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Highly Efficient Cardiac Differentiation of Human Embryonic Stem Cells for Cardiac Repair

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Highly Efficient Cardiac Differentiation of Human Embryonic Stem Cells for Cardiac Repair

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering and Small Scale Technologies

by

Silin Sa

Committee in charge:
Professor Kara McCloskey, Chair
Professor Ajay Gopinathan
Professor Marcos Garcia-Ojeda
Professor Valerie Leppert
Professor Michelle Khine

2013
Highly Efficient Cardiac Differentiation of Human Embryonic Stem Cells for Cardiac Repair

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The Dissertation of Silin Sa is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, Merced
2013
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Second, I would like to thank my advisory committee Professor Ajay Gopinathan, Professor Marcos Garcia-Ojeda, Professor Valerie Leppert and Professor Michelle Khine for their advice through the work in this dissertation.

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Fourth, I would like to thank all members of McCloskey lab, especially Dr. William Turner, Drew Glaser and Lian Wong for their help of my work.

On personal level, I would like to thank my mom, dad and husband for being a constant source of encouragement and admiration.
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1. Sa S, McCloskey KE. Stage-Specific Cardiomyocyte Differentiation Method for H7 and H9 Human Embryonic Stem Cells Control. Stem Cell Reviews and Reports. Aug 2012 (Epub ahead of print)


ABSTRACT

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2013
Heart failure is a leading cause of death in United States. One of the causes of heart failure is associated with the death or loss of cardiomyocytes (CMs). Since adult CMs do not regenerate, their death permanently compromises myocardial contractile function. Stem cell transplantation is one therapeutic strategy to replace damaged or lost myocardial tissue to restore cardiac function. Embryonic stem cells (ESCs) are an attractive population for cardiac repair because they can self-renew unlimitedly and differentiate into all cell types including CMs. Furthermore, ESC derived CMs can functionally integrate with the recipient organ and improve heart function after transplantation.

However, a major challenge in ESC-based cardiac therapies is that the differentiation efficiency of ESCs into CMs has been very low (~1%). And large numbers of cells are required for administration for each patient. In this study, a protocol for efficient generation of CMs from hESCs was explored by optimizing various staged components in the microenvironment. Specifically, I 1) developed Honeycomb Microwell chips to generate homogeneous EB for CM differentiation; 2) optimized Actvin A/BMP4 concentration for CM differentiation; 3) optimized effects of extracellular matrix (ECM) signaling and investigated some mechanisms of ECM signaling on CM differentiation. The optimized protocols reproducibly generate approximately 70% CMs from H7 and H9 hESCs. These hESC derived CMs can now be enriched and tested for their ability to enhance cardiac function in preclinical animal models and for utility in drug discovery for future study.
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Chapter 1. Introduction

1.1 Background

Heart failure is the leading cause of hospitalization and death of those over 65 years of age. In the United States alone, about 5 million people have heart failure which contributes to around 300,000 deaths each year.\(^1\) Heart failure is a condition where the heart cannot pump enough blood to meet the needs of the body. It is the final clinical manifestation of a variety of cardiovascular diseases, such as coronary artery disease, hypertension, valvular heart disease, or dilated cardiomyopathy.\(^2\) The presence of cardiomyocytes (CMs) apoptosis has been described in human end-stage heart failure and in experimental models of ischemic and dilated cardiomyopathy. CMs apoptosis is induced by a variety of factors that play a role in congestive heart failure, such as myocardial infarction, ischemia, mechanical stretch and pressure overload.\(^3\) Heart failure can also be precipitated through CM necrosis associated with Ca\(^{2+}\) overload.\(^4\) However, CMs are terminally differentiated throughout the life of the organ and the organism. Traditionally the heart has been viewed as an organ incapable of repairing itself after significant damage. Current therapies for cardiac disease include drug treatments, lifestyle modification, device therapy, and heart transplantation. The first three therapies only reduce or delay progression of the disease. The only standard therapy that addresses the fundamental problem of CM death is a heart transplant, but it is limited by donor supply. The number of available donor hearts approximates 2500 in the U.S. per year, well below the estimated 25,000 patients who would benefit from cardiac transplantation. Given these statistics, much attention has recently been directed at cell transplantation strategies as an alternative strategy to prevent advanced heart failure and restore cardiac function in injured hearts.\(^5\)

1.1.1 Stem Cell-based Cardiac Therapy

Stem cell transplantation is a type of cell therapy that introduces stem cells into damaged tissue in order to treat a disease or injury. Stem cell transplantation for treating heart conditions is a growing field of clinical research. There are two mechanisms of using stem cells in cardiac repair: first, inject stem cells which can differentiate into CMs or transplant stem cell derived CMs directly in the heart to replace damaged CMs\(^6\)-\(^13\); or second, inject stem cells which can differentiate into cardiovascular cell types to promote angiogenic processes and increase the neovascularization.\(^14\)-\(^16\) There is an intensive effort to develop stem cell–based strategies for cardiac repair. Adult stem cells, embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells are some of the potential sources of cardiac progenitors in cell-based cardiac therapy (Figure 1).
Figure 1. Potential sources of cardiac progenitors in cell replacement therapy for heart disease. Different types of stem cell and progenitor cell are being tested to improve cardiac function.5

Various sources of adult stem cells are being studied in preclinical models and early-stage clinical trials, including skeletal myoblasts (SkMs), bone marrow mononuclear cells (BMMNCs), mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs) and cardiac progenitor cells (CPCs) (Table 1).12-15, 17-19 Cardiac-based cardiac repair began with the transplantation of autologous skeletal muscle satellite cells (commonly referred to as myoblasts).20 SkMs are progenitor cells resident within skeletal muscle that can be isolated by skeletal muscle biopsies and expanded in vitro.21 They were the first cell type to be used clinically for cardiac repair owing to their preclinical efficacy, autologous availability, ability to be amplified in vitro and relatively good survival after implantation. However, these cells do not readily transdifferentiate into CMs and remain committed to a skeletal muscle fate.17 A recent randomized trial showed that myoblast injections combined with coronary surgery in patients with depressed left ventricular function failed to improve heart function.18 Thus, the future of this approach is currently unclear.

Adult bone marrow-derived stem cells are the most frequently used cell product in cardiac stem cell therapy. A heterogeneous subset, autologous BMMNCs, comprises small numbers of stromal or MSCs, HSCs, EPCs, and more committed cell lineages such as natural killer, T, and B lymphocytes.22 MSCs reside mainly in the bone marrow’s stromal compartment, the connective tissue-rich, nonhematopoietic region of the marrow.17 They are precursor cells able to differentiate into the cells of mesenchymal origin such as osteoblasts, chondrocytes,
adipocytes and skeletal muscles. It has been reported that human MSCs from bone marrow can be expanded in culture and have multipotent differentiation capacity in vitro.\textsuperscript{23} MSCs show evidence of differentiation into CMs if maintained under appropriate conditions in vitro or if injected into healthy or diseased myocardial microenvironment in experimental animals in vivo, but at an extremely low rate.\textsuperscript{12,14} EPCs find their origin in bone marrow and can be more easily isolated from peripheral blood.\textsuperscript{15} They are known to stimulate neovascularization at the myocardial ischemia border zone and enhance myocardial healing.\textsuperscript{19} Clinical studies suggest that cell-based therapy with EPCs can improve myocardial function.\textsuperscript{14} But differentiation of EPCs into CMs did not occur in vitro or in vivo, and probably have a role in promoting angiogenesis only.\textsuperscript{15}

Several laboratories have identified resident CPCs with the capacity to differentiate into CMs. The choice for CPCs to repair the heart seemed to be a logical one, since CPCs are programmed to acquire the myocyte, vascular smooth muscle cell and endothelial-lineages. CPCs were effective in preclinical models. Smits and colleagues identified a population of Sca-1+ CM progenitor cells capable of differentiating efficiently into beating CMs, endothelial cells, and smooth muscle cells. Transplantation of these CPCs or CPCs-derived CMs resulted in a positive effect on cardiac performance.\textsuperscript{13} However, clinical studies using cells have not yet been implemented. A complicated issue is the diverse phenotypes of the CPCs as reported in the literature and no one knows which CPCs are the most ideal for clinical treatment purposes.\textsuperscript{15}

Demonstration of successful engraftment in the injured heart has made cell transplantation, as a means to treat cardiac disease, a realistic possibility. The advantage of using adult stem cells for cardiac repair is that they are autologous cells, they can overcome the shortage of organ donor, as well as the requirement of immune-suppression after transplantation of allogeneic tissues. However, adult stem cells exhibit limited plasticity. By contrast, ESCs can propagate without differentiation in cell culture while maintaining the potential to differentiate into all three embryonic germ layers; ectoderm, endoderm and mesoderm. Therefore, their extensive capacity for differentiation has garnered tremendous interest for their use in regenerative medicine.

Other than adult and ESCs, there is a type of pluripotent cell artificially derived from a non-pluripotent cell called iPS cells. The generation of iPS cells from somatic cells has opened a new field for basic research and regenerative medicine applications. The formation of teratomas following transplantation of human iPS cells into mice has provided evidence for pluripotency.\textsuperscript{24} Zhang et al. reported recently that human iPS cells can differentiate into functional CMs, and thus iPS cells are a viable option as an autologous cell source for cardiac repair.\textsuperscript{25} The main advantage of iPS cells over ESCs is the possibility to generate patient-specific multipotent stem cells, thus avoiding the risk of immune rejection. However, this procedure is time-consuming and may preclude its wide use for treating acute myocardial infarction.\textsuperscript{16}
Table 1. Potential sources of adult stem cells in cell transplantation therapy for heart disease.

