Medium was changed 24 hours later to 2ml of serum-free N2B27, and refreshed every 2 days.

### 3.6 Electrophysiological measurements

#### 3.6.1 Optical mapping of action potential propagation in 2D monolayers

Optical mapping of action potential propagation was performed as previously described\(^{163,165}\). 2D monolayers were stained with 15 μM voltage-sensitive dye Di-4 ANEPPS (Invitrogen) for 5 minutes at room temperature and superfused with 35°C Tyrode’s solution. Tyrode’s solution consisted of (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, 0.33 NaH\(_2\)PO\(_4\), and 5 Glucose adjusted to pH of 7.4 with NaOH and to osmolarity-of 285 – 300 mOsm with sucrose. Samples were subjected to 30s long bursts of electrical stimulation delivered at 1.5x threshold voltage, at increasing rates from 1-8 Hz using a bipolar platinum electrode. At the end of each step, 2-5 s of activity were recorded at 1.2 KHz sampling rate using a 504-channel photodiode array (RedShirt Imaging). Spatial resolution of the recording was 750 μm. Local conduction velocities were calculated using a custom Matlab program by comparing the activation time (time of maximum upstroke of action potential) at each recording site relative to those of neighboring sites. Action potentials were measured at 80% repolarization\(^{164,165}\).
3.6.2 Optical micromapping of intracellular Ca\textsuperscript{2+} transients in 3D engineered tissues

Engineered tissue patches were detached from their PDMS molds and incubated for 40 min in 1.5ml serum-free N2B27 medium (Table 3.4) containing 6.7μg/ml Rhod2-AM (Invitrogen) with gentle (~0.5Hz) rocking in a standard cell culture incubator to ensure perfusion of Rhod2-AM throughout the tissue. Tissue patches were then washed and incubated in fresh N2B27 medium for 20 min to allow intracellular cleavage of the cell-penetrating ester moiety. Tissue patches were placed in a temperature-controlled chamber mounted on a Nikon fluorescence microscope with a 4x magnification objective and an excitation/emission of 560/600nm. Tissue patches were superfused with warm Tyrode’s solution and 10 μM blebbistatin was added to abolish contraction-induced artifacts\textsuperscript{166}. Tissues were electrically paced (at 1.5x threshold voltage) using a bipolar platinum wire electrode connected to a Grass stimulator. For optical mapping, an image intensifier (XR5, Photonis, The Netherlands) was placed in front of a 504-channel photodiode array (RedShirt Imaging) to enhance the signal from the microscope. In this setting, signals were recorded with 187.5μm spatial and 2.4 KHz temporal resolution. Alternatively, optical signals were recorded from a 7x7mm field of view by a fast EMCCD camera (iXon\textsuperscript{EM+}, Andor) at a higher spatial resolution (55.3μm) and 432Hz frame rate. Signals were analyzed using a custom Matlab program\textsuperscript{165}. 

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3.6.3 Sharp electrode intracellular recordings in 2D monolayers

22mm circular glass coverslips were prepared by spin-coating Sylgard 184 PDMS at 3000 RPM for 30s, followed by curing overnight at 65°C. PDMS-coated coverslips were sterilized by UV irradiation, coated with 30μg/ml fibronectin (Sigma) and placed in 12-well plates. Single-cell suspensions were prepared by enzymatic dissociation as previously described and seeded onto coverslips at a density of 0.5 million cells per well in a 2ml volume of serum-containing medium (Table 3.2). After 24 hours, medium was changed to serum-free N2B27, which was used to maintain the monolayers for up to 16 days, with medium change every alternate day. Sharp microelectrode recordings were carried out using an Axon Instruments Multiclamp 700B setup with an ROE200 motorized headstage (Sutter Instruments). Sharp microelectrodes were pulled from borosilicate glass (Sutter Instruments BF100-50-10) using a Model P-97 brown-flaming micropipette puller (Sutter Instruments) and backfilled with 3M KCl solution. The electrode tip resistance was 40 – 60MΩ. Pipet capacitance neutralization of 2–5 pF was applied as appropriate to ensure accurate representation of upstroke velocity. All measurements were performed at 35°C in Tyrode’s solution.
3.7 Active and passive force measurements in engineered cardiac tissues

3.7.1 Measurement of contractile force and passive tension

For mechanical measurements, tissue patches were immersed in warm Tyrode’s solution in a 37°C temperature-controlled chamber. One side of the square nylon frame surrounding tissue patch was fixed to the chamber while opposite side was attached to a sensitive force transducer (gift of Dr. Robert Dennis, UNC Chapel Hill) mounted on a computer-controlled linear actuator (Thorlabs). The two free sides of the frame were carefully cut using a pair of surgical scissors to allow application of unidirectional stretch. Tissue patches were gradually stretched in 4% increments relative to the baseline length up to a maximum of 24% stretch. At each length increment, the tissue patch was allowed to equilibrate for 1 minute. Field shock was then applied by a pair of platinum foil electrodes connected to a Grass stimulator, and the resulting force traces were recorded on a PC. Force traces were analyzed using a custom-made MATLAB program. At each applied tissue length, passive tension in the tissue patch was recorded as the signal baseline right before application of the electrical stimulus, thus allowing passive tension and active contractile force to be derived from a single set of measurements.
3.8 Molecular assays – Western blot and quantitative RT-PCR

3.8.1 Western blot

Following electrophysiological or contractile force testing, each tissue patch was lysed for half an hour on ice in 100μl buffer consisting of 87% RIPA, 10% glycerol, 5mM EDTA, 3% SDS and 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). During lysis, mechanical disruption was applied every 5 min using a disposable plastic pestle. Tissue lysates were cleared by centrifugation at 15,000xg for 10 min, following which 10μl of lysate was used to determine protein concentration using a standard BCA assay (Thermo Scientific). 10μl of 1M DTT (Sigma) was added to the cleared lysate, which was then distributed into aliquots containing 30-40μg of protein and frozen at -80°C for storage. An equal volume of 2x lamelli buffer (Bio-Rad) containing 5% ß-mercaptoethanol (Bio-Rad) was added to each sample and boiled for 10 min at 90°C to completely denature the protein before running on a 4-15% mini-Protean precast gradient SDS-PAGE gel (Bio-Rad). Gels were run for 2 hours at 100V. Wet transfer (20% methanol) was applied at 100V for 1 hour to immobilize proteins to 0.22μm nitrocellulose scaffolds (Santa Cruz). Nitrocellulose scaffolds were then carefully cut according to molecular weight using the protein ladder as a guide and blocked in 5% BSA. Primary antibodies used were Nav1.5 (1:2000, generous gift of Dr. Peter Mohler), Sarcomeric α-actinin (1:5000, Sigma), Kir2.1 (1:1000, Alomone Labs), ß-tubulin (1:10,000,
Abcam), Connexin-43 (1:5000, Abcam). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse (1:2000 respectively, Sigma). Bands were visualized using x-ray film (Amersham film, GE Healthcare Life Sciences) and densitometry was performed using ImageJ.

3.8.2 Quantitative RT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen) and quantified using Nanodrop (Thermo Scientific). 0.5 - 1μg of RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). PCR primers were designed to have an annealing temperature of 60°C using NCBI’s Primer-BLAST to amplify 150bp or smaller stretches of DNA (Table 3.5). Quantitative PCR was performed using iTaq Sybr Green Supermix with ROX (Bio-Rad) on an ABI 7900 HT machine. Relative gene expression changes were quantified using the 2-ΔΔCT method. Primer efficiencies were measured by serial dilution of cDNA template over four orders of magnitude; all primer sets used had efficiencies of 95 – 100%. Specificity of amplification was determined by melting curve analysis.
### Table 3.5: qPCR primers

<table>
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<th>Gene</th>
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<td>COL1A2</td>
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<td>AGCACCAACAGTCTCCAGA</td>
<td>118</td>
</tr>
<tr>
<td>COL3A1</td>
<td>TCCTGCTGGTGCTCTGACTG</td>
<td>AGAGAACCACACTGTGGCTCTG</td>
<td>148</td>
</tr>
</tbody>
</table>

### 3.9 Immunohistochemistry
3.9.1 Immunostainings

All samples were fixed in 4% PFA for 12 minutes (monolayers) or 30 minutes (tissue patches). Samples were permeabilized in 0.1% triton-X for 1 hour, then blocked in a solution of 0.8% BSA + 20% chick serum for 30 min. Primary antibodies were applied at a concentration of 1:100 – 1:400 at room temperature for 1 hour. Alexa-fluor conjugated secondary antibodies were applied at a concentration of 1:200 at room temperature for 1 hour. Images were captured using a Leica SP5 confocal microscope.

3.10 Conditioned media studies

3.10.1 Engineering of cardiac "micro-patch" tissues for conditioned media studies

3- and 7-mm ring-shaped nylon frames were punched out of a piece of fabric (Cerex Advanced Fabrics) using circular punchers. A small amount of PDMS was poured into each well of 24-well plates and allowed to set. PDMS-coated wells were sterilized using 70% ethanol, and a ring-shaped circular nylon frame was pinned in each well. To form engineered tissues, 35μl of cell suspension (Table 3.3) at a density of 2x10^6 cells/ml was added to the central portion of each ring and evenly distributed using a pipet tip to allow the gel solution to interface with the nylon mesh. The gel solution was allowed to polymerize for 45 min at 37°C, and cultured in 1ml of serum-free N2B27 medium for up to 14 days.
3.10.2 Conditioned media + use of small molecule inhibitor library

Cardiac fibroblasts were plated in serum-containing medium (Table 3.2) at a density of 400 cells/mm² on gelatin-coated T-75 tissue culture flasks (approximately 3x10⁶ cells per flask), and used to condition serum-free N2B27 medium (10ml per flask) for 48hr. Conditioned media was then collected in 15ml conical tubes, and any suspended cells or debris was cleared by centrifugation at 500xg for 10 mins. Conditioned media was then directly used for cell culture, or stored at 4°C for later use. Conditioned media was collected in this manner from the same flasks of cells three times (over 6 days in culture). No observable decrease in cardiac fibroblast cell density was observed during conditioning.

Micro-patches were pre-conditioned with small molecule inhibitor drugs (Table 3.6) for 4hrs before the introduction of conditioned medium. Conditioned medium containing inhibitor drugs was then added to the micro-patches. Media was changed every other day until assessment.
Table 3.6: Small molecule inhibitor drugs

<table>
<thead>
<tr>
<th>Small Molecule</th>
<th>Target Inhibited</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT04</td>
<td>Rho</td>
<td>0.25μg/ml</td>
</tr>
<tr>
<td>Y27632</td>
<td>ROCK</td>
<td>10μM</td>
</tr>
<tr>
<td>SP600125</td>
<td>JNK</td>
<td>10μM</td>
</tr>
<tr>
<td>SB203580</td>
<td>p38 MAPK</td>
<td>4.5μM</td>
</tr>
<tr>
<td>AG490</td>
<td>JAK2</td>
<td>10μM</td>
</tr>
<tr>
<td>CP690550</td>
<td>JAK1/2</td>
<td>5μM</td>
</tr>
<tr>
<td>U73122</td>
<td>PLC</td>
<td>5μM</td>
</tr>
<tr>
<td>Chloroethylthine Chloride</td>
<td>PKC</td>
<td>5μM</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK1/2</td>
<td>1μM</td>
</tr>
</tbody>
</table>

3.10.3 Assessment of spontaneous beating amplitude by video analysis

3, 10 and 14 days after the introduction of conditioned medium, 24-well plates containing micro-patches were mounted on a standard fluorescence microscope with live cell imaging heating chamber warmed to 37°C. For each micro-patch, 5 seconds of video at a frame rate of 25 FPS (4x magnification) was captured using a 1.3 megapixel eyepiece camera. Video files were then analyzed using a custom program written in Python. Video files were loaded into main memory frame-by-frame and converted into 8-bit greyscale. Although other studies have used video edge detection analysis to derive parameters such as fractional shortening in the assessment of contractile function\[167\], we found that this technique was not suitable for our system. Due to the
relatively low cell seeding density and homogenous nature of the micro-patches, there
was no ‘edge’ that we could conveniently use to assess beating intensity. Instead, we
treated each cell or (cluster of cells) within each micro-patch as beating independently of
its neighbors, and used greyscale intensity fluctuations as indicators of beating in each
part of the micro-patch. The time-varying greyscale signal intensity for each pixel was
detrended to correct for optical artifacts during recording. Greyscale amplitude for each
pixel was computed and used as a scalar representation of contraction intensity for that
pixel. The average contraction intensity in each video was determined by averaging the
contraction intensities over all pixels.

3.10.4 Quantitative Morphometry of Cell Spreading

Confocal microscopy was used to obtain image stacks from each micro-patch
stained using sarcomeric α-actinin (red channel) and DAPI (blue channel) using 1µm
step intervals to a total of 12µm depth. Three 20x fields of view were taken per micro-
patch. Image stacks were then flattened and analyzed using a custom program written
in Matlab. In brief, each color channel was converted to 8 bit greyscale, despeckled, and
subjected to thresholding such that only cellular areas (red channel) or nuclei (blue
channel) were shown. Cellular nuclei in each image were counted by using an algorithm
based on Hough transformation to recognize partially overlapping or closely-spaced
nuclei (“Detect circles with various radii in grayscale image via Hough Transform” –
Tao Peng, MATLAB Central File Exchange, http://tinyurl.com/cby393k). The total cellular area in each channel was then divided by the number of nuclei to measure the amount of cell spreading taking place in each micro-patch.
4. Development of stem cell-derived engineered cardiac tissue patch with advanced structure and function

4.1 Rationale

Advances in pluripotent stem cell research have provided investigators with potent sources of cardiogenic cells. Purified populations of cardiac myocytes have been successfully isolated from both mouse and human pluripotent stem cells by antibiotic selection or fluorescence sorting based on the expression of αMHC\textsuperscript{43,168,169}, Mlc-2v\textsuperscript{170,171} or NCX-1\textsuperscript{172}. These cells have been shown to express cardiac-specific markers, display striated sarcomeric arrangement, contract spontaneously, and fire sustained action potentials with waveforms reminiscent of atrial, ventricular or nodal cells. In addition, multipotent cardiovascular progenitors have been isolated by using Isl1\textsuperscript{42} and Nkx2.5\textsuperscript{173} as genetic markers, or by using Flk-1\textsuperscript{174} (KDR-1\textsuperscript{131} in humans), PDGF-α\textsuperscript{175} and CXCR-4\textsuperscript{176} as cell surface markers. Cardiovascular progenitors are capable of differentiation towards vascular smooth muscle and endothelial lineages, in addition to cardiomyocytes.

Despite the success in derivation of functional cardiomyocytes from pluripotent stem cells, tissue engineering methodologies to assemble these cells into aligned, 3-dimensional (3D) myocardial tissues capable of physiologically relevant electrical conduction and force generation have been lacking. Guo et al.\textsuperscript{177} and Tulloch et al.\textsuperscript{146} have engineered cardiac tissue constructs from mouse and human ES cell-derived
cardiac myocytes respectively, and reported maximum contractile forces of approximately 0.48mN and 0.15mN. Song et al.\textsuperscript{178} have used engineered cardiac tissues composed of neonatal rat cardiac myocytes as an \textit{in vitro} model to test the electrical integration of donor stem cell-derived cardiac myocytes with host tissue, and report average conduction velocities of approximately 5.5cm/s within the fluorescently labeled stem-cell derived graft. Zimmermann et al.\textsuperscript{179} engineered tissues composed of neonatal rat ventricular cells (NRVCs) that produced in excess of 2mN of force, a finding corroborated in a recent study by Ott et al.\textsuperscript{180} where decellularized rings of heart ECM re-seeded with NRVCs produced approximately 2mN of force. Similar findings were also reported by Black et al.\textsuperscript{181} and Kensah et al.\textsuperscript{182}. Bursac et al.\textsuperscript{138,141} reported that PLGA scaffolds seeded with NRVCs were capable of conducting action potentials at an average velocity of 20cm/s, which is comparable to neonatal rat ventricles. It is thus evident that engineered tissues composed of NRVCs outperform those composed of stem cell-derived cardiomyocytes by 4 – 14 times in contractile force generation, and by 4 times in conduction velocity.

Possible reasons for the under-performance of previous stem cell-derived tissue constructs include: (1) suboptimal culture media formulation to advance functional differentiation of cardiomyocytes \textit{in vitro}; (2) the presence of inappropriate contaminating cell types that may prevent cardiomyocyte intercellular contacts and/or
negatively affect their survival and differentiation; (3) the lack of adequate cell alignment that may enhance cell-cell contact formation between cardiac myocytes\(^{181}\) and increase longitudinal conduction velocity and force production; and (4) the absence of necessary supporting cardiac non-myocytes which may enhance survival, structural organization, and functional differentiation of cardiac myocytes. In the studies described in this chapter, we therefore set to develop a new method to reproducibly engineer highly functional 3D cardiac tissues starting from mouse ES cells (mESCs). Using our recently developed gel micro-molding technology\(^ {70}\), I hypothesized that pure populations of mESC-derived cardiac myocytes or their progenitors purified by antibiotic selection could be induced to form compact, aligned and highly differentiated cardiac tissues capable of generating contractile forces and conduction velocities comparable to those of engineered tissues composed of primary NRVCs.

4.2 Results

4.2.1 Optimization of high aspect ratio tissue mold fabrication.

Standard soft lithography techniques involve fabrication of a negative template of the final object using silicon wafer spun-coated with photoresist. The final object is then usually created by casting PDMS against the photoresist template. We found that it was impossible to generate well-formed high aspect-ratio negative templates using standard U.V. photolithography due to excessive clogging of the features with partially-
crosslinked photoresist that could not be removed by standard development procedures (Fig. 4.1A). Instead, we developed a procedure to generate a positive template of the tissue mold in photoresist with high fidelity (Fig. 4.1B,C; Fig. 4.2A). When used in conjunction with a PDMS double-casting technique, this allowed us to generate features with a minimum dimension of 200μm and a height of 1.5mm (an aspect ratio of 7.5:1; Fig 4.2B). A negative template was then cast in PDMS (Fig. 4.2C). The surface of the negative template was passivated by extensive silanization to reduce its surface energy. Finally, a positive PDMS replica of the original photoresist template was created by casting PDMS off the negative template. This method greatly facilitated production of PDMS tissue molds without tears or defects. Although not used in this dissertation, an alternative PDMS-casting technique was developed by Dr. Wayne Pfeiler and has been used successfully by our lab (Annex A). Using smaller PDMS tissue molds with a planar dimension of 7x7mm² allowed us to perform tissue engineering experiments in a relatively high throughput format in 12-well plates (Fig 4.2D). Larger tissue molds of 1.4x1.4cm² could also be fabricated and used within 6-well plates. Tissue patches engineered using both smaller (Fig. 4.2E) and larger molds (Fig. 4.2F) had the same size and distribution of elliptical pores.
Figure 4.1 Optimization of SU-8 photolithography parameters. (A) The use of negative tone photomasks in conjunction with standard soft-bake and post-exposure bake timings to generate recessed pits in SU-8 was unsuccessful. Poorly-developed 5μm-deep pits were the result. (B) Using positive tone photomasks and a longer 16hr soft-bake time after sequential deposition of SU-8 layers resulted in higher fidelity features. However, exceeding the glass transition temperature during post-exposure bake resulted in diffusion of the crosslinker, and yielded features that were wider (250μm) than desired (200μm). (C) Using a long 48hr post-exposure bake below the glass transition temperature resulted in features of the desired width.
Figure 4.2. Fabrication of 3-D cardiac tissue patches. (A) Eight master templates microfabricated in SU-8 photoresist on silicon wafer. (B) Optical profile of the microfabricated SU-8 template’s surface. The master template consists of an array of staggered high aspect-ratio ‘posts’ with hexagonal cross-sections. (C) Four negative polydimethylsiloxane (PDMS) templates obtained by casting against the SU-8 master. (D) The identical ‘PDMS tissue mold’ replicas casted from negative PDMS templates are cut to fit in standard 12-well culture plates and used for reproducible, high-throughput production of 7x7 mm² tissue patches. Each mold contains square Velcro frame that serves to anchor the patch. (E) Porous tissue patches are formed by polymerizing cell/fibrin gel mixture around high aspect ratio PDMS posts. The pores attain elliptical shape through a process of cell-mediated gel compaction and serve to: 1) promote diffusion of oxygen and nutrients enabling the generation of thicker viable patches (up to 300 µm), and 2) locally orient cells in a single direction throughout the entire patch. (F) Larger 14x14 mm² tissue patches are made using larger PDMS molds cut to fit in standard 6-well plates.

4.2.2 Structural and functional characterization of mESC-CM monolayers.

mESC-derived cardiomyocytes (mESC-CMs) were cultured on aclar coverslips as 20 mm diameter confluent monolayers in a defined, serum-free medium. After 1-2 weeks of culture, the mESC-CMs exhibited a cross-striated phenotype and robust intercellular expression of connexin-43 gap junctions and the mechanical coupling
proteins N-cadherin, zona occludens-1 (ZO-1), desmoplakin and plakophilin (Fig. 4.3A-E) known to support normal electrical and mechanical communication in the adult heart by stabilizing the formation of gap junctions.

**Figure 4.3.** mESC-CMs form highly functional 2D monolayers. (A-E), Under optimized conditions, pure mESC-CMs in 1-2 week old confluent monolayers exhibit well-defined sarcomeric structure and robust intercellular coupling via electrical (A) and mechanical (B-E) junctions. (F) Point pacing (pulse sign) in mESC-CM monolayers elicits rapid and uniform action potential spread. Small circles denote 504 recording sites. Isochrones of cell activation (white circles) are labeled in milliseconds. (G-H). Addition of 2% serum in culture media improves conduction velocity (CV) without affecting action potential duration (APD). The use of a higher concentration of FBS (G) during embryoid body differentiation resulted in higher average CV and lower APD regardless of the concentration of FBS during monolayer culture. *P < 0.05, one-tailed Student’s t-test; N = 6.

When locally stimulated by a point electrode, mESC-CMs responded with uniform action potential propagation across the entire monolayer (Fig. 4.3F) at velocities that varied with serum concentration used for EB differentiation and for monolayer culture (Fig. 4.3G, H). Under optimized culture conditions (20% and 2% FBS for EB differentiation and monolayers, respectively), the conduction velocity (CV) and action
potential duration at 80% repolarization (APD) in mESC-CM monolayers were 22 ± 1.2 cm/s and 84.4 ± 3.4 ms, respectively.

Genes associated with pluripotency (Oct4 and Nanog) were still expressed in embryoid bodies after 8 days of differentiation, but were undetectable in puromycin-purified mESC-CM monolayers, and did not re-emerge even after 28 days of culture (Fig. 4.4). Functional differentiation of mESC-CMs was accompanied by a robust, time-dependent activation of cardiac functional genes, with a notable increase in expression of genes coding for contractile proteins cardiac myosin light chain 2 (Myl2) and sarcomeric α-actinin (Actn2), calcium handling proteins calsequestrin (Csq2) and ryanodine receptor (Ryr2), and the fast sodium channel Nav1.5 (Scn5a).
Figure 4.4. Gene expression during mESC-CM differentiation. mRNA transcripts were isolated from differentiating EBs before application of puromycin (Day 8, D8) and after puromycin selection during monolayer culture (D14+). Transcripts analyzed by RT-PCR included the pluripotency markers Oct4 and Nanog; cardiac developmental markers Nkx2-5, Gata4, Tbx5, and Mef2c; Cardiac cytoskeletal markers Tnnt2, Myl2, Myl7, Myh6, Myh7, and Actn2; Calcium handling, ion channel, and intercellular coupling markers Casq2, Ryr2, Cacna1c, Scn5a, Gja1, and Gja7; and the positive control marker Gapdh. Notably, pluripotency markers were completely absent from monolayer cultures (D14+), showing that undifferentiated cells were eliminated by puromycin selection. Expression of cardiac sodium (Scn5a) and L-type calcium (Cacna1c), channel subunit, ryanodine receptor (Ryr2), and calsequestrin (Casq2) transcripts increased with time of monolayer culture, suggesting maturation of membrane electrical properties and Ca\(^{2+}\) release and storage machinery.
4.2.3 Structural characterization of mESC-CM tissue patches

After 3 weeks of culture, pure mESC-CMs in tissue patches survived, as shown by their expression of live dsRed, but failed to compact the surrounding fibrin hydrogel matrix and undergo spreading and alignment (Fig. 4.5A). Instead, although being cross-striated and coupled by connexin-43 gap junctions, the cells remained rounded and formed isolated clusters (Fig. 4.5B), and only occasionally aligned against the hydrogel boundary (Fig. 4.5C). Remarkably, even cells that were a short distance (approximately 20μm) from the hydrogel boundary remained rounded (Fig. 4.5C). The rounded cardiac myocytes in the patches exhibited local contractions, but were not capable of macroscopic, synchronized contractions.

We hypothesized that pure mESC-CMs (unlike cultured NRVCs that contain a fraction of non-myocytes) fail to form a functional 3D syncytia due to their inability to significantly compact and mechanically remodel surrounding hydrogel matrix. Based on the measured non-myocyte (predominantly fibroblast) fraction in cultured NRVCs, we co-encapsulated mESC-CMs with a small number of neonatal rat ventricular fibroblasts (3%, 6% or 12% of mESC-CMs). With time of culture, the mESC-CM/fibroblast patches significantly compacted, and the mESC-CMs in the presence of fibroblasts elongated, co-aligned, interconnected throughout the patch volume (Fig. 4.5D-F), and generated macroscopically synchronous contractions. Unlike mESC-CMs, neonatal rat ventricular
fibroblasts, exhibited predominantly random orientations within the co-cultured (Fig. 4.5F) patch. The degree of cardiac myocyte alignment within the co-cultured patch could be controlled by varying the length of the microfabricated tissue pores, with longer pores resulting in better alignment of cardiac myocytes (Fig. 4.5G,H)\textsuperscript{70,184}.

**Figure 4.5.** mESC-CMs in 3D cardiac patches spread and interconnect only in the presence of fibroblasts. (A) Pure mESC-CMs in 14 day old tissue patches appear round and clustered. Dotted ellipse delineates the pore boundary created by the PDMS mold. (B) mESC-CM clusters contain rounded, cross-striated cardiomyocytes (green) inter-connected with connexin-43 gap junctions (red). (C) Aligned cells are occasionally found only at the gel boundary (white arrowheads). (D) In the presence of neonatal rat ventricular fibroblasts (red), mESC-CMs (green) start spreading (white arrowheads) by culture day 7. (E) At day 14, mESC-CMs in co-cultured patches are extensively aligned. (F) Fibroblasts around formed cardiac bundles show no preferential alignment. (G,H) Degree of local cell alignment can be controlled by varying the length of microfabricated posts.

### 4.2.4 Functional Characterization of mESC-CM tissue patches

In contrast to patches containing only mESC-CMs (Fig. 4.6A), the 14 day old, 7x7 and 14x14 mm\(^2\) mESC-CM + fibroblast patches supported robust macroscopic tissue
contractions (Fig. 4.6B) when subjected to 2Hz field shock. All patches exhibited a positive force-length (Fig. 4.6C) and a negative isometric force-frequency relationship (Fig. 4.6D). The amplitudes of contractile force in 7x7 mm² patches ranged between 1.20 ± 0.40 mN (12% fibroblasts) and 1.96 ± 0.54 mN (3% fibroblasts).

Optical mapping using Rhod-2 calcium-sensitive dye revealed that mESC-CM + fibroblast patches supported fast, micro- and macroscopically uniform action potential propagation (Fig. 4.7A) with velocities that ranged between 17.8 ± 1.9 cm/s (12% fibroblasts) and 24.1 ± 1.4 cm/s (6% fibroblasts). The patches exhibited average Ca²⁺ transient durations of 243 ± 10 ms (Fig. 4.7B) and could be steadily captured by electrical stimulation at maximum rates that ranged between 4.3 ± 0.3 Hz (3% fibroblasts) and 5.7±0.3 Hz (6% fibroblasts).
Figure 4.6. Isometric measurements of contractile force in co-cultured mESC-CM + fibroblast patches. (A) Patches containing mESC-CMs alone produced no significant forces on 2Hz field shock electrical stimulation. Instantaneous peaks above background noise are stimulus artifacts. Red arrowheads represent electrical stimuli. (B) Patches containing an additional 3% of cardiac fibroblasts showed a dramatic increase in force production. (C) Co-culture tissue patches exhibited rising force-length relationships reminiscent of the Frank-Starling law in whole hearts. Percent elongation is shown relative to tissue length during culture (7 mm). (D) The force recordings are further analyzed to derive force-frequency relationships. Mean ± s.e.m.; N = 3.

Figure 4.7. Co-cultured mESC-CM+fibroblast patches exhibit advanced electrical function. (A) Optical mapping revealed that co-cultured tissue patches supported fast and uniform action potential propagation. The void spaces in the isochrone map correspond to
tissue pores. (B) Conduction velocity (CV) and calcium transient duration (CaD) as a function of the initial fraction of fibroblasts (e.g., $CV = 241 \pm 16$ mm/s for 6% fibroblasts). CV of 6% and 12% fibroblast conditions differed significantly. *$P < 0.05$, one-tailed Student’s t-test assuming unequal variances, N = 3.