<table>
<thead>
<tr>
<th>Source</th>
<th>Cell Type</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>Skeletal Myoblasts (SkMs)</td>
<td>• Preclinical efficacy • Autologous • Expandable in vitro • Good survival after implantation (^{17})</td>
<td>• Cannot transdifferentiate into CMs. (^{17}) • No significant benefit in patients with ischemic cardiomyopathy (^{18})</td>
</tr>
<tr>
<td>• Bone Marrow</td>
<td>Mesenchymal Stem Cells (MSCs)</td>
<td>• Expandable in vitro • Multipotent • Can differentiate into CMs in vitro or in vivo (^{12})</td>
<td>• Low differentiation efficiency (^{14})</td>
</tr>
<tr>
<td>• Fat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Umbilical blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Bone Marrow</td>
<td>Endothelial Progenitor Cells (EPCs)</td>
<td>• Stimulate neovascularization. (^{19}) • Enhance myocardial healing. (^{14}) • Promoting angiogenesis. (^{15})</td>
<td>• Cannot transdifferentiate into CMs. (^{15})</td>
</tr>
<tr>
<td>• Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>Cardiac Progenitor Cells (CPCs)</td>
<td>• Can differentiate into CMs. (^{13}) • Effective in preclinical models</td>
<td>• No clinical trials • Hard to determine ideal subpopulation for clinical treatments. (^{15})</td>
</tr>
</tbody>
</table>
Table 2. Advantages and disadvantages of adult stem cells, ESCs and iPS cells for cell transplantation therapy.

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Stem Cells</td>
<td>• overcome the shortage of organ donor</td>
<td>• limited plasticity</td>
</tr>
<tr>
<td></td>
<td>• no requirement of immune-suppression</td>
<td></td>
</tr>
<tr>
<td>Embryonic Stem Cells (ESCs)</td>
<td>• propagate without differentiation</td>
<td>• ethical concerns</td>
</tr>
<tr>
<td></td>
<td>• differentiate into all three germ layers</td>
<td>• immunologic incompatibility</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• formation of teratomas</td>
</tr>
<tr>
<td>Induced Pluripotent Stem Cells</td>
<td>• pluripotent</td>
<td>• time-consuming to generate new patient-specific line</td>
</tr>
<tr>
<td>(iPSCs)</td>
<td>• autologous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• patient-specific</td>
<td></td>
</tr>
</tbody>
</table>

1.1.2 ESC derived CMs for Cardiac Disease

Although the use of ESCs is limited by ethical concerns, immunologic incompatibility and the potential formation of teratomas, they still possess several features that make them an attractive source for cell-based cardiac therapy. ESCs have unquestioned cardiac potential. Doetschman et al. were among the first to show that, by removing mESCs from conditions supporting pluripotency, they can be induced to form cystic 3D aggregates, so-called embryoid body (EB), that will include foci of rhythmically contracting CMs. Subsequently, numerous laboratories have reported the successful derivation of CMs from hESCs, via EB differentiation.26

EBs are 3D cell aggregates comprised of three embryonic germ layers. After a few days of culture under appropriate conditions of cell density, culture medium along with growth supplements, spontaneously contracting areas appeared in 8% of the human EBs on day 20, containing CMs portraying structural and functional properties consistent with early-stage cardiac tissue.27 CMs derived from hESCs variably exhibit spindled, rod-shaped, spherical, and tri- or multiangular morphologies, and they have expected ultrastructural features, including sarcomeres and intercalated discs. Moreover, as hESC derived CMs mature during in vitro EB differentiation, the quantity of myofibrils increases and their arrangement changes from random to parallel alignment. As demonstrated by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemical studies, hESC-CMs express a number of early cardiac-specific transcription factors including Nkx2.5, GATA-4, myocyte enhancer factor 2C (MEF2C), and the T-box transcription factors Tbx-5 and Tbx-20. They also express sarcomeric proteins (e.g., \(\alpha\)-actinin, cardiac troponins I and T, sarcomere myosin heavy chain, atrial- and ventricular-myosin light chains, desmin, and tropomyosin) and other cardiac or muscle-specific proteins (e.g., atrial natriuretic peptide, creatine kinase-MB, and myoglobin). In addition,
hESC-CMs exhibit electrophysiological signatures that include cells with distinct nodal-, atrial-, and ventricular-like action potential (AP) properties, although the majority (82%) of cells are ventricular type (Figure 2). In conclusion, the hESC-CMs displayed structural and functional properties of early-stage CMs.

Figure 2. Ventricular, atrial and nodal APs. i. Typical APs (in millivolts) recorded from cells in the A-ventricle, B-SA node, and C-Atrium. ii. Ventricular (V), atrial (A) and nodal (N) APs of hESC-CMs. APs were recorded from a total of 125 hESC-CMs using perforated patch current clamp technology. The majority (82%) of cells were ventricular type. One cell showed clear pacemaker currents and was designated nodal type, whereas 22 cells were designated atrial type.

A number of animal studies have shown that hESC derived CMs can survive and proliferate after transplantation, form stable cardiac implants, and improved cardiac functions (Table 3). Although, hESC derived CM transplantation appears to be extremely promising as a potential new therapeutic strategy, severe obstacles need to be overcome before clinical application of hESC derived CMs becomes a reality. One of the challenges is the poor efficiency and insufficient numbers to treat adult human patients. Large numbers of cells (approximately 1-5 million cells per injection) are required for administration for each patient. Furthermore, multiple injections would be needed to bring stable functional improvement over a long
However, numbers of hESC derived CMs produced are still far fewer than needed to fulfill their full potential in cell-based approaches to regenerate CMs. The efficiency of EB-based differentiation of ESCs to CMs is low, typically lower than 1% from hESCs. Although various techniques have been used to direct and control the cardiomyogenic differentiation of hESCs in vitro (Table 4)\(^8,32-35\), significantly increasing the efficiency of CM differentiation and scaling up to cell numbers will still be required to produce CMs in sufficient numbers that would be adequate for treating a human heart.

Table 3. Summary of previous animal studies of hESC-CM transplantation into the myocardium.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cell type</th>
<th>Animal model</th>
<th>No. of Cells implanted</th>
<th>Follow-up</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kehat et al. (2004)(^7)</td>
<td>hESC-H9.2 derived CMs</td>
<td>Swine</td>
<td>40-150 beating EBs</td>
<td>1-3 weeks</td>
<td>The transplanted hESC-CMs paced the hearts of swine with complete atrioventricular block.</td>
</tr>
<tr>
<td>Laflamme et al. (2005)(^6)</td>
<td>hESC-H1 &amp; H7 derived CMs</td>
<td>Rat</td>
<td>5×10^6</td>
<td>4 weeks</td>
<td>The grafted hESC-CMs proliferated and exhibited substantial angiogenesis.</td>
</tr>
<tr>
<td>Laflamme et al. (2007)(^8)</td>
<td>hESC-H7 derived CMs</td>
<td>Rat</td>
<td>10×10^6</td>
<td>4 weeks</td>
<td>The engrafted human myocardium attenuated ventricular dilation and preserved regional and global contractile function after myocardial infarction.</td>
</tr>
<tr>
<td>Caspi et al. (2007)(^9)</td>
<td>hESC-H9.2 derived CMs</td>
<td>Rat</td>
<td>1.5×10^6</td>
<td>4 weeks</td>
<td>Transplantation of hESC-CMs results in the formation of stable CM grafts, attenuation of the remodeling process, and functional benefit.</td>
</tr>
<tr>
<td>Leor et al. (2007)(^10)</td>
<td>hESC-CM</td>
<td>Rat</td>
<td>0.1 mm pieces of beating myocardial tissue</td>
<td>2-4 weeks</td>
<td>hESC-CM transplantation can attenuate post-myocardial infarction (MI) scar thinning and left ventricular dysfunction.</td>
</tr>
</tbody>
</table>
Table 4. Various techniques and approaches to direct hESCs into the cardiomyogenic lineage in vitro.

<table>
<thead>
<tr>
<th>Techniques</th>
<th>References</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-azacytidine</td>
<td>Xu et al. (2002) 33</td>
<td>CM differentiation was enhanced by treatment of cells with 5-aza-2'-deoxycytidine.</td>
</tr>
<tr>
<td>Directed differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone morphogenetic protein 4 (BMP4) Activin A</td>
<td>Laflamme et al. (2007) 8</td>
<td>More than 30% CMs could be induced from monolayer and 80%–90% CMs could be enriched using density-gradient centrifugation.</td>
</tr>
<tr>
<td>Coculture/Conditioned media</td>
<td>Mummery et al. (2007) 35</td>
<td>Serum-free coculture with a mouse endoderm-like cell line (END2) yields cultures containing on average 25% CMs for two widely available hESC lines, hES2 and hES3.</td>
</tr>
<tr>
<td>Activin A basic fibroblast growth factor (bFGF)</td>
<td>Burridge et al. (2007) 36</td>
<td>Formation of human EBs in defined medium containing Activin A and bFGF resulted in 23.6% +/- 3.6% beating EBs, representing a 13.1-fold increase relative to mass culture (1.8% +/- 0.7%).</td>
</tr>
<tr>
<td>SB203580 (a small molecule inhibitor of p38 MAP kinase) END2 conditioned media</td>
<td>Graichen et al. (2007) 37</td>
<td>Doubled the yield of CMs from hESCs (from 12% to 25%) by enhancing induction of mesoderm.</td>
</tr>
<tr>
<td>BMP4</td>
<td>Takei et al. (2009) 34</td>
<td>The incidence of beating EBs at 25 ng/ml BMP-4 reached 95.8% on day 6 of expansion and then plateaued until day 20.</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>Tran et al. (2009) 32</td>
<td>EB and mesendoderm formation is enhanced, leading to greater differentiation toward CMs. The generated beating clusters are highly enriched with cardiomyocytes (50%).</td>
</tr>
</tbody>
</table>
1.1.3 Early Cardiogenesis

Myocardial cells are derivatives of the mesoderm, which emerges from the primitive streak during gastrulation. Cardiac progenitor cells are in the anterior region of the streak, at this stage their commitment to a cardiac fate remains plastic. Later, they leave the streak and migrate in an anterior-lateral direction to positions under the head folds, forming two groups of cells on either side of the midline. Myocardial markers are first detected at this stage. The cells then extend across the midline to form a crescent-shaped epithelium, the cardiac crescent, which fuses at the midline to form the early heart tube. This tube undergoes rightward looping, with its posterior region moving anteriorly. The heart is shaped by the looping process and by expansion of the myocardium, which leads to the formation of recognizable cardiac chambers (Figure 3).