4.3 Discussion

In this aim we have demonstrate that: (1) it is possible to reproducibly obtain large, anisotropic 3D cardiac tissue patches starting from pluripotent stem cells; (2) in vitro conditions can be optimized to enable mESC-CM monolayers and engineered tissue patches to attain levels of structural and functional differentiation similar to those of engineered tissues containing neonatal rat ventricular myocytes; and (3) cardiac fibroblasts are essential for mESC-CMs to form functional syncytia in 3D engineered tissue patches. Our results emphasize the use of 3D, rather than 2D, cell culture environments as a requisite setting for systematic studies of cardiac tissue formation in vitro.

4.3.1 Control over crosslinker diffusion in thick photoresist layers is critical for the development of high aspect ratio tissue molds

As has been reported, the relationship between crosslinker diffusion and the crosslinking of SU-8 in photoresist layers is a highly complex function of temperature and solvent concentration\textsuperscript{185,186}. However, even without complex and laborious empirical experiments to fully characterize our system, some knowledge of the general principles involved was invaluable in creating a working process: (1) crosslinker diffusion should be minimized relative to the rate of crosslinking; (2) the presence of residual solvent,
which is a common problem in thick photoresist layers\textsuperscript{186}, greatly increases the rate of crosslinker diffusion and interferes with crosslinking; (3) uncrosslinked SU-8 photoresist undergoes glass transition at approximately 50°C, which greatly increases the mobility of crosslinker molecules. Solvent transport during the development process has also been reported in a number of studies as being a source of imperfections in the fabrication of high aspect-ratio structures, suggesting that a protocol which maximized solvent transport would be most likely to succeed.

The protocol we developed was specifically designed to address these general principles: (1) sequential deposition and soft-baking of SU-8 layers, followed by a final long, overnight soft-bake, was used to reduce solvent content in the photoresist layers as much as possible; (2) a post-exposure bake temperature was chosen below the glass transition temperature of SU-8 so as to reduce the mobility of crosslinker molecules. To compensate for the lower crosslinking rate at the cooler temperature, a very long crosslinking time (48hrs) was used; and (3) photomasks were designed so as to create the positive template in SU-8 (consisting of raised posts, as opposed to recessed pits in the negative template) to allow maximum solvent transport during the development process. These modifications greatly improved the quality of the templates we were able to produce, and enabled fine spatial control over myocyte fiber alignment in 3D in our project\textsuperscript{187} as well as those of others\textsuperscript{70,188}.  

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4.3.2 mESC-CM monolayers are strongly coupled and possess mature electrophysiological characteristics

Our immunostaining assessment showed that mESC-CMs displayed both robust expression of connexin-43 gap junctions as well as high levels of mechanical coupling proteins located at intercellular borders. Collectively, these intercellular junction proteins enabled the formation of a well-coupled electro-mechanical syncytium, characterized by fast conduction velocity of propagation and synchronized contractions. In murine hearts, it has been shown that mechanical coupling of cardiac myocytes with N-cadherin, desmoplakin, plakophilin and ZO-1 complement the electrical integration of the heart, stabilizing gap junctions at intercalated discs and enabling strong contractile forces to be transmitted throughout the tissue without damaging cells^{189,190}.

Although we were unable to measure contractile force generation in our monolayer preparations due to rigidity of the culture substrate, our optical mapping experiments showed that mESC-CM monolayers possessed CV and APD similar to those in intact neonatal rat^{138} and mouse ventricles^{191,192} as well as monolayer cultures of primary NRVCs^{163,193}, suggesting physiologically-relevant levels of electrical maturation and intercellular coupling. These CV values are significantly higher than previously reported for mouse or human ESC-CMs (1-6 cm/s^{128,178,194-198}). These late fetal / early neonatal levels of electrical maturity in mESC-CMs were achieved in vitro after a total of 21-28 days, a period comparable to that of mouse embryonic development^{16,161}. 
We believe that these results reflect our careful optimization strategy where serum type and concentration during embryoid body differentiation, the onset and duration of antibiotic selection, and media formulation during subsequent cell culture were each individually varied to maximize an important tissue-scale functional parameter, conduction velocity. In addition, efficient derivation of highly purified cardiogenic cells through puromycin selection further contributed to the success of our approach.

4.3.3 Cardiac fibroblasts are necessary for mESC-CM alignment and the formation of functional syncytia in 3D culture environments

Although many techniques exist to induce structured cell growth on 2D surfaces, including use of aligned electro-spun nanofibers\textsuperscript{72} and micro-contact printing of ECM proteins\textsuperscript{165}, few methods exist to control cell orientation in 3D culture environments. Aubin et al\textsuperscript{199} have used a microfabrication-based method similar to our own to pattern cell spreading in gelatin methacrylate gels, but their technique is limited to relatively thin hydrogels, does not allow the engineered tissue to benefit from enhanced media perfusion, and has been only applied to proliferative non-cardiomyocytes. In contrast, our technique allows for the creation of engineered tissues with near-millimeter-thickness, enabling the creation of engineered cardiac tissues that may produce more physiologically-relevant contractile force magnitudes. More elaborate 3D cell patterning technologies based on 2-photon laser scanning photolithography\textsuperscript{200} have been recently
developed which may enable the patterning of cells on a finer scale than our technology allows, but our micro-molding technique remains a relatively simple, efficient and cost-effective method to pattern 3D engineered tissues.

In our experiments with hydrogels containing only mESC-CMs, we observed that cardiac myocytes in direct contact with the hydrogel boundary were capable of spreading and alignment (Fig. 4.5C), whereas myocytes within the bulk of the hydrogel remained rounded. This is consistent with previous reports\textsuperscript{201,202} showing that the alignment of cells, such as fibroblasts, in 3D hydrogels is initiated by contact with the hydrogel boundary in partially constrained hydrogels. The presence of cardiac fibroblasts within our engineered tissue patches likely facilitated gel compaction (Fig. 4.8) and formation of local stresses within the hydrogel matrix\textsuperscript{203}, which have been shown to align the matrix’s constituent protein fibers\textsuperscript{204}. The alignment of protein fibers in the presence of cardiac fibroblasts is likely responsible for the subsequent alignment of cardiac myocytes remote from the hydrogel boundary. Interestingly, however, the fibroblast-induced gel compaction has not affected alignment of fibroblasts per se, which remained randomly oriented within compacted hydrogel regardless of the presence of cardiomyocytes (Fig. 4.5.F and 4.8A-C).
Figure 4.8. Cell-mediated gel compaction in fibroblast and mESC-CM patches. A-C, Neonatal rat ventricular fibroblasts (fib) compacted fibrin gel between hexagonal posts (A1-C1) while remaining uniformly distributed and unaligned throughout the patch (shown by vimentin staining in A2-C2). The degree of compaction increased with seeding cell density (2e5, 8e5, and 6.5e6 fib/ml of gel in A, B, and C, respectively). Panels are shown at culture day 3.5 D, Cell-mediated gel compaction as a function of time in culture. For all cell densities, rapid gel compaction (measured as normalized bundle width relative to that before gel polymerization) occurred in the initial 2-3 days of culture. After culture day 6, patch dimensions remained relatively constant. The degree of final gel compaction, increased with fibroblast density reaching plateau at 2e6 fib/ml. Pure mESC-CMs yielded significantly less gel compaction than pure fibroblasts for the same number of seeded cells (shown for 6.5e6 cells/ml). Co-culture of small number of fibroblasts (2e5/ml, 4e5/ml, or 6e5/ml corresponding to 3%, 6% or 12%, respectively) with 6.5e6/ml of mESC-CMs, resulted in additional gel compaction (shown for co-culture with 4e5 fib/ml). This compaction yielded mESC-CM alignment and the formation of functional cardiac patch (Fig. 4.5E,F). Similar to mono-cultured
patches, fibroblasts in the co-cultured patches remained unaligned (Fig. 4.5F) despite significant gel compaction.

Importantly, this requisite need for non-cardiomyocytes in generating functional 3D cardiac syncytium could not be revealed if only standard 2D cultures of mESC-CMs were studied. In fact, studies from other groups that used pseudo-1D micropatterned cell strands suggested that even very small numbers of non-cardiomyocytes may have adverse effects on the formation of functional cardiac tissue by blocking electrical conduction between cardiomyocytes\textsuperscript{205}. This notion in 3D engineered tissues would however only hold if significant volume of engineered cardiac tissue was occupied by fibroblasts\textsuperscript{138}, as characteristic for fibrotic heart disease\textsuperscript{206}. Therefore, the 3D cell culture environment is a more valid and relevant setting for studies of functional cardiogenesis \textit{in vitro} than standard 2D cultures.

Other studies have also suggested the importance of fibroblasts for the adhesion and integration of cardiac myocytes onto avitalized tissue slices\textsuperscript{158} and collagenous scaffolds\textsuperscript{157}. More recent studies have further supported our findings, showing that fibroblasts are necessary for the successful formation of contractile mESC-CM cell sheets on temperature-responsive surfaces and in engineered 3D tissues. Specifically, Matsuura et al\textsuperscript{207} found that 10-50\% neonatal rat cardiac fibroblasts were required for the formation of a self-supporting 2D cell sheet composed of mESC-CMs, and Kensah et al\textsuperscript{198} found that 8 – 17\% γ-irradiated mouse embryonic fibroblasts were needed to form a functional
hydrogel-based 3D tissue. Several studies have shown beneficial effects of other stromal and vascular cells on the survival, spreading, and functional maturation of neonatal rat and ESC-derived cardiomyocytes\textsuperscript{152,154,156,157,208-210}. The mechanisms of synergistic, or potentially antagonistic, cardiomyocyte/non-myocyte interactions in the context of cardiac development and tissue engineering remain unknown and are likely to depend on non-myocyte type\textsuperscript{154,156,210}, age\textsuperscript{211} and number\textsuperscript{138} and involve different cell contact, paracrine, and extracellular matrix mediated effects.

**4.3.4 mESC-CM + fibroblast engineered tissue patches display advanced electromechanical function**

Importantly, our described tissue engineering approach yielded levels of electrical and mechanical function that significantly surpass those previously reported for mouse or human stem cell-derived cardiomyocytes and cardiac tissues\textsuperscript{128,178,194-198,210,212}. Moreover, even though our initial cell source consisted of undifferentiated mESCs, the obtained functional properties are comparable to those of engineered 2D monolayers\textsuperscript{163,213,214} and 3D tissues\textsuperscript{138,141,179,180} made starting from considerably more mature NRVCs. In particular, mature electrophysiological properties of engineered cardiac tissues including robust electrical coupling and high conduction velocities are of critical importance for the ability of implanted cardiac tissues to functionally integrate with host myocardium without causing conduction abnormalities and increasing the propensity for arrhythmia induction. Significantly higher CVs (4-5 fold) in our tissues
than previously reported, although still suboptimal, suggest high degree of functional maturation of mESC-CMs in this tissue-engineered 3D culture environment.

4.4 Summary and Implications

In the studies described in this chapter, we refined a microfabrication-based tissue engineering method that enabled the formation of highly reproducible 7x7mm$^2$ and 14x14mm$^2$ 3D engineered cardiac tissue patches with controllable cell alignment. We then created monoclonal mouse ES cell lines capable of yielding large numbers of highly purified cardiac myocytes, and established in vitro culture conditions that allowed for the evolution of optimal electrophysiological properties in cardiac myocyte 2D monolayers. By combining our novel tissue engineering approach with this cell source, we attempted to create engineered cardiac tissues composed of ES cell-derived cardiac myocytes. We observed that the cells failed to form a functional syncytium without the addition of supporting cardiac fibroblasts. After only 2 week of culture, the resultant engineered tissues possessed unprecedented electro-mechanical maturity, and were judged to be the equivalent of late fetal or early neonatal murine cardiac tissue based on the observed structural and electrophysiological properties.

These findings have important implications for stem cell-based cardiac tissue engineering. It is now relatively well-established$^{198,207}$ that fibroblasts greatly facilitate the formation of functional engineered cardiac tissues. When extending this finding to
engineered tissues composed of human ES or induced pluripotent stem cell-derived cardiac myocytes, it will be necessary to determine the type and quantity of supporting cells that give rise to engineered tissues of the highest function. Although human foreskin fibroblasts\textsuperscript{198}, vascular endothelial cells and mesenchymal stem cells\textsuperscript{146} have been all used to create engineered human cardiac tissues, the functional parameters have so far been relatively disappointing. We speculate that supporting non-myocyte cells of cardiac origin may provide optimal support for developing engineered tissues \textit{in vitro}.

Although we have managed to attain an unprecedented\textsuperscript{146,178,198,212} level of electromechanical function in our engineered tissues, we note that they are still relatively immature and resemble late fetal or early neonatal myocardium. As such, these engineered tissue patches do not yet represent an ideal replacement for damaged adult myocardium. Still, it is encouraging that the in vitro time period needed to attain this level of engineered tissue maturation (total of 3-4 weeks of differentiation) closely corresponded to that of normal mouse embryonic development \textit{in vivo}\textsuperscript{215,216}. Additional cues such as electrical\textsuperscript{217} or mechanical\textsuperscript{146} stimulation and/or longer time of culture may be necessary for engineered tissues to approach adult levels of functionality.
5. Pluripotent stem cell-derived cardiovascular progenitor cells: A single cell source for cardiac tissue engineering

5.1 Rationale

In the previous chapter, we showed\textsuperscript{187} that the addition of neonatal rat cardiac fibroblasts to pure mouse ES cell-derived cardiac myocytes was required for the \textit{in vitro} formation of highly functional engineered cardiac tissues. This finding has since been corroborated by studies from the Okano\textsuperscript{207} and Martin\textsuperscript{198} groups who used other types of fibroblasts and derived similar conclusions. However, the developmental origin of cardiac fibroblasts is still obscure\textsuperscript{49,50,218}, and no single marker has been found that can uniquely distinguish cardiac fibroblasts from other cell types in the body. As a result, it has so far been impossible to derive cardiac fibroblasts from pluripotent stem cells. This precludes studies that would directly explore interactions of developmentally matched stem cell-derived cardiac myocytes and cardiac fibroblasts in an \textit{in vitro} setting, but it does not limit the possibility of combining various cardiogenic and fibroblastic cells sources towards development of efficient cardiac therapies.