Figure 3. Morphogenesis of the mouse heart. a. Myocardial progenitor cells originate in the primitive streak (PS), from where they migrate to the anterior of the embryo at about embryonic day E6.5. b. These cells come to lie under the head folds (HF) and form the cardiac crescent, where differentiated myocardial cells are now observed (E7.5). c. The early cardiac tube forms through fusion of the cardiac crescent at the midline (ML) (E8). d. It subsequently undergoes looping (E8.5). e. By E10.5 the heart has acquired well-defined chambers, but is still a tube (upper panel, ventral view; lower panel, dorsal view). f. In the fetal heart (E14.5) the chambers are now separated as a result of septation and are connected to the pulmonary trunk (PT) and aorta (Ao), which ensure the separate pulmonary and systemic circulation of the blood, respectively, after birth. Deoxygenated blood enters the heart through the
right atrium (RA) and is pumped to the lungs through the pulmonary trunk by the right ventricle (RV). Oxygenated blood returns to the left atrium (LA) and is pumped by the left ventricle (LV), through the aorta, to the systemic circulation that serves the whole body. (g) Simplified version of myocardial differentiation. Activien/TGF-β signaling from the hypoblast initiates myocardial specification. FGF and BMP signaling initiates myocardial determination by turning on myocardial transcription factor Nkx2.5 and Gata4. Determination is quickly followed by myocardial cell differentiation which is promoted by other signaling factors such as Wnt. Anterior (A)–posterior (P) and right (R)–left (L) axes are indicated. AA, aortic arch; AVC, atrioventricular canal; IFT, inflow tract; IVC, inferior vena cava; IVS, interventricular septum; OFT, outflow tract; PLA primitive left atrium; PRA, primitive right atrium; PV, pulmonary vein; SVC, superior vena cava; Tr, trabeculae.

1.1.4 Cardiovascular Progenitors in the Heart

The heart is composed of diverse cell types: cardiac muscle, smooth muscle, conduction system, endothelial, valvular, and interstitial mesenchymal fibroblast cells. The lineage diversity in the heart is generated by the presence of mesoderm-derived, multipotent cardiovascular progenitors. A common primordial cardiovascular progenitor population derived from Brachyury+ (Bry+) mesodermal precursors gives rise to these cardiovascular progenitors. The Bry+ precursors may differentiate early during development (EB day 3.25) into Bry+, Flk-1+ hemangioblasts and, after a second wave of Flk-1 expression (EB day 4.25), into cardiac mesodermal precursors. Whether the common primordial cardiovascular progenitor cells are derived from the latter awaits further investigation. Islet-1 (isl-1), together with Nkx2.5 and Flk-1, defines multipotent isl-1+ cardiovascular progenitor cells (MICPs), which can give rise to CMs, smooth muscle cells, and endothelial cells, representing all three major cell lineages of the heart. Wnt/β-catenin signaling and Bmp-2/Smad-1 play critical roles in the specification, self-renewal, and differentiation of isl-1+ progenitors. A specific subset of precursors derived from the MICPs may function as bipotent progenitors, restricting their downstream progenies to smooth muscle and endothelial cells or smooth muscle and CMs. Nkx2.5 bipotent progenitors may account for CMs and/or smooth muscle cells. The transient intermediates that connect the bipotent progenitors to the differentiated progeny remain unknown. Additional candidate genes (shown here with red question marks) that may designate alternate transitional populations await further characterization (Figure 4).
1.2 Goal and Overview of the Dissertation

Heart failure is a leading cause of death and hospitalization in the developed world. One of the causes of heart failure is associated with the progressive death of CMs by apoptosis or necrosis. Since adult CMs do not seem to regenerate, their death permanently compromises myocardial contractile function. Even if self-repair mechanisms exist in the adult human heart, they are of insufficient magnitude to compensate for loss of CMs. Stem cell transplantation is one therapeutic strategy to replace damaged or lost myocardial tissue to restore cardiac function. Stem cells offer the possibility of repairing damaged organs like the heart, and there is an intensive effort to develop stem cell–based strategies for cardiac repair. Both adult
and ESCs are being studied in preclinical models, and early-stage clinical trials. Compared to adult stem cells with limited plasticity, ESCs are an attractive population for cardiac repair because they can be isolated and maintained by well-established protocols. Secondly, ESCs derived from the inner cell mass of blastocyst-stage embryos, are pluripotent and can self-renew and differentiate into all cell types including CMs. Furthermore, ESC derived CMs are viable human heart cells that can functionally integrate with the recipient organ after transplantation.

However, a major challenge in ESC-based cardiac therapies is that the spontaneous differentiation efficiency of ESCs into CMs is low, typically 1%–3% from mESCs and lower than 1% from hESCs, and large numbers of cells (approximately 1-5 million cells per injection) are required for administration for each patient.

Therefore, the goal of the work in this thesis is to optimize microenvironment signals for enhanced CM differentiation. Because differentiation of ESCs is directed by morphogenic cues comprising the intercellular and surrounding extracellular microenvironment, including exogenously administered molecules and endogenous factors produced by the ESCs, attempts to force EB aggregation, control stage specific soluble factor signals, and improve cell-extracellular matrix (ECM), cell–cell interactions influence differentiated cell phenotypes (Figure 5).

Figure 5. Microenvironmental factors direct ESC cell fate. The size of the EB, soluble factor signaling, cell-ECM interactions and cell–cell interactions can all influence ESC commitment.
This thesis focuses on optimizing 1) the homogeneous EB formation, 2) Activin A/BMP4 Signaling and 3) ECM signaling for efficient cardiac differentiation. The following chapter, Chapter 2, describes an ultra-rapid fabrication and culture method utilizing Honeycomb Microwells for the induction of uniform EBs from single cell suspension for CM differentiation; and then compares the quality of EB generated using our Honeycomb Microwells to EB generated using the more traditional Hanging Drop method, and the commercially available AggreWell™400 plate. Chapter 3 shows the Activin A/BMP4 stage-specific CM differentiation methods for two of the most prevalent and accessible hESC: H7 and H9. Chapter 4 presents the precise levels of ECM protein fibronectin (FN) and laminin (LN) signaling promoting highly efficient cardiac differentiation of hESC through Integrin/FAK/ERK signaling. The last chapter concludes this dissertation.
Chapter 2. Honeycomb Microwell for Uniform Embryoid Bodies for Cardiomyocyte Differentiation

2.1 Introduction

The formation of EBs is the principal step in the differentiation of ESCs. The term EB has been broadly applied to describe pluripotent cell aggregates induced to differentiate using a variety of different formation and culture methods. Generally, an aggregate of pluripotent stem cells, cultured in suspension, and capable of forming derivatives of all three germ lineages is regarded as an EB. When factors that maintain the stemness of ESCs are removed, ESCs are commonly differentiated in vitro by spontaneously self-assembling in suspension culture into EBs. EB differentiation begins with the formation of an aggregate of ESCs, the size of which is dependent on the number of cells which initially self-assemble via cell–cell adhesion receptors. The early differentiation of EBs occurs in two phases: within the first 2–4 days of suspension culture endoderm forms on the surface of EBs, giving rise to structures termed “simple EBs” (Figure 6A); subsequently, around Day 4, “cystic” EBs (Figure 6B) develop with the formation of a central cavity and differentiation of a columnar epithelium with a basal lamina. Upon continued in vitro culture, EBs give rise to all three germ layers (ectoderm, mesoderm, endoderm) and terminally differentiate into a wide variety of cell types, such as CMs, hematopoietic cells, neurons and pancreatic islet cells.

![Figure 6. Formation of monkey ES cell-derived EBs. Phase-contrast micrographs show simple EBs (A) and a cystic EB (B).](image)

2.1.1 EB Size Directs Cell Fate

The size of EBs is thought to be an important physical parameter capable of influencing the proportion of cells differentiating toward different lineages. EB size, which is primarily a function of the number of ESCs constituting each cell aggregate, impacts other environmental parameters affecting differentiation, such as the diffusion of soluble molecules and the extent of ECM-cell and cell–cell adhesive interactions. Ng et al. reported that hematopoietic differentiation was influenced by the number of cells seeded into each EB. A minimum human EB starting size (500 cells/EB) was required for myeloid differentiation to occur in over 90% of EBs and
that an intermediate size range (1,000 cells/EB) promoted erythroid cell differentiation.\textsuperscript{45} Park, J. et al. reported that the smaller (100 μm) mouse EBs showed the increased expression of ectodermal markers compared to the larger (500 μm) EBs, while the 500 μm EBs showed the increased expression of mesodermal and endodermal markers compared to the 100 μm EBs, confirming that the initial size of the aggregate is an important factor for ESC differentiation, and can affect germ layer selection as well as the extent of differentiation.\textsuperscript{46}

The importance of EB size in regulating cardiac differentiation is likely related to diffusion of critical substrates as well as the role of various chemical cues such as growth factors. Spatial gradients of a variety of signaling molecules are essential for normal cardiac embryological development. For example, factors secreted from neighboring anterior lateral endoderm such as BMPs promote cardiogenesis.\textsuperscript{47} Deterministic mechanisms in which noncanonical WNT pathway controls embryonic cardiac and vascular development as a function of EB size was reported. The smaller mouse EBs (150 μm) expressed high levels of WNT5a and endothelial cell differentiation while suppressing WNT11 expression. Meanwhile, the larger mouse EBs (450 μm) showed significantly increased expression of WNT11 and higher cardiogenesis with low expression of WNT5a at day 5 of EB formation (Figure 7).\textsuperscript{48} But for hESCs, medium size EBs (250 μm) generated the greatest percentage of contracting EBs and cardiac gene expression levels, comparing to small EBs (90 μm) and large EBs (350 μm).\textsuperscript{47} These varied results may represent differences between the species.