Nevertheless, an ideal cell therapy would still involve the use of only a single, well-characterized stem cell-derived cell source that can allow generation of all cell types needed for engineering of functional 3D cardiac tissues. In this chapter, we therefore assessed the maturation and electro-mechanical function of 2D monolayers and 3D
engineered tissues made using genetically-selected \( \textit{Nkx2.5} \) cardiovascular progenitor cells (CVPs) with potential to generate not only cardiac myocytes but also supporting vascular cells. We have adapted our protocols from the previous chapter for use with both ES and iPS-derived CVPs. iPS cells, in particular, are easier to obtain, are associated with fewer ethical issues\textsuperscript{219,220} and are therefore more clinically translatable than ES cells.

We hypothesized that multipotent CVPs would be capable of generating their own supporting cell populations, and might therefore form highly functional engineered cardiac tissues without the addition of fibroblasts. The CVPs have been previously shown to contribute to the endothelial and smooth muscle cell lineages in addition to cardiac myocytes\textsuperscript{37,42,131,174,221}. In particular, smooth muscle cells have been documented to bear some similarities to (myo)fibroblasts: (1) smooth muscle contractile proteins such as \( \alpha \)-smooth muscle actin and SM22\( \alpha \) can be induced in both cell types by TGF-\( \beta \)\textsuperscript{222}; (2) both smooth muscle cells and activated myofibroblasts contract in response to stimuli such as angiotensin and vasopressin\textsuperscript{223}; and (3) the two cell types are morphologically similar \textit{in vitro}\textsuperscript{224}. We therefore postulated that CVP-derived smooth muscle cells might reasonably substitute for cardiac fibroblasts and facilitate functional cardiogenesis \textit{in vitro}. 
5.2 Results

5.2.1 Derivation and characterization of cell lineages from Nkx2.5-puro selectable mouse ES and iPS cell lines

Mouse ES cells (D3) were used to generate a monoclonal cell line capable of mESC-CVP generation based on the methods described by Christoforou et al. In this line, stable EGFP and puromycin N-acetyl transferase expression were driven by a cardiac-specific Nkx2.5 enhancer element, allowing antibiotic purification of green Nkx2.5+ cells from differentiating mESCs. Mouse iPS (miPS) cells were derived from primary mouse embryonic fibroblasts by transduction with a dox-inducible, polycistronic lentiviral vector encoding the four “Yamanaka” factors Sox2, Klf4, Oct4 and Myc. Over the course of 14 days in culture, round, compact colonies with refractile borders characteristic of pluripotent stem cells grew from the surrounding fibroblasts (Fig. 5.1A). After doxycycline withdrawal, colonies that maintained this characteristic morphology were hand-picked, expanded, and characterized for their expression of pluripotency markers by immunostaining for Pou5f1, Fut4 and Nanog (Fig. 5.1B,C). A pluripotent miPS cell line was determined as one which expressed these markers and which maintained stable doubling time and morphology. In collaboration with Dr. Christoforou and Malathi Chellappan, miPS cell lines were further assessed by quantitative RT-PCR for endogenous expression of transcription factors associated with pluripotency (Fig. 5.1D). miPS cells were found to express similar levels of Pou5f1, Sox2...
and Klf4 as ES cells, but expressed significantly higher levels of Nanog and significantly lower levels of Rex1 and Myc.

One miPS cell line was chosen and subcloned to express Nkx2.5-puro and Myh6-Red2-1/SV40-neo transgenes by electroporation. Colonies were picked under neomycin selection and screened for genetic integration by embryoid body differentiation (Fig. 5.2A). Positive clones were selected according to their expression of dsRed in contractile areas after selection with puromycin (Fig. 5.2B). These areas were confirmed to be highly enriched for the cardiac protein α-actinin (Actn2, Fig. 5.2B).

Puromycin-selected cells could be dissociated as single cells and plated as 2D monolayers on PDMS-coated glass coverslips (Fig. 5.2C). Under standard culture conditions, the majority of puromycin-selected cells were EGFP⁺ (mES-CVPs) or dsRed⁺ (miPS-CVPs), and positive for cardiac markers, indicating their cardiac myocyte fate. A reduction in cell seeding density or the addition of VEGF induced the proliferation of SMA⁺ smooth muscle cells and VWF⁺ endothelial cells, revealing the tri-potent capacity of the CVPs (as shown for mES-CVPs in Fig. 5.2D). 2D monolayers of puromycin-selected CVPs differentiated into cardiac myocytes that displayed prominent sarcomeric α-actinin striations, and were strongly coupled with connexin-43 gap junctions (Fig. 5.2E) as well as ZO-1 (Fig. 5.2F) and N-cadherin (Fig. 5.2G) mechanical junctions.
Figure 5.1 Derivation of iPS cells. (A) Doxycycline-inducible polycistronic lentiviral vectors were added to primary mouse embryonic fibroblasts. Over time, colonies with morphology characteristic of pluripotent stem cells appeared in culture i.e. heaped, compact with refractile borders. Doxycycline was withdrawn mid-way through the reprogramming process, and colonies which maintained stable morphology were picked after 14 days. Reprogrammed iPS cell lines stained positive for pluripotency markers Pou5f1 (B), Fut4 (B), and Nanog (C). (D) PCR primers designed to amplify only endogenous transcripts showed endogenous expression of pluripotency-related genes, indicating successful iPS reprogramming. *, ** and *** indicate $p < 0.05$, 0.01 and 0.0001, N = 3.
Figure 5.2. CVPs differentiate to well-coupled cardiac myocytes, smooth muscle and endothelial cells in 2D monolayers. (A) Candidate CVP-selectable cell lines were screened by embryoid body differentiation. Successful miPS cell lines displayed red fluorescent protein expression levels of genes coding for cardiac myocyte calcium handling (5.2.2 Genes related to calcium handling and contractile functions are upregulated in miPS-CVP monolayers)

Expression levels of genes coding for cardiac myocyte calcium handling (Atp2a2, Cacna1c, Casq2, Pln, Ryr2 and Slc8a1) were all strongly upregulated after 6 days in monolayer culture (Fig. 5.3). The expression pattern of genes encoding contractile proteins differed between protein isoforms with expression of ventricular-specific Myl2 being increased and Myl7 (atrial-restricted in later development) being decreased after 6 days in monolayer culture (Fig. 5.4). We also detected a switch in myosin heavy chain
isoforms, with expression of *Myh6* increasing relative to that of the fetal slow-twitch *Myh7* (Fig. 5.4).

![Figure 5.3](image)

Figure 5.3 Expression of Ca\(^{2+}\) handling genes as a function of differentiation time. A miPS CVP-selectable cell line was differentiated in embryoid bodies (until day 8), selected with puromycin for 3 days, dissociated, and cultured as monolayers until day 14. Key calcium handling genes were expressed with the onset of CVP differentiation at day 6, and were greatly upregulated with monolayer culture. SERCA pump (ATP2a2), L-type calcium channel (Cacna1c), Calsequestrin (Casq2), Phospholamban (Pln), Ryanodine receptor (Ryr2), Sodium-calcium exchanger (Slc8a1). *, significantly different from day 8, N = 3.

![Figure 5.4](image)

Figure 5.4 Expression of genes coding for cardiac contractile proteins as a function of differentiation time. Although *Myl7* dominated *Myl2* expression during embryoid body culture, monolayer culture of CVPs was associated with an increase in *Myl2* expression and decrease in *Myl7*, consistent with differentiation of CVPs towards distinct cardiac lineages. Similarly, although slow twitch *Myh7* expression was higher in embryoid body culture, monolayer cultures showed a higher expression of *Myh6*, indicating maturation of the cardiac lineage. *, significantly different from day 8, N = 3.
5.2.3 Optical mapping and intracellular electrode recordings show electrophysiological maturation of CVP monolayers

Since the Nkx2.5 enhancer element used to derive our cell line has been shown to have transient expression in differentiating cardiac myocytes, we chose to optimize for the number of days of puromycin selection. CVP monolayer conduction velocity was observed to be maximal after 3 days of puromycin selection, and APD increased and stabilized at approximately 80ms with any selection duration longer than 2 days (Fig. 5.5A). Thus, 3 days of puromycin selection was chosen as the optimal selection duration. After optimum puromycin selection, CVP monolayers exhibited an increase in CV and decrease in APD with time in culture (Fig. 5.5B), and after 14 days exhibited conduction properties comparable to those of pure mESC-CM monolayers (see Chapter 4.2.2) and NRVC monolayers (Fig. 5.5B). Simultaneously, the rate of spontaneous activity in CVP monolayers increased to $2.72 \pm 0.20$ Hz by culture day 5, followed by a gradual decrease to 0 Hz (no activity) by day 14 (Fig. 5.5C), while the maximum rate of cell capture by point electrical pacing steadily increased to 7 Hz by culture day 16.

Next, we performed intracellular electrode recordings on dsRed+ cardiac myocytes present in miPS-CVP monolayers. With time in culture, we observed no significant change in resting potential or action potential amplitude (Fig. 5.6B,C), but found a significant increase in action potential upstroke velocity from $149.3\pm3.5V/s$ to $179.5\pm9.3V/s$, and a significant decrease in APD$_{80}$ from $77.2\pm3.2ms$ to $60.9\pm1.7ms$ (Fig.
Overall, we found that action potential traces from CVP-derived cardiac myocytes became more abbreviated and triangular-shaped over time (Fig. 5.6A), indicative of progressive cardiac maturation.

Figure 5.5 Electrophysiological properties of CVP monolayers as assessed by optical mapping. (A) Electrophysiological parameters in 14-day old monolayers made using CVPs purified by selection with 5µg/ml puromycin (puro). The three-day puromycin selection yielded optimum conduction velocities (CV = 24.2 ± 0.4 cm/s) and was used in all further studies. *P < 0.05 relative to corresponding parameters for 2-day puromycin selection; N = 3-6 patches per group. Functional cardiac differentiation of CVPs with time in culture was associated with increased CV and decreased APD (B), as well as decreased spontaneous beating rate (closed diamonds) and increased maximum rate of electrical pacing (open squares) yielding steady 1:1 capture (C). *, significantly different from day 2; **, significantly different from day 7. N = 3-5 patches per group.
Figure 5.6 Electrophysiological characteristics of miPS-CVPs at 7 and 14 days of monolayer culture. (A) Representative action potential traces at day 7 and day 14. (B-C) With time of culture, maximum diastolic potential (B) and action potential amplitude (C) remained unchanged, while maximum action potential upstroke velocity (D) increased. *, significantly different from day 7. N=4 – 6.
5.2.4 CVPs form highly functional engineered tissues without the addition of fibroblasts

We then applied the same tissue engineering strategy as described in the previous chapter to fabricate 3D cardiac tissue patches starting from the mES-CVP cell source alone. In contrast to pure mESC-CMs that remained rounded in 3D culture, the cardiomyocytes that differentiated from pure CVPs spread, aligned, and interconnected throughout the entire hydrogel volume (Fig. 5.7A-C). Small amounts of endothelial and smooth muscle cells that differentiated from CVPs were found interspersed between the cardiomyocytes and concentrated near the free gel boundaries (Fig. 5.7A). After 14 days in culture, the CVP patches supported continuous and uniform action potential conduction (Fig. 5.7D) with velocities of 19.2 ± 0.4 cm/s and Ca\textsuperscript{2+} transient durations of 300 ± 10 ms (Fig. 5.7E), comparable to values measured in mESC-CM/fibroblast patches (see section 4.2.4). The CVP patches also exhibited a negative force-frequency and positive force-length relationship with maximum contractile forces of 1.28 ± 0.11 mN (Fig. 5.7F).
Figure 5.7. CVPs can autonomously form a functional 3D cardiac tissue patch. A, Three-week old tissue patch with aligned cardiomyocytes (red), and dispersed endothelial (blue) and smooth muscle (green) cells. SMA; smooth muscle actin. VWF; Von Willebrand factor. B-C, CVP derived cardiomyocytes in tissue patches are cross-striated and robustly coupled via electrical (B) and mechanical (C) junctions. D, A snapshot of a uniformly propagating action potential (see Supplementary movie 5) induced by a point electrode. E-F, After 3 weeks in culture, CVP patches exhibit high CVs (E), significant contractile forces, and physiological force-frequency and force-length relationships (F). N=3 patches per group.

5.3 Discussion

Although multipotent CVPs have been derived using a variety of genetic\textsuperscript{42} and cell surface markers\textsuperscript{131,174}, we chose to use genetically-selected Nkx2.5\textsuperscript{+} CVPs because: (1) unlike cell surface markers, Nkx2.5 has well-established function in this context as a critical cardiac transcription factor\textsuperscript{226}, ensuring specificity of selection; (2) Nkx2.5 is the earliest marker of both the primary and secondary heart fields\textsuperscript{162}, as opposed to Isl1.
which marks only the secondary heart field. Nkx2.5 would thus be expected to yield higher cell numbers; and (3) both Nkx2.5+ ES cell lines and transgenic mice have been reported in the literature, suggesting that the technology is robust and reproducible.

5.3.1 iPS cells show similar morphological and differentiation characteristics as ES cells

There have been various suggestions in the literature that constant overexpression of iPS reprogramming factors can lead to abnormal cell behavior including delayed cardiac differentiation. For our studies, we have used doxycycline withdrawal to silence the expression of exogenous reprogramming factors. By carefully designing primers to amplify only endogenously expressed transcripts, our quantitative RT-PCR results showed that endogenous expression of pluripotency-related transcription factors was upregulated, indicating successful reprogramming (Fig. 5.1D). Similar to others, we found that iPS cells do not have identical gene expression to ES cells (Fig. 5.1D), but still have the morphological hallmarks of pluripotency (Fig. 5.1A-C), as well as the ability to differentiate into beating cardiac myocytes. Embryoid body differentiation of iPS cells followed similar kinetics as ES cells, with Nkx2.5 being upregulated significantly by day 6 of differentiation (Fig. 5.8). Embryoid bodies showed dsRed expression confined to spontaneously contracting areas by day 8 (data not shown), in agreement with previous experiments with mouse ES cells.
Figure 5.8 Temporal expression of Nkx2.5 in differentiating iPS embryoid bodies. Nkx2.5 was significantly upregulated at day 5-6 in differentiating embryoid bodies, and thus puromycin selection was initiated at differentiation day 6. *, significantly different from day 4. N = 3.

5.3.2 CVP cardiac tissue patches show maturation of functional and Ca handling properties with time of culture

Although embryonic cardiac myocytes contract spontaneously in the absence of pacemaker cells, the adult ventricle is quiescent once nodal cells have been electrically isolated or ablated. Fundamentally, this difference exists because embryonic cardiac myocytes have immature calcium handling. As a result, there are localized spontaneous releases of calcium ions from intracellular stores, which in turn generate inward plasmalemmal current through the sodium-calcium exchanger. This triggers the L-type calcium channels, causing cell-wide synchronized calcium induced calcium release\textsuperscript{229}. 

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This mechanism of spontaneous activity has also been described in ES cell-derived cardiac myocytes\textsuperscript{32}.

In this study, we have observed that the spontaneous beating rate of CVP monolayers increases from 0 to approximately 3 Hz by culture day 5, before gradually becoming quiescent. In contrast, the maximum capture rate was observed to increase monotonically over time (Fig. 5.5C). This suggests that increasing electrophysiological maturity is not isolated to only plasmalemmal currents and ion channels, but includes the proper development of intracellular calcium handling. This is corroborated by our quantitative RT-PCR experiments which showed a large increase in the expression of key calcium-handling genes with time in culture (Fig. 5.3). Notably, key calcium handling proteins such as calsequestrin, junctin and triadin have been reported to be absent in spontaneously beating human ES cell-derived cardiac myocytes\textsuperscript{30}, contributing to their immature calcium handling characteristics. Our results suggest that calcium handling in CVP monolayers is more advanced than in human ES cell derived cardiac myocytes, which may be attributable to the shorter developmental time scale of mouse cells.

Nevertheless, calcium handling in CVP engineered cardiac tissues is clearly not fully-developed. Consistent with other reports\textsuperscript{230}, CVP engineered cardiac tissues showed a negative force-frequency relationship between 0.5 – 5.5Hz (Fig. 5.7F) which is
characteristic of slow or incomplete calcium cycling into the sarcoplasmic reticulum. In contrast, mouse embryonic heart slices have been reported to display a positive force-frequency relationship. The maturation of ES cell-derived cardiac myocyte calcium handling is an ongoing topic of active research, with attempts to over-express key calcium handling proteins meeting with limited success. Spontaneous calcium release due to immature calcium handling poses a potential safety risk in the context of cell therapies because it could result in the spontaneous firing of ectopic beats from implanted cells and the initiation of re-entrant arrhythmias.

5.3.3 Functional and gene expression assays suggest CVP monolayers and engineered tissues reach a developmental stage resembling that of late fetal or early neonatal mouse ventricle

The observed trends in macroscopic electrical properties (i.e., CV increase, APD decrease, maximum upstroke velocity increase) suggested that CVP monolayers initially resembled early embryonic cardiac tissue, but with time of culture, significantly matured to acquire a functional phenotype of late fetal or early neonatal ventricular muscle. Specifically: CV has been reported to increase from 7cm/s in early embryonic to 21cm/s in late fetal mouse ventricles; APD has been reported to decrease from 100ms to 74ms; and maximum upstroke velocity has been reported to increase from 88.2V/s to 113.2V/s. This is consistent with our observed changes in CV (2.75±0.05 cm/s at day 7 to 17.4±3.12 cm/s at day 14) and APD (77.2±3.2 ms at day 7 to
60.9±1.7 ms at day 14). The maximum upstroke velocity that we observed at either 7 or 14 days in culture (149.3±3.6 V/s and 179.5±9.3 V/s respectively) exceeded the range reported in literature for fetal ventricular myocytes, and is instead more consistent with values reported in neonatal mouse ventricles (196 V/s)\(^ {235}\).

Furthermore, embryonic and fetal hearts display uniform distribution of connexin-43 and N-cadherin junctions\(^ {236,237}\). With further cardiac development into adulthood, these electrical and mechanical junctions predominantly localize at the ends of cardiomyocytes (in specialized regions called intercalated disks). Both in CVP monolayers and engineered tissues we observed punctate uniform pattern of connexin-43 and N-cadherin staining. Along with near-neonatal values of CV (19.2±0.4 cm/s) in cardiac patches, these results further imply that functional maturity of CVPs in our 2-week 2D and 3D cultures corresponded to that of the late fetal or early neonatal cardiomyocytes.

In addition to functional analysis, quantitative RT-PCR showed that there was a switch in the dominant isoform of myosin heavy-chain being expressed in CVP monolayers with time in culture. The ratio of *Myh6/Myh7* gene expression increased from 0.11 on day 0 of monolayer formation to 1.29 on day 6 (Fig. 5.4). This switch in myosin heavy chain isoforms is a well-documented feature in mouse heart development, and occurs between E17 and birth\(^ {16\text{1}}\). This is in agreement with the estimated near-
neonatal developmental state of our CVP monolayers based on their functional properties. Interestingly, we also observed an increase in Myl2 expression relative to Myl7 (Fig. 5.4). Myl7 is ubiquitous in early embryonic cardiac myocytes but becomes progressively more restricted to the atria with development; in contrast, Myl2 expression is restricted to ventricular myocytes. This suggests that even in the absence of looping or septation, CVP-derived cardiac myocytes may be predominantly fated to a ventricular phenotype. However, since we measured the electrophysiological properties of CVP-derived cardiac myocytes within well-coupled monolayers, we were not able to distinguish chamber-specific action potential phenotypes.

5.3.4 CVP-derived non-myocytes support the formation of highly functional engineered cardiac tissues

Our study\textsuperscript{187} and others\textsuperscript{146,156,198,207} have confirmed that supporting non-myocytes such as cardiac or non-cardiac primary mouse embryonic fibroblasts are supportive of the formation of functional engineered tissues. Notably, recent efforts to engineer tissues using human ES cell derived cardiac myocytes have suggested a similar conclusion. Kensah et al\textsuperscript{198} showed that addition of human dermal fibroblasts facilitated the integration of genetically purified human ES cell-derived cardiac myocyte bodies into functional engineered cardiac tissues. Kim et al\textsuperscript{208} showed that accompanying non-myocytes in differentiating embryoid bodies enhanced the electrophysiological maturation of human ES cell-derived cardiac myocytes. Similarly, in studies where
directly differentiated human ES cell-derived cardiomyocytes have been used to create engineered tissues, they comprised only a fraction of the cells used for tissue formation\textsuperscript{238,239}. Our own recent experiences in this regard indicate that even SIRPA\textsuperscript{+} ES cell fractions that predominantly contain cardiac myocytes and autonomously form functional tissues also contain a fraction of endothelial and smooth muscle cells (Zhang et al, unpublished). These studies collectively suggest that supporting fibroblasts or vascular cells (such as those derived from CVPs) are required to support the formation of functional engineered cardiac tissues made of either mouse or human pluripotent cells.

### 5.4 Summary and implications

Although we and others have found that cardiac fibroblasts are necessary for the formation of 3D functional engineered cardiac tissues, it is currently not possible to derive human cardiac fibroblasts from ES or iPS cells for use in engineering of functional human cardiac tissues. Adult human cardiac fibroblasts could potentially be used together with human iPS-derived cardiomyocytes for autologous therapies, however they can only be obtained through Invasive cardiac biopsies and likely in relatively small numbers. Some groups have attempted to circumvent this limitation by utilizing fibroblasts from non-cardiac cell sources, such as primary mouse embryonic fibroblasts in mouse engineered tissues and human foreskin fibroblasts in human engineered
tissues\textsuperscript{198}. However, we note that sub-optimal functional parameters measured in these studies, in particular conduction velocity, suggest that cells of non-cardiac origin may be less suited for cardiac tissue engineering. In particular, Kensah et al\textsuperscript{198} reported low conduction velocities of 2-3 cm/s in engineered tissues containing PMEFs, and low velocities of up to 4.9 cm/s in engineered tissues containing human foreskin fibroblasts. Contrastingly, high conduction velocities reported in Chapters 4 and 5, as well as our group’s unpublished data by Zhang et al. (up to 25.1 cm/s using SIRPA\textsuperscript{+} human ES cell-derived cardiac myocytes), suggest that supporting cells of cardiac origin may be the appropriate choice for functional cardiac tissue engineering. Furthermore, global expression profiling of endothelial cell and fibroblasts have shown that their characteristics can vary greatly depending on the organ of origin or anatomical location\textsuperscript{159,240}. Furthermore, generation of functional murine cardiac myocytes by genetically induced fibroblast transdifferentiation has been shown to be more efficient when using fibroblasts from heart rather than skin or tail\textsuperscript{241,242}. Thus, it is plausible that cardiac fibroblasts differ from fibroblasts of other organs, and that they are particularly suited for engineering of functional cardiac tissues. Of note is however, that vascular (endothelial and smooth muscle) cells rather than fibroblasts are the dominant non-myocyte cell type in the heart during early development, and thus, they would be also
expected to provide trophic and other cues for functional cardiomyogenesis \textit{in vitro} and \textit{in vivo}.

In this chapter, we have shown that it is possible to use a single well-characterized, pluripotent stem cell-derived source to engineer highly functional cardiac tissues. This overcomes the limitations discussed above although cardiac fibroblasts may be only a small fraction of the supporting cells in this case, with smooth muscle cells and to less extent endothelial cells being the main supporting cell type. Similarly, in the context of human ES cells, the recent discovery that extracellular marker SIRPA marks differentiating cardiac myocytes but also vascular cells\textsuperscript{243} may obviate the need to use transgenic CVP-producing cell lines as described in this chapter. In fact, our recent experience has shown that MACS-selected SIRPA\textsuperscript{+} cell fractions can be utilized to autonomously form highly functional human cardiac tissues, that also contained a percentage of endothelial and smooth muscle cells (Zhang et al, unpublished). However, of note is that SIRPA is not restricted to the cardiac lineage during development\textsuperscript{244,245}, and can be thus used to select for cardiovascular progenitors only along with well-optimized directed differentiation protocols\textsuperscript{243}. Nkx2.5, being restricted to the cardiovascular lineage, may present a more reproducible alternative (albeit with use of genetic modification) for different pluripotent stem cell lines, since differentiation protocols based on this marker are generally line-specific.
The studies conducted in these two chapters raise an interesting question: what characteristics define a suitable supporting cell type for functional cardiac tissue engineering? In the next chapter, we investigate in more detail the roles of cardiac fibroblasts, and in particular their developmental stage, in regulating engineered cardiac tissue function.
6. Cardiac fibroblast developmental stage-dependent effects on stem cell-derived engineered cardiac tissue function

6.1 Rationale

Although the precise developmental origin of cardiac fibroblasts remains unknown\textsuperscript{218}, there is evidence that they play a critical role\textsuperscript{39} in normal heart development. Cardiac muscle gains mass during development through the simultaneous processes of cardiac myocyte division (hyperplasia) and increases in the size of individual myocytes (hypertrophy)\textsuperscript{39,64,246}. Although both hyperplasia and hypertrophy take place as part of normal development\textsuperscript{64}, fetal cardiac myocytes remain predominantly hyperplastic until shortly after birth, i.e., Day 4 post-partum in mice\textsuperscript{246}. Embryonic cardiac growth is thus driven to a large extent by hyperplasia – in particular, the proliferation of compact myocardium, a key step in the formation of full-thickness ventricular wall, has been found to be driven, among other cues, by cardiac fibroblast-secreted paracrine factors\textsuperscript{39,247}. In contrast, adult cardiac fibroblast secreted paracrine factors have been shown to contribute induction of cardiac myocyte hypertrophy in a variety of disease\textsuperscript{55,248-250} and non-disease\textsuperscript{39,251,252} contexts. This suggests that developmental changes in cardiac fibroblasts may proceed in tandem with changes in cardiac myocytes and facilitate the normal developmental processes, and/or mediate pathological remodeling.
In the context of tissue engineering, Xi and Pfannkuche et al. showed that avitalized myocardial tissue\textsuperscript{158} as well as collagen-based tissue-engineered constructs\textsuperscript{157} benefitted from pre-conditioning with primary mouse embryonic fibroblasts. Pre-conditioning improved the attachment and subsequent ability of cardiac myocytes to convey contractile forces to their attachment substrates. A study by Matsuura et al.\textsuperscript{207} indicated that cardiac myocytes require the presence of cardiac fibroblasts to form self-supporting, detachable cell sheets. Our own study\textsuperscript{187} showed that cardiac fibroblasts are required for the formation of highly functional engineered cardiac tissues. Finally, Kensah et al.\textsuperscript{198} have found that fibroblasts are essential for the formation of functional iPS cell-derived engineered cardiac tissues of mouse or human origin. It is therefore evident that: (1) engineered and native cardiac tissues share the same critical dependence on fibroblast-mediated signaling; and (2) at least some aspects of this signaling can be recreated in vitro regardless of the species or origin of the fibroblasts used for this purpose.

Freshly-isolated mESC-derived cardiac myocytes are known to have immature structural and functional properties similar to outflow tract cardiac myocytes at embryonic E12.5\textsuperscript{253}. However, under optimized culture conditions, we have shown (Chapters 4 and 5) that monolayers and engineered tissues consisting of these cells structurally and functionally remodel over time to attain a more mature phenotype\textsuperscript{187}

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characteristic of late embryonic or early neonatal cardiac tissues. Specifically, cardiac myocytes in our 3D culture system start out having a rounded shape, but over the course of 14 days in culture, they become progressively more elongated and interconnected with connexin-43 and N-cadherin junctions, and become electrophysiologically more mature and capable of conducting action potentials at ~20 cm/s. Overall, the 2-week culture of mESC-derived engineered cardiac tissues appears to recapitulate multiple aspects of cardiac development between embryonic E12.5 and the perinatal period but only in the presence of supporting non-cardiomyocytes.

Whether cardiac fibroblasts of different developmental stages have distinct effects upon engineered cardiac tissue formation and function (either through direct cell-cell contact or paracrine action) has not been investigated. We therefore explored this question in a same-species setting (rather than using neonatal rat fibroblasts shown in Chapter 4), by isolating cardiac fibroblasts from: (1) E13.5 mouse embryos; or (2) adult mice, and co-culturing them with mESC-derived cardiac myocytes to form engineered tissues. Electrophysiological properties of the engineered cardiac tissues were assessed by optical recording of intracellular calcium transients, and active (contractile) and passive mechanical properties were studied using previously described force measurement tests. Immunostaining and Western blot analyses were performed to
assess structural and protein-expression changes in the engineered tissues made using the two fibroblast populations. To determine the effects of cardiac fibroblast conditioned medium on mESC-derived cardiac myocytes, we fabricated and utilized miniature engineered tissue patches (“micro-patches”). This allowed us to carry out higher-throughput paracrine and drug conditioning studies whilst using fewer cells. Selected small molecule inhibitor drugs were then applied to elucidate the intracellular pathways involved in the fibroblasts paracrine factor-enhanced cardiomyocyte spreading and contractile activity. Lastly, quantitative RT-PCR was used to screen cardiac fibroblasts for expression of genes coding for paracrine factors that likely mediated the observed effects on cardiomyocytes. These studies revealed mechanisms by which heterocellular interactions between cardiac myocytes and fibroblasts may act to facilitate the formation of functional myocardium with important implications for cardiac development and tissue engineering therapies.