Figure 7. Schematic of the mechanism and role of WNT signals to control cardiogenic and endothelial cell differentiation. WNT5a and WNT11 played an important role in regulating endothelial and cardiogenic cell fates.\textsuperscript{48}

2.1.2 Controlling EB Size

There are several methods for inducing the formation of EBs from ESCs. The three basic methods are liquid suspension culture in bacterial-grade dishes, culture in methylcellulose semisolid media, and culture in hanging drops. Liquid suspension culture and methylcellulose semisolid media culture give rise to EB populations which are heterogeneous in size, morphology and thus epigenetic expression,
rendering them unsuitable for potential clinical applications. Hanging drop is the only conventional method to produce homogeneous cell aggregates by dispensing a defined number of ESCs in physically separated droplets of media suspended from the lid of a Petri dish. Individual EB form within each drop via gravity-induced aggregation of the cells and although EBs created by the hanging drop method can be subsequently introduced to suspension batch culture, the technique is not easily amenable to scale up for production of large numbers of EBs.\textsuperscript{41} Figure 8 illustrates the typical protocol of the hanging drop method for the generation of ESC derived CM.\textsuperscript{42, 49} EBs formed by the hanging drop method have been used to generate a broad spectrum of cell types, including neuronal cells, lymphoid, hematopoietic cells, CMs, smooth muscle cells, chondrocytes, renal cells, adipocytes, hepatocytes, insulin-producing cells, and gametes.\textsuperscript{42}

![Diagram](image)

**Figure 8.** Protocol for the generation of ESC derived CMs. Plate 15–30 μl drops containing 400–1000 ESCs on the lid of petri dishes in regular arrays. Invert the lid and place it over the bottom of a petri dish filled with PBS to prevent the drops from drying out. When the lid is inverted, each drop hangs and the ESCs fall to the bottom of the drop. Incubate the petri dish with hanging drops for 2 days. The ESCs aggregates into a single EB. Once these EBs form, they keep proliferating and differentiating. Harvest EBs and transfer the suspension of EBs into bacterial-grade dishes and cultivate them for 5 days. Plate 7-day-old EBs on gelatin-coated tissue culture plates.\textsuperscript{42, 49}

The initial size of EBs can also be controlled through the geometric size of microwells that spatially define the number of ESCs within individual aggregates.
Microfabrication offers advantages for EB formation for high throughputs because it provides a homogeneous surface and control of the cellular microenvironment, and micropatterned control of ESC colonies can dictate the size of EBs which affects the differentiation of the cells towards particular germ lineages. Various microwell designs have reported to form homogeneous EBs using forced aggregation and the size of the resulting EBs is further controlled by the concentration of the cells seeded into each well. EBs may then be extracted from the microwells with gentle pipetting and transferred to suspension culture for differentiation. However, most microfabrication approaches require complex steps, such as photolithography and anisotropic etching of silicon wafers, thus limiting their accessibility and adoption.

Recently, STEMCELL Technologies introduced the AggreWell™400, the first commercially available plate for the generation of standardized EBs from ESCs and iPS cells. This product was followed by the AggreWell™800, which is a plate containing larger sized-wells for generating larger EBs. Together, these plates allow the generation of large quantities of uniform-sized EBs. Although the wells of the AggreWell are cone-shaped, and not spherical, the system allows very nice control over the size of EBs by altering the number of cells added to each well.

### 2.1.3 Honeycomb Microwell for Uniform EB formation

Here we report an ultra-rapid fabrication method of closely arrayed microwells in a honeycomb configuration of customizable and well-controlled size negating the need for photolithography altogether. We achieve rounded bottom wells not easily achievable with standard microfabrication methods but critical to achieve spherical EBs and then CM. Furthermore, by simply controlling the size of the microwells and the concentration of the cell suspension we can control the initial size of the cell aggregate, thus influencing lineage commitment.

We then compare EB generated using 1) Honeycomb Microwells, 2) the commercially available AggreWell™400, and 3) the more traditional Hanging Drop method. We compare the efficiency, viability, quality, and control of EB sizes. Results show that the microwell and the AggreWell plates are both able to efficiency generate EBs of well-controlled sizes, but the round-bottomed Honeycomb Microwell generates more circular-shaped EBs. Additionally, the cone-shaped EBs from the AggreWell plate exhibit a significant reduction in the formation of the primitive endoderm in the EBs.

### 2.2 Materials and Methods

#### 2.2.1 Culture of mESC

E14 mESCs transfected with green fluorescence protein (GFP) expression linked to the myosin heavy chain (MHC) promoter (courtesy of Conklin Lab, UCSF) were maintained in Knockout Dulbecco’s modification of Eagle Medium (DMEM:
Gibco) supplemented with 15% Knockout Serum Replacement (KSR; Gibco), 100 mg/mL of penicillin-streptomycin (Invitrogen), 1 mM L-glutamine (Gibco), 0.1mM nonessential amino acids (NEAA; Invitrogen), 0.1mM betamercaptoethanol (Calbiochem) and 1000 U/mL leukemia inhibitory factor (LIF; Chemicon) and plated on tissue cultured plates coated with 0.1% Gelatin (Sigma-Aldrich). When the mESC on the tissue culture plates were 80% confluent, they were detached using trypsin/EDTA (0.1%/1 mM), and dissociated into single cells using a pipette to assure uniform distribution of cells during the loading process. mESC were then spun down and re-suspended in differentiation medium, which has the same composition as ESC medium with the exclusion of LIF and supplemented with 20% KSR and 10 ng/ml bone morphogenic protein4 (BMP4; R&D System).

2.2.2 hESC Culture

H9 hESCs were maintained in mouse embryonic fibroblast (MEF)-conditioned medium supplemented with 5ng/ml basic fibroblast growth factor (bFGF, Sigma) and plated on tissue cultured plates coated with Matrigel (Becton Dickenson). When the hESCs on the tissue culture plates were 80% confluent, they were detached using Accutase (Invitrogen), and dissociated into single cells using a pipette to assure uniform distribution of cells during the loading process. The human ESCs were then spun down and re-suspended in high glucose Dulbecco’s Modification of Eagle Medium (DMEM; Gibco) supplemented with 20% Knockout Serum Replacement (KSR; Gibco), and 25 ng/ml bone morphogenic protein-4 (BMP-4; R&D System). The Y-27632 ROCK inhibitor, final concentration of 10 μM, was added during EB formation to enhance cell survival during EB formation.

2.2.3 Fabrication of Honeycomb Microwells

Honeycomb Microwell patterns were drafted in the drafting software AutoCAD (AudoDesk). In order to achieve a range of microwell diameters, we accounted for the 60% reduction in size after shrinking of the pre-stressed polystyrene sheet. To minimize the spacing between wells, well patterns were placed in a staggered position as to minimize free surface area (Figure 9). Next, well patterns were printed onto biaxially prestressed PS sheets (Grafix Inc.) using a laser-jet printer (Hewlitt Packard 2600N) (Figure 9A1). These PS molds were then heated to 155 °C for approximately 5 minutes to form high-aspect micromolds (Figure 9A2). After molding with Polydimethylsiloxane (PDMS) using standard procedures (Figure 9A3), the microwells, designed to fit in standard 24 well plates, are achieved (Figure 9A4). After removing PDMS from micromolds, the Honeycomb Microwells were bonded to glass slides (to prevent floating) and inserted into standard 24-well culture plates (Figure 9B). By repeated printing of the well patterns (by reinsertion into the laser-jet printer), the size and depth of the microwell patterns can be adjusted through the increase of ink deposition. By pieptting cells in, uniform aggregates in Honeycomb Microwells results in uniform EBs similar to the hanging drop method (Figure 9C).
2.2.4 Generation of EBs Using Honeycomb Microwell

To load cells, the bottom of the Microwell was bonded to a piece of cover glass (Fisherbrand) using an O₂ plasma machine (SPI Supplies) and placed into each well of a standard 24-well plate containing 500 µL of differentiation medium. The initial 500 µL assisted in preventing air bubbles within the well and enhanced the adherence of the cover glass to the plate. Next an additional 500 µL of differentiation medium was placed into the well and was pipetted gently to remove any remaining air bubbles on the PDMS surface. ESCs were added at concentrations of $1.75 \times 10^5$ cells/ml concentration to the 200-µm, 300-µm, and 400-µm Honeycomb Microwells for EBs approximately 150, 250, and 500 cells each. For larger EBs with 1000 and 2000 each, we used $3 \times 10^5$ cells per ml and $6 \times 10^5$ cells per ml concentration respectively in the 400-µm Honeycomb Microwell. The $1.75 \times 10^5$ cells/ml concentration was also used for the 200-µm and 300-µm Honeycomb Microwells. To achieve uniform EB sizes, 1 mL of the ESCs were then gently pipetted and dispensed drop-wise into each well of a 24-well plate. To prevent convective effects within each well of the 24-well plate, which may disrupt the
uniform distribution, ESCs were allowed to settle into the Honeycomb Microwells at room temperature for 15–30 minutes before being transferred into the incubator.

2.2.5 Generation of EBs Using AggreWell™

The manufacturer’s instructions from the AggreWell manual version 1.0.0 were initially followed for generating EBs. Accordingly, 1 mL of differentiation medium was placed into each well of an AggreWell™400 plate (Stem Cell Technologies) and then centrifuged the AggreWell™400 plate at 3000g for 10 minutes in a swinging bucket rotor that was fitted with a plate holder to remove any small bubbles from the AggreWell. ESCs were then added at concentrations of 3 × 10⁵ cells/ml, 6 × 10⁵ cells/ml, and 1.2 × 10⁶ cells/ml to each well to make EBs with 500, 1000, and 2000 cells respectively. The AggreWell™400 plate was centrifuged at 200g for 3 minutes to capture the cells in the wells. According to instructions, aggregates should be harvested 24 hours after adding the ESCs to the AggreWell™400 plate. Because some aggregates tended to break up if transferred to suspension culture after only 24 hours, we increased this time to 48 hours in the AggreWell plate before transfer to suspension culture.

2.2.6 Generation of EBs Using Hanging Drops

For the generation of EB with 500, 1000, and 2000 cells using hanging drops, approximately 100,000, 200,000, and 400,000 ESCs were suspended in 3mL of differentiation medium without LIF, respectively. Using a micro-multi-channel pipette, micro-drops were pipetted onto the lid of a culture dish. The lid was inverted and placed on the culture dish. Within 24 hours, the ESCs aggregated in the drops into the initial stage of EB formation.

2.2.7 Verification of the Number of Cells per EB

On day 2, 100 EBs were collected from each Honeycomb Microwell, AggreWell, and Hanging Drop plates. The 100 EBs in each group were pooled together and then dissociated into single cells. The total number of cells from each of the 100 EB-pooled samples was counted using a hemacytometer, and then this number was divided by 100 to calculate the average number of cells per EB.