6.2 Results

6.2.1 Cardiac fibroblasts purified by MACS show minor activation towards myofibroblast phenotype

Although previous work by our group\textsuperscript{163,164} and others\textsuperscript{254} have shown that cardiac fibroblasts from neonatal rat cardiac tissue can be purified by pre-plating onto tissue culture plastic, in our hands both fetal mouse cardiac fibroblasts and myocytes were similarly adhesive to tissue culture plastic (data not shown), therefore pure mouse
cardiac fibroblasts could not be readily pre-plated out. Instead, similar to methods by Ieda et al.\textsuperscript{39} and Hudon-David et al.\textsuperscript{53}, we have isolated pure CD90\textsuperscript{+}/CD31\textsuperscript{-} cardiac fibroblasts by MACS. This allowed us to obtain relatively large numbers of cardiac fibroblasts (0.17x10\textsuperscript{6} cells per engineered tissue patch) needed for the described tissue engineering studies.

Specifically, using MACS we enriched the percentage of CD90\textsuperscript{+}/CD31\textsuperscript{-} fetal cardiac fibroblasts from a low purity of 68.1±7.3\% to a relatively high purity of 84.5±4.0\% (Fig. 6.1A-C). We also found that adult cardiac cells isolated using our enzymatic digestion protocol consisted almost entirely of CD90\textsuperscript{+}/CD31\textsuperscript{-} cardiac fibroblasts (86.9±4.4\%), thus obviating the need for further MACS purification. In all cases, only a small population of CD31\textsuperscript{+} endothelial cells (0.86 – 2.42\%) could not be depleted by MACS.

**Figure 6.1**: FACS analysis of magnetically sorted (MACS) fibroblasts. MACS was effective at separating CD31\textsuperscript{-}/Thy1\textsuperscript{-} fetal cardiac cells (A) from CD31\textsuperscript{-}/Thy1\textsuperscript{+} fetal cardiac fibroblasts (B). (C) Percent Thy1\textsuperscript{+}/CD31\textsuperscript{-} cardiac fibroblasts in fetal and adult cardiac cells before MACS and fetal cells after MACS purification (N=4). A low percentage of CD31\textsuperscript{+} Thy1\textsuperscript{+} endothelial cells was present in both fetal and adult cardiac cells (Fetal = 1.48±0.6\%, Adult = 0.05±0.05\%).
Fetal+MACS = 0.86±0.5%, Adult = 2.42±1.0%, N=4). Values shown are mean ± SEM. *, significantly different from Fetal (non-MACS) group.

Immunostaining was performed in adult and fetal cardiac fibroblasts after isolation and purification. We found that isolated cells were morphologically homogenous and stained negative for cardiac (sarcomeric α-actinin, Fig. 6.2A2,A3), endothelial (VWF, Fig. 6.2A3, 6.3A3) and smooth muscle (SM-MHC, Fig. 6.2B2,B3) markers. All cardiac fibroblasts stained positive for mesodermal marker vimentin (Fig. 6.2C2,C3). Similar to our previous studies with neonatal rat fibroblasts\textsuperscript{163}, smooth muscle actin (SMA, Fig. 6.2B3, 6.3B3), a marker of myofibroblast phenotype\textsuperscript{257} was only detected at very low levels in cultured mouse cardiac fibroblasts.
Figure 6.2. Phenotypic characterization of cultured adult cardiac fibroblasts. Adult cardiac fibroblasts were negative for cardiac marker sarcomeric α-actinin (SAA), endothelial marker von willebrand factor (VWF) and smooth muscle marker smooth muscle myosin heavy chain (SM-MHC). Virtually all cardiac fibroblasts stained positive for vimentin, and no connexin-43 (CX43) gap junctions were observed between any fibroblasts. Using a highly specific anti-Smooth Muscle Actin (SMA) antibody (Abcam), we determined that the amount of adult fibroblast activation to myofibroblast phenotype was minimal. Scale bar: 200μm.
Figure 6.3. Phenotypic characterization of cultured fetal cardiac fibroblasts. Similar to adult cardiac fibroblasts (Fig. 6.2), fetal cardiac fibroblasts were negative for cardiac marker SAA, endothelial marker VWF and smooth muscle marker SM-MHC. Fibroblasts stained positive for Vimentin, and were not coupled with CX43 gap junctions. Using a highly specific anti-SMA antibody (Abcam), we determined that the amount of fetal fibroblast activation to myofibroblast phenotype was minimal. Scale bar: 200μm.

6.2.2 Cardiac fibroblasts modulate the structure and function of engineered cardiac tissue patches in a developmental stage-dependent manner

Similar to studies in Chapter 4, the encapsulation of only pure mESC-CMs (Fig. 6.4A) in tissue patches (without fibroblasts) resulted in minimal gel compaction and rounded cell morphology that persisted throughout the culture duration (Fig. 6.4B). Addition of fibroblasts yielded hydrogel compaction that was more pronounced for fetal
(Fig 6.4C) than for adult (Fig. 6.4D) fibroblasts. Furthermore, fetal cardiac fibroblasts consistently localized to the outer layer of engineered patches (Fig. 6.4C), whereas adult cardiac fibroblasts tended to be interspersed between the cardiac myocytes (Fig. 6.4D). Finally mESC-CMs in tissue patches co-cultured with fetal fibroblasts appeared to be more spread, aligned, and to have more abundant connexin-43 expression (Fig. 6.4E) than those co-cultured with adult cardiac fibroblasts (Fig. 6.4F).

Figure 6.4. Structural characterization of co-cultured tissue patches. (A) A representative 1-week old monolayer of puromycin-selected mESC-CMs. (B) Pure mESC-CMs remain rounded after 14 days of tissue patch culture. The absence of vimentin staining demonstrates purity of cardiomyocytes. (C-D) Z-stack confocal images showing that mESC-CMs elongated and interconnected within tissue patches when co-cultured with fetal (C) or adult (D) cardiac fibroblasts. (E-F) mESC-CMs express more connexin-43 (Cx43) gap junctions in patches made with fetal (E) than adult (F) cardiac fibroblasts.
Similar to our studies using neonatal rat cardiac fibroblasts, optical mapping of co-cultured cardiac tissue patches made of mESC-CMs and adult or fetal cardiac fibroblasts revealed that they were capable of sustaining continuous action potential conduction during point pacing (Fig. 6.5A). In contrast, engineered cardiac tissue patches containing only mESC-CMs and no cardiac fibroblasts were unable to sustain any action potential propagation, and responded to neither point pacing nor field shock (Fig. 6.5A). Interestingly, CV of patches made with fetal cardiac fibroblasts (9.86±0.88 cm/s) was higher than that of patches made with adult fibroblasts (4.87±1.18 cm/s), while Ca^{2+} transient duration was lower (209.2±39.3 ms for fetal vs. 344.9±83.7 ms for adult), but without reaching statistical significance (Fig. 6.5B, p = 0.1).
Figure 6.5. Electromechanical characterization of co-cultured tissue patches. (A) Representative $\text{Ca}^{2+}$ transient activation map in a tissue patch made of mESC-CMs and fetal cardiac fibroblasts. Unlike fibroblast-containing patches (top trace), tissue patches without fibroblasts were not excitable and showed no calcium transient activation (bottom trace). (B) Action potential conduction velocity (CV) and $\text{Ca}^{2+}$ transient duration (CaD) in tissue patches made with fetal and adult cardiac fibroblasts. (C) Contractile force traces during 3Hz field shock stimulation in tissue patches with adult (top), fetal (middle), or no (bottom) cardiac fibroblasts. (D-E) Physiological active (D) and passive (E) force-length curves in patches made with adult and fetal cardiac fibroblasts. *, significantly different – C. Fib. **, significantly different between + adult and + fetal C. Fib at the same length. &, significantly different from + adult C. Fib maximum force at different length.

Similar to studies in Chapter 4, tissue patches with added cardiac fibroblasts (adult or fetal), but not those without fibroblasts, generated significant contractile forces (Fig. 6.5C) upon electrical stimulation. From average force-length relationships (Fig. 6.5D), tissue patches containing fetal fibroblasts produced significantly higher baseline
(0.5 mN vs. 0.15 mN) and maximum contractile force (0.90±0.07 mN vs. 0.63±0.09 mN) than those containing adult fibroblasts. In addition, the ascending part of force-length curve in patches containing adult fibroblasts was steeper, reaching maximum force at 12% stretch, compared to 20% stretch for patches containing neonatal fibroblasts. Finally, with applied stretch, tissue patches made with adult cardiac fibroblasts generated more passive tension than those made with fetal fibroblasts or no-fibroblast controls (9.53±1.26 mN vs. 3.35±0.52 mN vs. 1.54±0.54 mN, at 24% stretch, Fig. 6.5E).

6.2.3 Cardiac fibroblasts alter expression of cardiomyocyte proteins relevant for tissue patch electrical and mechanical function

By performing Western blot analysis, we found that the patches containing cardiac fibroblasts (either fetal or adult) exhibited significantly increased expression of cardiomyocyte proteins relevant for electrical and mechanical function as compared to patches made of pure mESC-CMs (Fig. 6.6A). The addition of cardiac fibroblasts in the tissue patch significantly increased the expression of sarcomeric α-actinin (SAA), a microfilament protein vital for the attachment of actin filaments to the Z-lines in myocytes (Fig. 6.6B), with tissue patches containing fetal cardiac fibroblasts having 1.92-fold increased SAA expression over tissue patches containing adult cardiac fibroblasts (Fig. 6.6B). We also found that patches containing fetal fibroblasts exhibited 1.63-fold and 2.73-fold higher expression of Nav1.5 (Fig. 6.6C) and connexin-43 (Fig. 6.6D) than patches containing adult fibroblasts, while no significant difference was found for the
expression of Kir2.1 (Fig. 6.6E). Collectively, these results demonstrated that 3D co-culture of cardiac fibroblasts and mESC-CMs significantly enhanced the expression of cardiac functional proteins and that this effect was augmented when using fetal compared to adult cardiac fibroblasts.

Figure 6.6. Western blot analysis of co-cultured tissue patches. (A) Representative blots for sarcomeric α-actinin (SAA), voltage-gated sodium channel (Nav1.5), connexin-43 (Cx43), and inward rectifying potassium channel (Kir2.1). Each band represents protein isolate from an independent tissue patch. Quantified expression of (B) SAA, (C) Nav1.5, (D) Cx43 and (E) Kir2.1, proteins relevant for cardiac electrical function. *, significantly different from patches without cardiac fibroblasts; **, significantly different from patches containing adult cardiac fibroblasts. Mean ± SEM, N = 4-6 patches each.
6.2.4 Conditioned media from cardiac fibroblasts increase cardiomyocyte spreading in engineered micro-patches

To assess the effects of fibroblast paracrine signaling on mESC-CM structure and function in a relatively high-throughput fashion, we fabricated 3D micro-patches that contained small numbers of pure mESC-CMs. Consistent with our studies in Chapter 4, mESC-CMs in the micro-patches remained viable but rounded (Fig. 6.7A). Addition of cardiac fibroblast-conditioned media yielded spreading of mESC-CMs with apparently stronger effects induced by fetal than adult paracrine factors (Fig. 6.7B&C). Quantitative morphometry of cardiac myocytes in immunostained micro-patches revealed that this effect was statistically significant (Fig. 6.7E). Furthermore, micro-patches conditioned with fibroblast media were simultaneously incubated with small molecule inhibitor drugs targeted against specific intracellular pathways (Table 3.6). Our hypothesis was that the inhibition of a particular signaling pathway might block fibroblast effects on mESC-CM size, thus indicating its importance in cardiac fibroblast–myocyte signaling and suggesting possible growth factors or cytokines that might mediate this effect. From the small molecules tested, the addition of PD0325901, an inhibitor of the MEK-ERK pathway (but not SP600125, an inhibitor of JNK pathway or SB203580, an inhibitor of p38 pathway (not shown)), significantly reduced the amount of cardiac myocyte spreading to control levels in fetal cardiac fibroblast conditioned micro-patches (Fig. 6.7D-E).
Figure 6.7. Quantitative morphometry of cell spreading in conditioned mESC-CM micro-patches. (A-C) Representative immunostainings of cardiac micro-patches in the absence (A) or presence of adult (B) or fetal (C) conditioned media and PD0325901 (D). (E) The number of sarcomeric α-actinin+ pixels (red) per nucleus (blue) from immunostaining images was quantified for each group as an index of cell spreading. The size of fibroblast-conditioned mESC-CMs was significantly increased relative to unconditioned cells. This effect was reversed by the addition of PD0325901. *, significantly different from all other groups. Mean ± SEM, N = 3 micro-patches per group.

6.2.5 Conditioned media from cardiac fibroblasts enhances the contractile activity of mESC-CMs in a developmental stage, time, and MEK-ERK dependent manner

Visual inspection of micro-patches suggested that the amplitude of their spontaneous contractions was greater in the presence of conditioned media from fetal than adult fibroblasts. To quantify the contraction amplitudes in different micro-patches, we recorded videos of spontaneous contractions and measured maximum absolute grayscale value and standard deviation in individual pixels over a 5-s recording period. The patches containing mESC-CMs that contracted more vigorously showed a higher
standard deviation and maximum absolute greyscale values (Fig. 6.8A). The range of greyscale values was relatively consistent in consecutive video frames instead of cyclic in nature, suggesting that the beating in the patch was asynchronous and uncorrelated among different patch areas (Fig 6.8A). We also plotted the histograms of all greyscale amplitudes (Fig. 6.8B) in each video and used mean histogram value as the parameter that quantified the intensity of contraction observed in each micro-patch.

Figure 6.8. Quantitative analysis of spontaneous contractions in engineered cardiac micro-patches when exposed to cardiac fibroblast conditioned media. (A) Scatter plot showing the range of greyscale values in each frame of each video. Note that since detrending was applied, all greyscale values in each video average out to zero. Standard deviations (represented by the central white zone) were calculated independently from frame to frame and were found to be consistent for each video analyzed, indicating steady beating over time. Relative to no conditioned media group (Not Conditioned) the contraction intensity was more increased in the presence of fetal than adult conditioned media. (B) Similarly, histogram distribution of changes in pixel intensity over all pixels and time points, shows higher increase in contraction intensity (evidenced by right-shifted histogram) in the presence of fetal vs. adult fibroblast conditioned media. When the MEK1/2 inhibitor PD0325901 is applied to
fetal or adult cardiac fibroblast conditioned micro-patches, contraction intensity was reduced to baseline (Not Conditioned) values.

From this analysis, we found that the contraction intensity in all micro-patches increased over time in culture, with fetal fibroblast conditioned media inducing larger increase than adult fibroblast conditioned media at all time points (Fig. 6.9). The addition of 10nM of MEK1/2 inhibitor PD0325901 significantly decreased the intensity of spontaneous contractions in patches conditioned with adult or fetal cardiac fibroblasts to the same level as in unconditioned patches (Fig. 6.10), an effect that was not observed during application of other tested small molecules, including JNK and p38-MAPK inhibitors (Fig. 6.11).

![Figure 6.9. Contraction intensity of fibroblast-conditioned cardiac micro-patches with time in culture. Conditioned media by fetal fibroblasts enhanced contractile activity of mESC-CMs more than that of adult fibroblasts. *, significantly different from unconditioned patches. **, significantly different from fetal cardiac fibroblast conditioned patches. Mean ± SEM, N=3 micro-patches per group.](image)
Figure 6.10. MEK1/2 inhibitor PD0325901 blocks the increase in contraction intensity caused by application of fibroblast conditioned media. *, significantly different from unconditioned patches. Mean ± SEM, N=3 micro-patches per group.

Figure 6.11 Increase in contraction intensity is blocked by PD0325901, but not inhibitors of JNK (SP600125) or p38 (SB203580). *, significantly different from unconditioned micro-patches. Mean ± SEM, N=4 micro-patches per group.
6.2.6 Fibroblast expression of genes coding for paracrine factors acting via MEK-ERK pathway

Our small molecule inhibitor experiments implicated the MEK-ERK pathway as critical to the enhancement of cell spreading and spontaneous contractility in micro-patches. We therefore, designed a quantitative RT-PCR (qPCR) assay to determine if the expression of genes encoding paracrine factors known to act via the MEK-ERK pathway was differently expressed in fetal and adult cardiac fibroblasts (Table 3.5). Out of 22 assessed genes, 6 showed a significant difference in expression (Fig. 6.12). Of these, only 2 factors, PDGF-C and TNF-α, were significantly upregulated (rather than downregulated) by 7.84 and 14.7-fold, respectively, which suggested that their secretion may have caused the observed paracrine effects of fetal fibroblasts. Furthermore, expression of genes coding for extracellular matrix proteins collagen I, collagen III or fibronectin did not differ between fetal and adult cardiac fibroblasts (data not shown).
Figure 6.12: Quantitative RT-PCR of genes coding candidate paracrine factors. qPCR was performed to quantify the relative expression in fetal vs adult cardiac fibroblasts of genes coding paracrine factors known to act via the MEK-ERK pathway. Inset: PDGF-C and TNF-α are significantly upregulated, whereas PDGF-D, FGF-2, NGF and BDNF are significantly downregulated in fetal vs. adult cardiac fibroblasts. *, significantly different between fetal and adult fibroblasts. Mean ± SEM, N = 4 micro-patches per group.

6.3 Discussion

Although there has been a wealth of literature describing the interaction between cardiac fibroblasts and myocytes, in most cases the focus has been on the compensatory or pathological effects of cardiac fibroblasts in disease states including myocardial infarction and heart failure. In diseased adult heart, cardiac fibroblasts are believed to mediate the formation of reactive or replacement scar tissue and through different (and mostly unknown) paracrine, cell-matrix, and direct contact effects influence the function of surrounding cardiac myocytes. In the context of heart
development, different studies have described signaling events between non-cardiac cells and cardiomyocytes that contribute cardiac organogenesis. For example, at the very early stages of mouse development (E7.25), the primitive streak and visceral endoderm provide commitment signals to cardiac progenitor cells to enable their differentiation into functional cardiomyocytes. This finding was used to enhance the capacity of mouse and human ESCs to differentiate towards cardiac myocytes by their co-culture with a visceral endoderm-like cell line. Similarly, the endocardium and epicardium are known to signal to the myocardium via several paracrine pathways involving neuregulin, FGF, endothelin and retinoic acid all of which have been shown to support either cardiac myocyte specification or maturation.

To the best of our knowledge, there has been only one other study examining the developmental stage-dependent effects of cardiac fibroblasts on myocytes. In 2D co-culture studies, Ieda et al. showed that fetal (but not adult) murine cardiac fibroblasts induced a potent proliferative effect on embryonic cardiac myocytes. This effect appeared to be mediated by fibroblast-secreted HB-EGF, and was dependent on β1-integrin engagement of cardiac myocytes. On the other hand, the presence of adult (and to a lesser extent, fetal) cardiac fibroblasts tended to increase the apparent size of cardiac myocytes, which is a commonly-used indicator of hypertrophy. In our study, we chose to focus on the functional consequences of fibroblast-myocyte signaling in 3D
engineered tissues as a culture substrate more representative of native cardiac environment. We discovered that cardiac fibroblast developmental stage has a strong effect on the structure, function, and protein expression of mESC-CMs in engineered cardiac tissues. Specifically, mESC-CM spreading and expression of connexin-43 gap junctions appeared to be increased in 3D co-cultures with fetal compared to adult cardiac fibroblasts. Furthermore, mESC-CMs in 3D engineered tissues exhibited higher conduction velocities and contractile force amplitudes when co-cultured with fetal compared to adult cardiac fibroblasts. Underlying these functional effects were significant differences in cardiac myocyte protein expression critical to the initiation and propagation of action potentials (Nav1.5, connexin-43) and contractile force generation (sarcomeric α-actinin). These results could be at least in part attributed to the finding that fetal and adult cardiac fibroblast paracrine factors had significantly different effects on 3D cultured mESC-CMs. Specifically, media conditioned by fetal cardiac fibroblasts induced a greater degree of cardiac myocyte spreading and more vigorous spontaneous contractions than media conditioned by adult cardiac fibroblasts, and these differences appeared to be mediated via the MEK-ERK pathway. Finally, our qPCR screen for growth factors and cytokines known to act via the MEK-ERK pathway have indicated two potential candidates (PDGF-C, TNF-α) which may mediate the observed differences.
6.3.1 MACS-purified cardiac fibroblasts show minor activation towards myofibroblast phenotype

In vitro culture has been documented to significantly increase the purity of cardiac fibroblasts. Hudon-David et al.\textsuperscript{53} reported that adult cardiac fibroblasts isolated using a method similar to ours were >90% Thy1\textsuperscript{+} at passage 0, which is in agreement with our finding that cell isolates from adult hearts were 86.9% Thy1\textsuperscript{+}/CD31\textsuperscript{-} even without MACS purification. The small difference in percentage may possibly be accounted for by the presence of Thy1\textsuperscript{+}/CD31\textsuperscript{-} endothelial cells in Hudon-David’s cell preparations. Most adult cardiac myocytes are highly sensitive to Ca\textsuperscript{2+} overload and shear forces and do not survive the described cell isolation procedure. Any cardiac myocytes that do attach are not proliferative, and are rapidly diluted out by the proliferating cardiac fibroblasts. Although fetal cardiac myocytes tolerate the isolation procedure well, we have observed that they typically do not survive the process of freezing and thawing. Thus in frozen/thawed cardiac cell isolates, we observed a higher (68.1\%) than previously reported\textsuperscript{39,54} percentage of Thy1\textsuperscript{+}/CD31\textsuperscript{-} fetal cardiac fibroblasts, which we then further purified using MACS.

Our cardiac non-myocytes contained a small fraction of cells that were weakly SMA\textsuperscript{+} and SMMHC (specific marker of smooth muscle phenotype). This indicated the occurrence of some degree of spontaneous transformation of fibroblasts towards a myofibroblast phenotype that likely occurred due to culture on rigid substrates\textsuperscript{50,55,257} or
hyperoxia\textsuperscript{260} as previously reported in multiple studies. However, this SMA expression was much lower than in cardiac fibroblasts exposed to TGF-β1 (Fig. 6.13), a strong inducer of myofibroblast phenotype both \textit{in vitro} and \textit{in vivo}\textsuperscript{259,261,270}. Therefore, we feel confident that myofibroblast activation in our fibroblasts was minimal, and that functional effects on cardiac myocytes largely reflected true age-dependent differences in fibroblast phenotype present \textit{in vivo}.

![Figure 6.13 Isolated cardiac fibroblasts show virtually no myofibroblast activation. (A) Adult and fetal (not shown) cardiac fibroblasts cultured in the presence of 10ng/µl TGF-β for 3 days show significant upregulation of SMA. In contrast, adult (B) and fetal (C) cardiac fibroblasts cultured under standard conditions used for this chapter show minimal expression of SMA. Images were taken using identical illumination and 1s integration time.](image)

6.3.2 \textbf{Engineered cardiac tissue function is modulated by cardiac fibroblasts in a developmental stage-dependent manner}

The most important finding of this chapter is that cardiac patches containing fetal cardiac fibroblasts were functionally superior to those containing adult fibroblasts as evidenced by their 2.02-fold higher conduction velocity and 1.43-fold higher maximum force of contraction. These functional differences were associated with a 2.73-fold, 1.63-fold, and 1.92-fold increase in connexin-43, Nav1.5, and sarcomeric α-actinin protein.
expression, respectively. Overall, these findings suggested that fetal cardiac fibroblasts induced a greater degree of functional differentiation in engineered cardiac tissue patches than adult fibroblasts.

Our studies also showed that patches containing adult cardiac fibroblasts generated significantly higher passive tension and steeper rising phase of active force-length relationship than those containing fetal cardiac fibroblasts. Notably, contrary to Ieda et al\textsuperscript{39}, our qPCR results indicate that there was no significant difference in the expression of genes coding for ECM components such as collagen type I, collagen type III and fibronectin between fetal and adult cardiac fibroblasts. Cardiac fibroblasts, on the other hand, are known to become stiffer with age\textsuperscript{271}.

6.3.3 Media conditioned by fetal cardiac fibroblasts enhance mESC-CM spreading and spontaneous contractile activity within engineered tissue patches

Cardiac fibroblasts are known to signal to cardiac myocytes extensively through a variety of paracrine mechanisms both in development\textsuperscript{39} and disease\textsuperscript{55,250,260}. Paracrine signals are amplified and integrated by intracellular pathways that convey these signals to the nucleus. We therefore further studied if the functional differences found in direct 3D co-cultures of mESC-CMs with fetal vs. adult cardiac fibroblasts could be attributed to age-dependent differences in fibroblast paracrine action. We observed that paracrine factors secreted by fetal cardiac fibroblasts enhanced mESC-CM spreading and intensity
of spontaneous contractions more than the adult fibroblast factors. Enhanced cardiac
myocyte spreading and contractions by fibroblast paracrine factors have been
previously documented for neonatal rat cells in standard 2D cultures\textsuperscript{55,163,260}, and our
results in 3D culture environment are in agreement with these studies. Furthermore, our
results agree with Xi et al\textsuperscript{158}, who showed that seeding mouse embryonic fibroblasts
together with mESC-CMs promoted cardiac myocyte attachment and force transmission
to the substrate. Assuming that the stiffness of ECM in which sparse mESC-CMs were
embedded in our study likely did not change due to addition of paracrine factors,
suggests that the more vigorous contractions in the presence of fetal fibroblast
conditioned media were likely a result of increased cardiomyocyte force production
capacity.

To attempt to narrow the list of possible paracrine factors that are most
responsible for the above observed phenomena, our strategy was to inhibit pathways
which are known to be involved in development and hypertrophy, and to see which
inhibitors were capable of reducing the effect of cardiac fibroblast conditioned media
relative to unconditioned control. High affinity small molecule inhibitors exist for these
pathways because they are often activated in disease states and make good therapeutic
targets, thus lending a high degree of specificity to our assays. Using this strategy, we
found that cardiac fibroblasts signal more strongly to cardiac myocytes than adult
cardiac fibroblasts via paracrine factors that act predominantly via the MEK-ERK pathway. Although ERK has been shown to be involved in pathological hypertrophy\(^\text{251}\), it is also shown to be highly important in cardiac development. Indeed, cardiac-specific overexpression of the MEK led to an increase in mouse heart to body weight ratio and contractile performance, but did not result in any pathology or premature death\(^\text{272}\). On the other hand, p38 and JNK are specialized mediators of stress or injury\(^\text{273}\), and are commonly known as stress-activated protein kinases. Notably, our small molecule experiments showed that unlike ERK inhibition, the inhibition of p38 or JNK could not reverse the effects of fetal fibroblast conditioned media on mESC-CMs. These results, in conjunction with the observed benefits of fetal fibroblasts on engineered cardiac patch contractile function, suggested that when exposed to fetal fibroblast paracrine factors, mESC-CMs underwent physiological rather than pathological hypertrophy.

### 6.3.4 Candidate fetal fibroblast paracrine factors that affected cardiomyocyte function

To elucidate fibroblast paracrine factors that exerted the described functional effects on mESC-CMs, we assessed the expression of genes coding for a select set of secreted factors and cytokines that have been documented in the literature as acting via the MEK-ERK pathway. PDGF-C, a potent mitogen of cardiac fibroblasts\(^\text{274}\), was the most upregulated gene (7.87-fold) in fetal vs. adult cardiac fibroblasts. PDGF-C is not expressed in cardiac myocytes\(^\text{274}\). PDGF-C has also been found to be necessary for
epicardial development in avian hearts\textsuperscript{275}. In the context of engineered tissues, PDGF receptor activation (by media supplementation with PDGF-BB, an isoform of PDGF) in neonatal rat cardiac myocytes prevented their apoptosis and enhanced their contractile force production\textsuperscript{276}, without inducing hypertrophy or proliferation. In our study, secreted PDGF-C may have played a similar role in enhancing cardiac functionality in 3D mESC-CM fibroblast co-cultures. On the other hand, PDGF-D, which was found to be significantly downregulated in fetal vs. adult cardiac fibroblasts, is known to be active in kidney, eye and brain development\textsuperscript{277}, however, it does not have a documented role in cardiac development.

Although cardiac overexpression of TNF-\(\alpha\) by genetic manipulation\textsuperscript{278} or in disease\textsuperscript{260} has been associated with cardiac hypertrophy and impaired cardiac function, it also known to have a cardio-protective effect\textsuperscript{279} and there is evidence that it increases cardiogenic differentiation of ES cells\textsuperscript{280,281}. TNF-\(\alpha\) is mainly expressed in interstitial cells such as macrophages, but is also expressed in cardiac myocytes after injury\textsuperscript{282}. However, the lack of evidence that TNF-\(\alpha\) has a role in cardiac development (TNF-\(\alpha^{-}\) mice show no cardiac abnormalities\textsuperscript{283}) suggests that it is probably not responsible for the enhancements in tissue patch function reported in this chapter.