2.2.8 Characterization of EB Size and Shape

EBs were formed via Honeycomb Microwell, AggreWell, and Hanging Drop methods and imaged at 24- and 48 hours after seeding. The EBs were imaged in live mode with a digital camera attached to an inverted microscope (Fisher Scientific) and operated by imaging software (Micron, Westover Scientific). From these images, the diameters and perimeters were measured from 100 randomly selected EBs formed from each method. From this data, the shape factor, aspect ratio, and circularity of the EBs were also calculated.
2.2.9 Cell Viability

EBs formed by Honeycomb Microwell, AggreWell, and Hanging Drop methods were harvested after 48 hours. Cell viability was determined by using a live/dead kit for mammalian cells (Invitrogen). Both whole EBs and single cells dissociated from the EBs were incubated in 2 M calcein-acetoxyethyl ester (AM) and 4 M ethidium homodimer in phosphate buffer saline (PBS) for 20 min at 37°C. After the incubation period, the stained cells were analyzed by Flow Cytometer LSR II (Becton Dickenson) and whole EBs imaged by laser scanning confocal microscopy. Live and dead cells were indicated by calcein AM (green) and ethidium homodimer (red) respectively.

2.2.10 Confocal Microscopy

Laser scanning confocal microscopy was performed on a Nikon digital eclipse C1 confocal microscope equipped with a Nikon eclipse TE2000U inverted microscope using a 20× air objective for imaging. Laser beams with 488nm excitation wavelengths and 515/30 nm band pass (BP) emission filters were used for Calcein AM and GATA-4 labeled cells. The EBs were also stained with mouse anti-GATA 4-Alexa Fluor® 488 following permeabilization with 0.7% Triton X100 for 15 minutes. Laser beams with 543nm excitation wavelengths and 590/50 nm BP emission filters were used for ethidium homodimer-labeled EBs. Laser beams with 633nm excitation wavelengths and 650 nm long pass emission filters were used for Draq5 labeled EBs. For EBs approximately 180 µm in diameter, typically 45 images were acquired at 4 µm slice intervals, each slice being the average of three laser scans. Z-stacked images were processed by using the Java-based image analysis program ImageJ (http://rsb.info.nih.gov/ij/). Z-projected images were assembled for each time point to produce a single image based on the sum of pixel brightness values through the image stack (ImageJ: z-project).

2.2.11 Flow Cytometry

Individual cells were isolated from EBs with cell dissociation buffer and stained for calcein-AM (green) and ethidium homodimer (red) for analyzing live and dead cells within the EBs. Cells were also stained with mouse anti-nestin-PE (R&D System), mouse anti-brachyury-PE (R&D System) and mouse anti-GATA 4-Alexa Fluor® 488 (Becton Dickenson) for 60 minutes after fixation and permeabilization with 0.7% Triton X100 for 15 minutes.

2.2.12 Statistics

For statistical significance, data was acquired and pooled from 100 EBs for each treatment group and all treatment groups for generating EBs were repeated at least 3 times each. Statistical significance was measured using a Student’s T-test individually between all groups.
2.3 Results

According to the technical manual version 1.0.0 for the AggreWell plate, EBs may be harvested from the AggreWell plate after 24 hours. However, after only 24 hours, the cells had not yet aggregated sufficiently to be removed as EBs (Figure 10 A and B) and often broke apart into smaller clusters once transferred to suspension cultures (not shown). Therefore, we also examined the ESC aggregates after 48 hours in both AggreWell and Honeycomb Microwell and found that, after 48 hours, the cells had aggregated enough to be removed and transferred into suspension cultures without breaking apart (Figure 10 C and D). Additionally, we calculated aspect ratio of the EBs in the wells using the diameters of the long- and short-axes for both the AggreWell and Honeycomb Microwell plates (Figure 10E). The long- and short-axis diameters were measured from EBs formed via AggreWell and Honeycomb Microwell plates. No significant differences were observed between the calculated aspect ratios for AggreWell and Honeycomb Microwell. Both the AggreWell and Honeycomb Microwells are able to generate equivalent EB. Note that after 48 hours, the aspect ratios of the EBs formed using both Honeycomb Microwell and AggreWell are both approaching 1, indicating a more circular formation compared with after only 24 hours (* and ^ indicate comparisons with P < 0.0001).

2.3.1 Circularity of EB

After transferring EBs into suspension culture, we noticed morphological differences that were for larger sized EBs ~ 1000 cells per EB (Figure 11). The methods for generating EBs with 500- and 1000-cell EB were optimized for the Honeycomb Microwell, AggreWell and Hanging Drop and imaged after transferring cells into suspension culture after 48 hours. Figure 11 depicts EBs at 500 cells per EB generated using the Honeycomb Microwell (Figure 11A), AggreWell (Figure 11B) and Hanging Drop (Figure 11C), and the larger EBs at 1000 cells per EB generated using the Honeycomb Microwell (Figure 11D), AggreWell (Figure 11E) and Hanging Drop (Figure 11F). Specifically, the larger EBs formed using the AggreWell plate appeared to retain the cone-shape from the bottom of the well plate (Figure 11E). The smaller EBs with 500-cell EBs appear to be approximately equivalent in size, shape, and cell density for all three generation methods, whereas the EBs containing 1000 cells per EB generated using the AggreWell appear to be more densely packed, smaller in size, and exhibit cone-shaped peaks (indicated by arrows) that reflect the cone-shaped morphology of the wells in the AggreWell plate. EB were also generated at 2000 cells per EB in Honeycomb Microwells (Figure 11G), AggreWell plates (Figure 11H), and Hanging Drops (Figure 11I) in order to test the limits of the well plates. Note that at 2000 cells per EB, the AggreWell plate generated smaller, denser, and more distinctly cone-shaped (indicated by arrows) EBs compared with the other methods.
Figure 10. The aspect ratios of EB formed in the Honeycomb Microwell and AggreWell plates increase after 48 hours compared with after only 24 hours. Images of the EB formed in the AggreWell plates and the 400-µm sized Honeycomb Microwells. A) Cell aggregates in AggreWell 24 hours after seeding. B) Cell aggregates in Honeycomb Microwell 24 hours after seeding. C) Cell aggregates in AggreWell 48 hours after seeding. D) Cell aggregates in Honeycomb Microwell 48 hours after seeding.
hours after seeding. Note that the cells seeded 48 hours prior formed larger aggregates that appear to be more circular compared with after only 24 hours. E) Aspect ratios of the EBs were also calculated. The long- and short-axis diameters were measured from EBs formed via AggreWell and Honeycomb Microwell. No significant differences were observed between the calculated aspect ratios for AggreWell and Honeycomb Microwell. Note that after 48 hours, the aspect ratios of the EBs formed using both Honeycomb Microwell and AggreWell are both approaching 1, indicating a more circular formation compared with after only 24 hours (* and ^ indicate comparisons with P < 0.0001). 56

Figure 11. Representative images depicting the morphology of EB flipped into suspension after 48 hours. The methods for generating EB with 500- and 1000-cell EB were optimized for the Honeycomb Microwell, AggreWell and Hanging Drop and imaged after transferring cells into suspension culture after 48 hours. Images depict EB at 500 cells per EB generated using the A) Honeycomb Microwell, B) AggreWell and C) Hanging Drop, and the larger EB at 1000 cells per EB generated using the D) Honeycomb Microwell, E) AggreWell and F) Hanging Drop. The smaller EB with 500-cell EB appear to be approximately equivalent in size, shape, and cell density for all three generation methods, whereas the EB containing 1000 cells per EB generated using the AggreWell appear to be more densely packed, smaller in size, and exhibit cone-shaped peaks (indicated by arrows) that reflect the cone-shaped morphology of the wells in the AggreWell plate. EB were also
generated at 2000 cells per EB in G) Honeycomb Microwells, H) AggreWell plates, and I) Hanging Drops in order to test the limits of the well plates. Note that at 2000 cells per EB, the AggreWell plate generated smaller, denser, and more distinctly cone-shaped (indicated by arrows) EB compared with the other methods.\footnote{56}

We then calculated the circularity, $C$, a more sensitive measurement than aspect ratio, for the 2 day old EBs following transfer into suspension culture. The cross-sectional area and perimeter of each cell aggregate was acquired using Image J. From each image (Figure 12A), a high contrast black and white image (Figure 12B) was generated. Image J then created a high resolution outline of the cell aggregates (Figure 12C), and, running this outline through a mean filter, then smoothed the outlines of the cell aggregates (Figure 12D). A radius of 20 pixels was used in order to remove excess surface aberrations in the aggregate outlines. This allows one to measure the general shape of the cell aggregate without incorporating the natural surface topology of the EB. Cross-sectional area, $A$, and perimeter, $P$, values were finally measured from these smoothed outlines and then used to calculate the circularity of individual cell aggregates (Figure 12E).

$$C = 4\pi \times \frac{A}{(P^2)}$$

The circularity calculations indicate that at 500-cell EB, the Microwell (comparison noted by $^\wedge$, $P<0.0001$) and AggreWell ($^*$, $P<0.0001$) are both superior to the Hanging Drop, and the Microwell is superior to the AggreWell ($^\#$, $P<0.01$) while at 1000 cells per EB, the Microwell forms more circular EBs compared with both the AggreWell ($^{**}$, $P<0.0001$) and Hanging Drop ($^{\wedge}$, $P < 0.0001$), but the Hanging Drop is superior to the AggreWell ($^{##}$, $P < 0.01$) (Figure 12 F and G). Taken together, the data indicate that the Honeycomb Microwell and AggreWell plates generate more circular-shaped EB compared with the Hanging Drop for the smaller sized-EB, but that the Honeycomb Microwell generated more circular larger sized EBs.\footnote{56}

2.3.2 Controlling EB sizes

The diameters of the EB generated in the 200-, 300- and 400-μm sized Honeycomb Microwells (Figure 13A) indicate that the size of the Microwells dictate the size of EBs, small 500-cell EB (Figure 13B) and large 1000-cell EBs (Figure 13C) were then measured following generation in the 400 μm sized Honeycomb Microwells, AggreWells, and Hanging Drops. The diameters of the EBs were measured after 2 and 7 days for the 200μm, 300μm and 400 μm Honeycomb Microwells (Figure 13A), and using the Honeycomb Microwell, AggreWell, and Hanging Drop to generate both the smaller EBs (500 cells; Figure 13B) and the larger EBs (1000 cells; Figure 13C). All three methods of EB formation generated EBs with well-controlled sizes (Figure. 13A and B) as evident from the small standard deviations in these EBs.
The EBs were moved to suspension cultures after 2 days and allowed to grow further. The diameters of the EBs were measured again after 7 days. We noted that the diameter of the EBs generated in the AggreWell plate were smaller than the EBs in the Microwell at both day 2 and 7 (* and **, P<0.0001) or Hanging Drop (^ and ^^, P<0.0001). The differences between all diameters were statistically significant (Figure 13A and B; ^, P<0.0001, except differences between the 400μm-sized Microwell and AggreWell, noted by # were significant at P<0.001) on day 2. At day 7, all differences were statistic (Figure 13A and B, *, P<0.0001, except differences between the 400μm-sized Microwell and AggreWell were significant at ##, P<0.1). More importantly, the small standard deviations in the sizes indicate the ability for all methods to control the EB sizes by adjusting cell numbers. After 5 days with unconstrained growth, the variation in sizes of the EBs had increased, but has remained proportional to the original EB sizes.