Gene expression of FGF-2 was found to be 3.8-fold downregulated in fetal vs. adult cardiac fibroblasts. FGF-2 is a potent angiogenic factor that has been used in
animal models of heart disease as well as in clinical trials\textsuperscript{284} as a therapeutic agent to improve blood flow. There is evidence that FGF signaling is important in the specification\textsuperscript{285-288} and regulation\textsuperscript{289} of cardiac progenitors. In differentiating ES cells, anterior primitive streak cells differentiate to cardiac myocytes in the presence of BMP4 and FGF2\textsuperscript{290}. However, FGF2\(-/-\) mice do not display a diseased cardiac phenotype, possibly due to the overlapping functions of different FGF isoforms in development\textsuperscript{288}.

FGF-2 is also known to be upregulated in injury models and to contribute to compensatory cardiac myocyte hypertrophy\textsuperscript{291}. Given the facts that our experiments in this chapter used already specified and differentiated mESC-CMs and that we observed cardiac myocyte hypertrophy despite FGF-2 downregulation, it is unlikely that different expression of FGF-2 in fetal vs. adult cardiac fibroblasts had any functional impact on mESC-CMs.

Expression of BDNF and NGF genes were found to be both downregulated in fetal vs. adult cardiac fibroblasts, and are known to play a strong role in the maintenance and proper functioning\textsuperscript{292} of cardiac neurons. Chronic electrical stimulation to control ventricular beating rate was found to increase the expression of BDNF and NGF in mongrel dogs, and was associated with an increased neuronal cell size and parasympathetic tone\textsuperscript{293}. In a model of chronic heart failure, changes in the expression levels of BDNF and NGF were associated with altered function of sympathetic nerve
endings. BDNF has also been implicated as a pro-angiogenic factor, and has been used in experimental therapies to improve blood flow by stabilizing small intramyocardial vessels. However, neither of these factors have known direct impacts on the development of cardiac myocytes, and thus are probably incidental to the other observations made in this chapter.

Ieda et al. has previously shown that fetal cardiac fibroblasts signal to embryonic cardiac myocytes via HB-EGF. Moreover, they found that unlike fetal cardiac fibroblasts, adult cardiac fibroblasts preferentially induced myocyte hypertrophy over proliferation. We on the other hand, find that media conditioned by adult cardiac fibroblasts increase apparent myocyte size in engineered tissues less than media conditioned by fetal cardiac fibroblasts. Furthermore, we did not find any effect of fibroblast conditioned media (fetal or adult) on the proliferation of mESC-CMs in micro-patches, with numbers of cells in interphase remaining unchanged and relatively low (not shown). Finally, although we find that HB-EGF gene expression is on average upregulated (1.22±1.85 fold, N=4) in fetal vs. adult cardiac fibroblasts, we do not find that this difference is significant. These differences in observations may exist because: (1) Ieda et al. used primary E12.5 – 13.5 fetal cardiac myocytes and examined proliferation after 3 days, whereas we used mESC-derived cardiac myocytes and performed measurements after 3 – 14 days; (2) Fibroblasts and cardiac myocytes in Ieda’s study
were analyzed in co-culture, while our conditioning studies were performed in separated fibroblast and cardiac myocyte monocultures; (3) Cardiac myocytes may behave differently in a 3D environment utilized in our studies than on the 2D surfaces used in Ieda’s study.

6.4 Summary and Implications

In the previous two chapters, we have shown that: (1) supporting neonatal rat cardiac fibroblasts mediate the formation of functional stem cell-derived engineered cardiac tissues; and (2) multipotent pluripotent stem cell-derived cardiovascular progenitor cells are able to autonomously form functional cardiac tissues without the addition of cardiac fibroblasts. Given the central importance of non-myocytes in native myocardium and primary and pluripotent stem cell-derived engineered cardiac tissues, we hypothesized that mESC-CM function could be modulated by altering the developmental stage of supporting cardiac fibroblasts. Our data show that cardiac fibroblasts from an early developmental stage (E13.5 fetal) are better able to mediate the in vitro formation of engineered functional myocardium than adult cardiac fibroblasts. Moreover, we show that paracrine factors from fetal cardiac fibroblasts (e.g. PDGF-C, TNF-α), known to act via the MEK-ERK pathway, augment spontaneous contractile activity and cell spreading in engineered cardiac tissues, suggesting that fibroblast paracrine signaling, at least in part, may underlie the structural and functional...
reorganization of a collection of disperse, single cardiac myocytes into a well-interconnected, contractile 3D syncytium.

In general, this study implies that the re-creation of a milieu consistent with the developmental stage of the nascent myocardium assists in the formation of functional cardiac tissue. Although the presence of the appropriate supporting cell types is one aspect of the milieu, others such as conditioning with the biomimetic levels of mechanical stress\textsuperscript{198} and substrate stiffness\textsuperscript{299,300} may be expected to yield further functional improvements. For example, Kensah et al. observed that stepwise growing static stretch designed to emulate the growing diastolic load in the developing heart improved the formation of elongated cardiac tissue when compared with cyclic or static stretch\textsuperscript{198}. Signaling molecules such as T3\textsuperscript{153} or IGF-1\textsuperscript{301-303}, which originate from different glands\textsuperscript{304} or organs and are temporally modulated during development, may also play important roles in functional cardiogenesis and need to be explored. We note that some tissue engineering strategies have relied on the addition of non-cardiac adult cells such as mesenchymal stem cells\textsuperscript{305}, endothelial cells\textsuperscript{306} and circulating endothelial progenitor cells\textsuperscript{307} to enhance the formation of vascular structures or cellular integration. In addition, recent human ES cell-based cardiac tissue engineering studies, used neonatal dermal\textsuperscript{156} or foreskin fibroblasts\textsuperscript{198} as supporting cells; however, these cells are still non-cardiac and at a much more advanced developmental stage than human ES-CMs. Our
study suggests that the developmental stage of supporting cardiac non-myocyte populations relative to that of cardiac myocytes is an important factor to be taken into account in the engineering of functional cardiac tissue with optimal performance.

6.5 Limitations and future work

Although the experiments in this chapter were performed to the best of our ability given our constraints in manpower and resources, we acknowledge that there were some methodological drawbacks that limited the power of our findings.

1. Using small molecule drugs, we were able to inhibit only a relatively small number of pathways compared to the large number of pathways that are known to be involved in cardiac development and hypertrophy\textsuperscript{251,308}. Moreover, some pathways e.g. SMAD-mediated TGF-β signaling, are difficult to block using currently available small molecules due to the large number of independent receptors that mediate signal transduction in these pathways\textsuperscript{309}.

2. We were not able to generate dose-response curves for each drug used. Pull-down\textsuperscript{310,311} or ELISA-based assays\textsuperscript{312} exist to test the activation of many of the pathways blocked in this chapter, and should be performed to minimize off-target effects and ensure the efficacy of each drug at the concentration used.

3. Our gene expression study was only able to probe a relatively small number of genes. Although we attempted to increase the power of our screen by choosing
paracrine factor genes known to act via the MEK-ERK pathway, we acknowledge this list is incomplete. We note that microarrays are able to probe a much larger set of genes, and have been effectively used in similar studies\textsuperscript{39,313} to elucidate the paracrine interactions between different cell types. They therefore present a superior alternative to our qPCR-based screen.

4. No direct verification of paracrine factor concentrations was conducted in this study.

5. No direct verification of the importance of PDGF-C or TNF-\textalpha in inducing spontaneous contractions or cell spreading was conducted in this study.

To address these limitations, we suggest that the following experiments be implemented:

1. High throughput gene expression assays such as microarrays should first be used to assess differences in paracrine factor secretion between fetal and adult cardiac fibroblasts. Although it is possible that some paracrine factors may be secreted at different levels despite having the same gene expression level, microarrays present a more comprehensive hypothesis-generation step than using a collection of small molecule drugs as we did in this chapter. This is because signaling pathways may be promiscuous, leading to results that may be difficult to interpret and limiting our ability to infer the identity of
important paracrine factors. Furthermore, small molecule inhibitors have dose-dependent and off-target effects, which may lead to experimental artifacts.

2. Based on the identity of differentially regulated paracrine factor genes, a more targeted pathway inhibition assay should be designed to corroborate the microarray findings using a smaller number of inhibitor drugs. Dose-response curves for each inhibitor drug can be generated either by (a) Assaying for the extent of phosphorylation of a specific downstream target in Western blots e.g. phospho-ERK1/2 in the MEK-ERK pathway; (b) Pull-down based methods; (c) ELISA-based methods. This pathway inhibition assay would present a useful step in reducing the number of candidate paracrine factors for further study.

3. The concentration of candidate paracrine factors in conditioned media should be determined by ELISA.

4. Finally, direct verification of the involvement of particular paracrine factors should be performed. Taking PDGF-C as an example:

   a. siRNA can be designed and delivered via lentiviral vectors to knock down PDGF-C expression in fetal cardiac fibroblasts\(^9\). Conditioned media can then be collected from knockdown fetal cardiac fibroblasts as well as normal fetal cardiac fibroblasts, and their effects on micro-patches can be compared.
b. Recombinant PDGF-C can be used to supplement adult cardiac fibroblast conditioned media. The effect of supplemented adult cardiac fibroblast conditioned media can then be compared against unsupplemented adult and fetal cardiac fibroblast conditioned media.

c. Recombinant PDGF-C can be added to unconditioned media and applied to micro-patches to see if PDGF-C alone is sufficient to induce spontaneous contractions and cell spreading in micro patches.
7. Future Directions

While the experiments described in this dissertation advance our basic understanding of engineering of functional cardiac tissues from pluripotent stem cells, they also suggest additional questions and directions that could be explored in future studies.

The microfabrication-based tissue engineering approach that we developed in Chapter 4 could potentially be used to generate human iPS cell-based tissue patches with more complex biomimetic geometry. Fig. 8.1 shows one possible proof-of-concept application of this technology: diffusion tensor magnetic resonance imaging is used to probe muscle fiber orientations in an infarcted heart, allowing fiber orientations in the damaged region to be determined. Personalized photomasks representing the precise geometry and dimensions of the damaged region can then be designed and printed, allowing the fabrication of engineered tissue patches with matching geometry to be produced. This ability to control local cardiac fiber orientation in engineered tissues to mimic native myocardial structure is expected to result in more efficient electrical integration of the engrafted tissue into the host heart, thus reducing the likelihood of arrhythmia.
Figure 7.1 Tissue engineering of a personalized cardiac patch. Described microfabrication-based approach enables engineering of cardiac tissue patches with personalized fiber orientation and geometry, that can facilitate restoration of native myofiber architecture. DT-MRI image obtained from\textsuperscript{315}

We envision that our finding that Nkx2.5\textsuperscript{+} CVPs are capable of autonomously forming engineered cardiac tissues will be applicable to currently available human ES cell lines\textsuperscript{316}, since the analogous cell population has already been isolated\textsuperscript{316}. Although work from our own lab (Zhang et al, unpublished) indicates that SIRPA\textsuperscript{+} cell fractions are also progenitor-like in nature insofar as they contain differentiating smooth muscle and endothelial cells\textsuperscript{243}, we note that this cell population is still relatively poorly defined. The non-cardiac fraction in this population may in some cases represent cells fated to other organ systems since SIRPA is not cardiac-restricted\textsuperscript{244,245}, thus raising the possibility that the use of SIRPA\textsuperscript{+} cells may result in more line and differentiation protocol-specific variation in engineered tissue properties. For potential clinical applications, we suggest that Nkx2.5-selectable cassettes may be integrated into a safe
harbor gene locus in human ES and iPS cell lines using zinc finger\textsuperscript{117} or TALEN\textsuperscript{118}-mediated homologous recombination approaches.

Our finding that compared to adult cardiac fibroblasts fetal fibroblasts mediate the formation of more functional engineered cardiac tissues made from pluripotent stem cell derived cardiac myocytes suggests that optimal culture conditions for functional cardiogenesis \textit{in vitro} may require different cellular components and environmental conditions of the forming tissue to be developmentally consistent. In support of this concept, others have observed that emulation of the growing systolic and diastolic pressures in the developing heart\textsuperscript{198} or media supplementation with developmentally upregulated hormones\textsuperscript{304} improves the function of engineered cardiac tissues\textsuperscript{153}. Future improvements towards engineering of functionally more mature (i.e., adult-like) cardiac tissues may require the ability to vary (with culture time and in controlled fashion) different culture parameters including substrate stiffness\textsuperscript{75,300}, fluid flow conditions\textsuperscript{319,320} or electrically-induced contraction sequence\textsuperscript{217,321} to further mimic the dynamic environment of the developing heart.

The incorporation of cardiac fibroblasts into our engineered tissues raises the interesting possibility that we may be able to create more useful \textit{in vitro} human models (using human iPS cell-derived cardiac myocytes or progenitors) of fibrosis, which can occur under disease states such as hypertension\textsuperscript{322,323}. Unlike 3D engineered tissues, 2D
monolayers are inadequate models because the presence of small numbers of fibroblasts or other non-conductive vascular cells can interrupt action potential propagation\textsuperscript{235}. Moreover, there is no easy way to simulate hemodynamic loading conditions in 2D monolayers. In contrast, 3D engineered tissues can be statically or dynamically loaded\textsuperscript{146,198}, enabling pathological conditions e.g. hypertrophy induced by increased afterload\textsuperscript{324} to be simulated. Our experimental paradigm also allows cardiac fibroblasts to be separated from cardiac myocytes and maintained in an equivalent 3D environment as an adjacent tissue patch. This system would allow identical mechanical loading to be applied separately to the two cell populations so that loading-dependent paracrine effects can be studied. Such experiments would be useful for basic scientific studies, and may also be useful for testing different drug or gene therapies.
Appendix A

Alternative PDMS casting method (Dr. Wayne Pfeiler) – useful for casting PDMS features that are highly prone to tearing

After fabrication of SU-8 templates on silicon, the silicon wafers were placed into clean 10cm petri dishes and a fluidized solution of 4% agarose (BioRad), heated to ~50°C, was poured into the dish to cover the features (to a depth of ~3cm from the silicon base). Care was taken to remove any bubbles in contact with the silicon or SU-8 surfaces with a 10μl pipet tip whilst the agarose was still a fluid. Petri dishes were then left to cool to 37°C in the humidified environment of a tissue culture incubator to avoid shrinkage of the agarose due to water evaporation. Solidified agarose was carefully removed from the silicon wafer, forming negative templates. The negative templates were placed feature-side-up in fresh petri dishes. Mixed, degassed PDMS was then poured into the negative templates, and bubbles removed using a 10μl pipet tip. Curing of PDMS was done overnight at 37°C in a humidified tissue culture incubator. The following day, the PDMS could easily be detached from the agarose without any tearing by breaking the agarose mold or melting under heat.
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**Presentations**

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