Figure 12. The EB formed in the Honeycomb Microwell are more circular compared with EB formed in AggreWell and Hanging Drop. Images of EB were further processed for determining the circularity, a more sensitive measurement of roundness, using ImageJ software. Images include: A) bright field image, B) high contrast image, C) rendered outline of cell aggregate, D) outline modified with mean
filter, and E) composite image of original bright field image with modified outline overlay. Scale bar is 50 µm. F) Tables indicating cell numbers (with standard deviations in cell numbers), seeding concentration and circularity values for the targeted 500 cells/EB and 1000 cells/EB. G) Graph depicting the circularity values calculated from the area and perimeter measurements of the inside of the modified outline. The data indicates that at 500-cell EB, the Microwell (comparison noted by ^, P<0.0001) and AggreWell (*, P<0.0001) are both superior to the Hanging Drop, and the Microwell is superior to the AggreWell (#, P<0.01) while at 1000 cells per EB, the Microwell forms more circular EB compared with both the AggreWell (**, P<0.0001) and Hanging Drop (^^, P < 0.0001), but the Hanging Drop is superior to the AggreWell (##, P <0.01).

Figure 13. Honeycomb Microwell, AggreWell and Hanging Drop all generate well-controlled sized EB. The diameters of the EB generated in the A) 200-, 300- and 400-µm sized Honeycomb Microwells indicate show that the size of the Microwells dictate the size of EBs, B) small 500-cell EB and large 1000-cell EB were then
measured following generation in the 400 μm sized Honeycomb Microwells, AggreWells, and Hanging Drops. The EB were moved to suspension cultures after 2 days and allowed to grow further. The diameters of the EB were measured again after 7 days. The differences between all diameters were statistically significant (Fig. A and B; ^, P<0.0001, except differences between the 400μm-sized Microwell and AggreWell (noted by # were significant at P<0.001) on day 2. At day 7, all differences were statistic (Fig. A and B, *, P<0.0001, except differences between the 400μm-sized Microwell and AggreWell were significant at ##, P<0.1). More importantly, the small standard deviations in the sizes indicate the ability for all methods to control the EB sizes by adjusting cell numbers. C) We also examined the EB sizes for the larger EB using only the largest 400 μm-sized Microwell. We noted that the diameter of the EB generated in the AggreWell plate were smaller than the EB in the Microwell at both day 2 and 7 (* and **, P<0.0001) or Hanging Drop (^ and ^^, P<0.0001).

Figure 14. The EB formed in the Honeycomb Microwell and AggreWell, and Hanging Drops contain viable cells. The larger 1000-cell EB generated using A) 400-μm sized Honeycomb Microwells, B) AggreWell plates, and C) Hanging Drops were stained with calcein-acetoxymethyl ester (green) for live cells and ethidium homodimer (red) for dead cells on day 2 and imaged using z-stacked confocal microscopy. In order to quantify the numbers of live and dead cells, the small 500-cell EB and large 1000-cell EB were dissociated into single cells and analyzed using a flow cytometer. D) The viability (% of live cells) in EB formed via AggreWell,
Honeycomb Microwell and Hanging Drops was consistently greater than 90% for all methods. The differences in viability of cells in EB between formation methods was not generally significant, but cells in the EB formed in the Microwell (*, P<0.1) and AggreWell (^, P<0.1) both contained more viable cells compared with the cells in the EB formed via Hanging Drop.

Figure 15. Primitive Endoderm, Ectoderm and Mesoderm in EB. The larger EB generated using A) 400-μm sized Honeycomb Microwells, B) AggreWell plates, and C) Hanging Drops were stained on day 2 with primitive endoderm marker, GATA-4
(green), and cell nucleus, Draq5 (blue) and imaged using z-stacked confocal microscopy. Note that the GATA-4 expression in the EB formed using the AggreWell plate is only present on the cone-shaped tip (indicated by arrow) rather than throughout the surface layer of the EB. These were then quantitatively analyzed by staining germ layers for FACS analysis on both days 2 and 7 with D and E) mesoderm marker, brachyury, (F and G) primitive ectoderm marker, nestin, and (H and I) primitive endoderm marker, GATA-4. Note that the GATA-4 expression in the EB formed using the AggreWell plate is most significantly reduced compared with the GATA-4 expression in EB formed using the Microwells and Hanging Drops (* and **, P<0.01) and (#, P<0.05).  

2.3.3 Cell Viability in EB

Cell viabilities in the EBs after 2 days of growth in the Honeycomb Microwell, AggreWell, and Hanging Drop were measured using a live/dead (red/green) staining kit. Z-stacked confocal microscopy images revealed a high number of live cells (green) compared with dead (red) cells (Figure 14A, B, and C). The EBs were then disaggregated, stained and analyzed by flow cytometry, plotted as histograms expressing the calcein fluorescent dye indicating live cells (not shown). The percent viability (% of live cells) in the EBs formed via AggreWell, Honeycomb Microwell and Hanging Drops was consistently greater than 90% for all methods. The differences in viability of cells in the EBs of the different formation methods was not generally significant, but cells in the EBs formed in the Microwell (*, P<0.1) and AggreWell (^, P<0.1) both contained more viable cells compared with the cells in the EBs formed via Hanging Drop. The data also indicates that after 48 hours, the EBs formed in the AggreWell and Honeycomb Microwell still contained greater than 95% live cells (Figure 14D).  

2.3.4 Primitive Mesoderm, Ectoderm, and Endoderm in EB

While evaluating our phase contrast images, we also noticed that the large and extra-large EBs formed using the cone-shaped bottomed AggreWell plate (Figure 15E and H respectively) did not generate the same distinct primitive endoderm layer on the exterior surface seen in the small EBs (Figure 15A-C) or the larger EBs formed in the Microwell (Figure 15D) and the Hanging Drop (Figure 15F). Therefore, we stained the large EBs for the presence of primitive endoderm marker, GATA-4 (green), and cell nucleus, Draq5 (blue), on 2 day. The EBs were imaged using z-stacked confocal microscopy. We found that the EBs formed by AggreWell (Figure 15B) expressed endoderm marker GATA-4 only at the cone-shaped tips (indicated by arrow) while the EBs formed by Microwell and Hanging Drop expressed the primitive endoderm maker evenly throughout the surface of the EB (Figure 16A and C). 

We proceeded to quantitatively examine the development all three germ layers at day 2 and 7, using brachyury for mesoderm differentiation (Figure 15D and E), nestin to identify the primitive ectoderm (Figure 15F and G) and GATA-4 for
primitive endoderm marker (Figure 15H and I). The percentage of cells expressing the early mesoderm marker, brachyury, ranged from 42-47%, but did not differ significantly between EBs formed from Microwell, AggreWell, or Hanging Drop (Fig. 15D and E). The percentage of cells expressing, nestin, the marker for primitive ectoderm, was less than 50% in the AggreWell on day 2, compared with the Microwell and Hanging Drop expressing over 55% nestin positive cells (Figure 15F). However, the nestin expression by day 7 was comparable in the EBs from Microwells and AggreWells at 50%, and slightly lower in the Hanging Drop (Figure 15G). Conversely, GATA-4 expression in the EBs formed using the AggreWell plate is most significantly reduced compared with the GATA-4 expression in EBs formed using the Microwells and Hanging Drops (* and **, P<0.01) and (#, P<0.05) on day 2 (Figure 15G). Although the cone-shaped morphology of the EBs in the AggreWell do recover after transfer to suspension cultures (not shown), the GATA-4 development of the primitive endoderm in these EBs does not recover and remains below 1% compared with 6-8% expression in EBs from Microwell and Hanging Drop (Figure 15I).

2.3.5 EBs from hESCs

Perhaps most importantly, with the application of Rho-associated kinase (ROCK) inhibitors, which permits the single cell dissociation of hESCs, this approach can also be used for human EBs formation from single cell suspension, the Microwell and AggreWell plates are able to generate EBs from single hESCs with greater efficiency than conventional Hanging Drop Methods (Figure 16).

This is a significant advancement in the field, as the generation of human EBs from single cells is notoriously challenging. We expect the success of these well plates in generating EBs from single cells is due to the physical aggregation of individual cells that bring the cells into closer contact with one another compared with a Hanging Drop.

2.4 Conclusions and Discussion

We report a novel method for the fabrication of Honeycomb Microwells utilizing a laser-jet printer and replica molding. In addition, we have shown functional application of the devices for the induction of uniform EBs of tunable sizes. By printing microwell patterns onto pre-stressed PS sheets and through heat induced shrinking, high aspect micromolds are generated. By molding PDMS onto the PS masters, honeycomb-shaped microwells are formed. In addition, through the inherent fabrication method, we are able to generate rounded bottom wells which facilitate the formation of spherical EBs and which are potentially much less restrictive to diffusive transport.

By varying the size of the honeycomb wells, we are able to control the initial number of cell aggregates thus enabling control of the rate of both human and mouse EB growth and differentiation, which has been shown to affect lineage commitment.
Notably, Honeycomb Microwells can be integrated into standard cell culture plates providing a low cost, robust method of high-throughput EB culture applicable in both academic and industrial settings.

Because the differentiation of stem cells within an EB is thought to recapitulate, at least in part, the developing embryo, the non-uniform shape of EB in the larger EB from the AggreWell plate is assumed to be an undesirable factor. We have shown that the endoderm marker, GATA-4, does not develop normally with a cone-shaped EB. On day 2 of development, the GATA-4 is concentrated at the cone-shaped tips of the AggreWell EB and is reduced throughout the remaining surface of the EB. Although the cone-shape of these EBs do recover into a spherical shape after transfer from the AggreWell plates into suspension culture, the GATA-4 expression remains significantly reduced in these EBs compared with EBs formed using the Microwell and Hanging Drop. Most importantly, use of the AggreWell plate for initial EB formation would most likely limit the derivation of endodermal tissue products, like cells in the gastrointestinal and respiratory tracts, and should be avoided in these protocols.

This study is the first indicating that the shape of an EB can influence the fate of the differentiating ESCs, however; the initial size of an EB is already known to be an important physical parameter influencing the proportion of cells differentiating towards some specific lineages. In addition, the shape of individual human mesenchymal stem cells affects their differentiation efficiency towards adipocyte or osteoblast lineages. Therefore, it is not surprising that the shape of an EB might play a role in the fate of the differentiating cells within the EB.

Figure 16. EB generation from hESC. We were also able to successfully generate EB from single human ESC using both the A) Honeycomb Microwell and B) AggreWell plates more readily compared with conventional the C) Hanging Drop method.
Chapter 3. Activin A and BMP4 Signaling for Efficient Cardiac Differentiation of H7 and H9 Human Embryonic Stem Cells

3.1 Introduction

Functional CMs differentiated from hESCs offer a potentially unlimited cell source for therapies in cardiac disease, regenerative cardiovascular medicine, and the study of human cardiac development. However, large numbers of cells are required for administration for each patient. Unfortunately, commonly used EB formation protocols for hESCs cardiac differentiation generates a low efficiency of approximately 1-8% CMs. Other than EB methods, many different approaches have been described recently for directed and efficient cardiac differentiation of hESCs. These methods include co-culture with END2 (mouse visceral endoderm-like cell) stromal layers and differentiation of hESCs in monolayer culture with high levels of Activin A and bone morphogenetic protein 4 (BMP4).

3.1.1 Role of Activin and BMP in Cardiac Differentiation and Development

Cardiac development is a dynamic process that is controlled by multiple signaling pathways and transcription factors which have been implicated in the development of specialized cardiac subtypes. More than a decade ago it was reported that two members of the transforming growth factor(TGF)-β family Activin A and BMP4 induce cardiac differentiation. Activin was originally discovered as a peptide responsible for the activity of pituitary follicle-stimulating hormone. It was first reported that Activin A has ability to induce mesodermal tissues in early Xenopus embryos in the early 1990s. Activin has been demonstrated to induce all mesodermal tissues in a dose dependent manner. Low levels (0.1ng/ml) of activin cause presumptive ectoderm explants to differentiate into ventral mesodermal tissues, including mesenchyme, coelomic epithelium (same as mesothelium), and blood-like cells, whereas increased medium concentrations (1ng/ml) of activin cause explants to differentiate into various mesodermal tissues such as mesenchyme, muscle, coelomic epithelium and secondary-induced neural tissues. At a higher concentrations (10ng/ml) induced dorsal tissue, such as notochord. At a even higher concentration (100ng/ml), the animal cap can form heart (Figure 17). However, it is unlikely that activin itself can induce cardiac muscle due to the fact that activin did not play any role during early mouse embryogenesis.

Other TGF-β family members, the BMPs, have also been shown to play important roles in cardiac development. BMPs are multi-potential proteins that regulate a plethora of cellular functions during development and adult life. It has been reported that BMPs play a critical role in cardiac development and are one of the inducers of cardiac differentiation, not only in the heart-forming regions, but also in the recruitment of CMs at the distal borders of the heart. During the formation of the four-chambered heart, BMPs are crucial in the regulation of septovalvular development. BMPs also play an important role in both self-renewal of stem cells and their differentiation into CMs. CM formation can be divided into four stages: (1)
pluripotent stem cell formation, (2) precardioblast stage, (3) cardioblast stage and (4) CM stage. In each of these stages, BMPs play an important role. The cells in the pluripotent stem cell stage express POU5F1 (Oct4), SOX2 and NANOG and self-renewal of this population is regulated by BMPs. BMPs stimulate the expression of inhibitor of differentiation genes, which in turn, reduce the levels of MAPK1 and MAPK14 that are involved in maintaining the undifferentiated cells. In order to allow differentiation into precardioblasts, BMPs needed to be transiently inhibited by NOG. After this transient BMP inhibition, BMPs can stimulate the up-regulation of cardiac transcription factors, like NKX2-5 and GATA4, thereby promoting the transition of precardioblasts into cardioblasts. During the final differentiation step, cardioblasts into cardiomyocytes, BMPs signaling via SMAD and MAP3K7 was again found to activate the expression of sarcomeric proteins which consequently leads to the differentiation of cardioblasts into rhythmically contracting cardiomyocytes (Figure 18).  

Figure 17. Diagrammatic representation of the animal cap assay and explants after activin treatment. Depending on the activin concentration, many mesodermal tissues from ventral type mesoderm (low concentration of activin treatment) to dorsal type mesoderm (middle or high concentration of activin treatment) are induced, high concentration of activin also induced the beating heart in the explant and the combination of activin and retinoic acid induced the renal tubules.
Figure 18. Role of BMPs in the differentiation of embryonic stem cells into CMs. Differentiation of totipotent ESCs into CMs can be divided into four stages. In the first stage, pluripotent stem cells, the cells are undifferentiated and have the potential to differentiate into several cell types, including CMs. During this stage, BMPs are important in self-renewal. BMP signaling was found to down-regulate MAPK1 (ERK) and MAPK14 (p38) and to stimulate expression of the transcription factors POU5F1 (Oct4), SOX2, NANOG and ID, which are important to retain the rather undifferentiated state of the cells. When these cells are transiently exposed to Noggin they can become committed to develop into cardiomyocytes by BMP. This stage is referred to as the pre-cardioblast stage. Subsequent stimulation by BMPs, which is transduced by both SMAD and MAP3K7 signal pathways, induces expression of cardiomyocyte-enriched transcription factors. These cells are referred to as cardioblasts. Ongoing stimulation of these cardioblasts by BMPs, which mediated via SMAD, MAP3K7 and PIK3 signal transduction pathways, induces the expression of sarcomeric proteins which consequently leads to the differentiation of cardioblasts into rhythmically contracting CMs.

3.1.2 Stage-Specific Optimization of Activin/BMP4 Signaling for Cardiac Differentiation of hESC

Studies on the signaling pathways for establishing cardiac fate indicate that Activin A and BMP4 signaling pathways work together to regulate induction of cardiac mesoderm. Activin A was shown to mediate mesoderm differentiation and BMP4 specifically acts within cardiogenic regions on the emerging mesoderm for enhancement of expression of cardiogenic genes leading to CM differentiation. Following the incorporation of Activin A and BMP4 supplementation into medium formulations in high density cultures on Matrigel, 35-40% Nkx2.5-positive CMs were generated from hESC. Then, most recently, Kattman et al. reported that each ESC line required individual titration of Activin A/BMP4 signaling for efficient cardiac differentiation. This group showed that the specification of cardiac mesoderm and CMs in different cell lines is determined by very precise levels of Activin A/BMP4 signaling, and that can be monitored by the KDR/PDGFR-α expression in early mesoderm commitment. Using these stage-specific optimization strategies, cultures containing over 60% CMs from H1 and HES2 hESC
lines can be generated. However, the published stage-specific optimizations were not conducted on two of the most prevalent and accessible hESC lines, the H9 and H7 hESCs, used in 10% and 37% of hESC publications, respectively. Therefore, we set out to generate our own stage-specific protocols for the H7 and H9 cell lines. Based on the Kattman, et al., groundbreaking cardiac differentiation scheme, we set out to examine: 1) the kinetics of differentiation and 2) define the optimal Activin A/BMP4 signaling for cardiac cell fate determination in the H7 and H9 hESC lines. We also use a basal RPMI medium instead of the more expensive StemPro-34, and show that our protocols can reproducibly generate cultures containing over 50% cardiac cells without cell sorting.

3.2 Materials and Methods

3.2.1 hESC Culture and Differentiation

Native H7 hESCs (WA07, WISC Bank) and H9 hESCs - transfected with green fluorescence protein (GFP) expression linked to the α-MHC promoter (courtesy of Bernstein Lab, UCSF) - were maintained in MEF-conditioned medium supplemented with 5 ng/ml bFGF (Sigma) and plated on tissue cultured plates coated with Matrigel (BD) according to previously published protocols. When the hESCs on the tissue culture plates were 80% confluent, they were detached using Accutase (Invitrogen), and dissociated into small clumps (3-5 cells). The cells were then spun down and re-suspended in 2ml Roswell Park Memorial Institute (RPMI; Invitrogen) medium supplemented with 2% B27 (Invitrogen). To generate EBs, a 400-µm Honeycomb Microwell microchip was placed into each well of a standard 24-well plate and 6×10^5 cells were added to the Microwell. Cells then settle into the bottom of microwells by gravity, facilitating the formation of large numbers of uniformly-sized EBs. Ten µM of ROCK inhibitor (Y-27632; EMD) was added during EB formation to enhance cell survival. After 24 hours, uniform EBs were formed in Microwell and then transferred to ultra-low attachment plates (Corning) in RPMI/B27 media. Then EBs were dissociated into small clusters (3-5 cells) by gentle pipetting 1×10^5 cells were seeded as monolayer into individual wells of a 96-well flat bottom plate (BD) coated with 0.5% gelatin in RPMI/B27 media (Figure 19A). Cell numbers were estimated by dissociation of parallel culture with trypsin (Invitrogen). The growth factors were added with the following sequence: day 0-1, varying concentration of BMP4 (0-5 ng/ml); day 1-4, varying concentration of Activin A (0-10 ng/ml), varying concentration of BMP4 (30-100 ng/ml) and 5ng/ml bFGF; day 4-8, 150ng/ml DKK1 and 10ng/ml VEGF; day 8-14, 5ng/ml bFGF and 10ng/ml VEGF. All growth factors were purchased from R&D Systems, Inc. The medium was replaced daily.
3.2.2 Flow Cytometry and Fluorescent Microscopy

EBs and cardiac cells generated from hESC differentiation experiments were dissociated into single cells by incubation with cell dissociation buffer (enzyme free, PBS-based, Invitrogen). The cells were stained with the following antibodies: anti-KDR-phycoerythrin (Biolegend), anti-PDGFR-α-allophycocyanin (R&D), anti-cardiac Troponin I (cTnI) (US Biological) and anti-Nkx2.5 (R&D). For cell surface markers, staining was carried out in PBS with 1% BSA. For intracellular proteins, immunostaining was carried out on cells fixed with 4% paraformaldehyde (Tousimis Research Corporation) in PBS and permeabilization with 0.7% Triton X100 for 15 minutes. Stained cells were analyzed on an LSRII flow cytometer (BD). For fluorescent microscopy, day 14 hESC-derived cardiac monolayers were fixed and permeabilized as above. Cells were stained for cTnI (US Biological) and DAPI (Calbiochem). Mouse IgG1 and IgG2b (Sigma) were used as isotype controls.

3.2.3 Statistics

Data are presented as mean ± standard deviation for two independent sets of experiments. All experiments were repeated a minimum of 2 times. Statistical significance was measured using a nonparametric Wilcoxon T-test to individually compare between all groups.

3.3 Results

The three stages involved in the differentiation of hESCs to CMs include: 1) the formation of a primitive-streak; 2) the induction and specification of cardiac mesoderm; and 3) final cardiovascular population expansion. By optimizing the number of days for EB induction, BMP4 concentration in the first 24 hours of differentiation, and Activin A/BMP4 concentrations from day 1-4, the protocols (Figure 19) described here can reproducibly high numbers of H7 and H9 hESCs towards the cardiac lineage.

3.3.1 Optimal Dissociation Day Promotes the Generation of Cardiac Mesoderm

The efficient generation of cardiac mesoderm during EB formation is prerequisite for cardiomyocyte differentiation. According to Kattman, et al., EB containing large KDR/PDGFR-α populations can generate highly enriched cardiomyocyte populations without the need for cell sorting. Takei, et al. showed that the expression of Brachyury, an early mesodermal marker, increased continuously until day 4 of EB suspension culture followed by a sudden reduction in expression by day 5. Therefore, we hypothesized that the induction and specification of cardiac mesoderm in EBs is related to the number of days in which the EBs are in suspension culture, and that the specific time in which the EBs contain the highest percentage of cardiac mesodermal cells is also the day in which the cells should be transferred from EB suspension culture to monolayer cultures for optimal expansion of the cardiovascular lineages.
EBs were generated from small clumps (3-5 cells) in Honeycomb Microwells at day 0 with 2ng/ml BMP4 and then transferred to suspension culture with 0ng/ml Activin A and 30ng/ml BMP4. EBs were collected from day 4 to day 7 and then dissociated into single cells for FACS analysis of KDR/PDGFR-α in order to evaluate the day in which the EBs contained the highest number of cardiac mesodermal cells. Results indicate that the number of KDR+/PDGFR-α+ cells in both H7 and H9 EBs increased over time, reaching an optimum number before reducing their expression if maintained within the EBs for longer. H7 EBs collected on day 5 express the highest percentage (14.9%±2.3%) of cardiac mesoderm monitored by the coexpression of KDR and PDGFR-α (Figure 20A), whereas H9 EBs collected on day 6 contain the highest yield (15.8%±0.6%) of KDR+/PDGFR-α+ population (Figure 20B) (*, P< 0.1).

Figure 19. Scheme for H7 (B) and H9 (C) hESC cell culture manipulation and methods. (A) Embryoid bodies (EB) were generated from small clumps (3-5 cells) in Honeycomb Microwells and then transferred to suspension culture. At indicated time points, EBs were dissociated into small clumps (3-5 cells) and plated down as monolayer on gelatin in 96-well flat bottom plate. Overall optimized differentiation
schemes for the first 14 days of cardiac induction of H7. The optimized stage-
specific differentiation scheme for generating cardiac cells from (B) H7 and (C) H9
hESC is also included.

3.3.2 Optimal BMP4 Concentration (First 24 Hours) for Generating Cardiac
Mesoderm

The first stage for cardiac differentiation from hESCs is the formation of a
primitive-streak-like population and cardiac mesoderm which can be induced by
Activin A and BMP4. Kattman et al. reported that optimal concentrations of
BMP4 (0.5 - 5 ng/ml) during the first 24 hour of EB formation varied between the
different ESC lines. Therefore, we next investigated the effect of BMP4
concentration (0ng/ml, 2ng/ml and 5ng/ml) in the first 24 hours on cardiac
mesoderm generation from hESC followed by transfer to suspension culture with
0ng/ml Activin A and 30ng/ml BMP4 for four days for H7 and five days for H9. H7
EBs were then collected and dissociated into single cells for FACS analysis on day
5. H9 EBs were collected and dissociated into single cells for FACS analysis on day
6. Results show that both H7 and H9 EB treated with 2 ng/ml of BMP4 contained
highest percentage (~14%) of KDR⁺/PDGFR-α⁺ population, whereas higher
concentrations (5 ng/ml) of BMP4 hindered the yield of cardiac mesoderm from both
H7 (Figure 2C) and H9 (Figure 2D) (*, P< 0.1).

![Figure 20. Optimization of day for EB dissociation and initial BMP4 treatment.](image)

EBs were generated from small clumps (3-5 cells) in Honeycomb Microwells at day
0 with 2ng/ml BMP4 and then transferred to suspension culture with 0ng/ml Activin
A and 30ng/ml BMP4. EBs were collected from day 4 to day 7 and then dissociated
in to single cells for FACS analysis of KDR/PDGFR-α.  

(A) H7 EBs collected on day 5 expressed highest percentage of KDR+/PDGFR-α+ cells.  (B) H9 EBs collected on day 6 contained the highest percentage of KDR+/PDGFR-α+ cells.  The optimization of initial (first 24 hours) BMP4 concentration in RPMI/B27 medium is also included for (C) H7 EBs and (D) H9 EBs with 0 ng/ml, 2 ng/ml and 5 ng/ml BMP4 treatment in the first 24 hours and then transferred to suspension culture with 0ng/ml Activin A and 30ng/ml BMP4.  H7 EBs were collected and dissociated into single cells for FACS analysis on day 5.  H9 EBs were collected and dissociated into single cells for FACS analysis on day 6.  Both H7 and H9 EB treated with 2 ng/ml of BMP4 contained highest percentage of KDR+/PDGFR-α+ cells (*, P< 0.1).

3.3.3 Optimal Combinations of Activin A and BMP4 for the Induction of Cardiac Mesoderm and Cardiomyocytes

In order to determine the combinations of Activin A and BMP4 from day 1 to day 4 in generating large KDR+/PDGFR-α+ populations, and subsequently, high numbers of CMs, EBs were cultured in suspension with varying concentrations of Activin A (0 – 10 ng/ml) and BMP4 (30 – 100 ng/ml) from day 1 to day 4.  Half of the induced H7 and H9 EBs were analyzed by flow cytometry on day 5 and day 6, respectively, for the presence of KDR and PDGFR-α.  The other half of the H7 and H9 EBs were dissociated into small clumps and replated as monolayer on day 5 and day 6, respectively.  The monolayer cells were then analyzed by flow cytometry for the presence of cardiac cells on day 14.  In the absence of Activin A, the yield of KDR+/PDGFR-α+ population was low in both H7 and H9 hESC lines, while increasing the concentration of Activin A resulted in the generation of larger KDR+/PDGFR-α+ populations.  Likewise, in the absence of BMP4, a very small number of KDR+/PDGFR-α+ cells were generated (data not shown); however, high concentrations (100 ng/ml) of BMP4 inhibited the Activin A induced KDR+/PDGFR-α+ populations (Figure 21 and 22).  Treatments at 6 ng/ml Activin A and 30 ng/ml BMP4 resulted in the emergence of the greatest KDR+/PDGFR-α+ populations (~24%) in H7 (Figures 21A and 22A), and H9 (Figures 21B and S22B) required higher concentrations of both of Activin A (10 ng/ml) and BMP4 (60 ng/ml) to generate the optimal KDR+/PDGFR-α+ populations (~34%).  The percentage of KDR+/PDGFR-α+ cells in red is significantly greater compared with data in blue (P<0.1), but not black.

Analysis of cardiac specific markers, cTnI and Nkx2.5, in H7 and H9 monolayers at day 14 produced optimal numbers at 6 ng/ml Activin A and 30 ng/ml BMP4 and 10 ng/ml Activin A with 60 ng/ml BMP4 for H7 and H9, respectively (Figures 21 C-F).  Under the optimal stage-specific culture conditions, differentiated H7 monolayer cultures (day 14) contain approximately 50% cTnI+ (Figures 21C and 23A) and 38% Nkx2.5+ (Figures 21E and 23B) cells; whereas H9 monolayer cultures contain approximately 55% cTnI+ (Figures 22D and 24A) and 54% Nkx2.5+ (Figures 21F and 24B) cells.  Again, the percentages of cTnI+ and Nkx2.5+ cells in red are
significantly greater than those in blue (P<0.1), but not black. Immunostaining of H7 and H9 monolayers with cTnI at day 14 also confirm that the hESCs were directed towards the cardiac lineage (Figure 25). Lastly, monolayers of differentiated H7 and H9 exhibit spontaneous beating at day 13, showing that the H7 and H9 hESCs had developed into functional CMs.  

A summary of our methodologies is presented in Figure 19 B and C. The results indicate that our suggested protocol for generating CMs from H7 includes: 2ng/ml BMP4 for the first 24 hours of EB formation, 6ng/ml Activin A and 30ng/ml BMP4 from day 1 to day 4, with transfer from EBs to monolayer culture on day 5. For H9, 2ng/ml BMP4 is used on day 0 (first day of EB formation), and then 10ng/ml Activin A and 60ng/ml BMP4 from day 1-4, with transfer to monolayer culture on day 6. On day 4, both protocols require switching to a medium supplemented with 150 ng/ml DKK1 and 10 ng/ml VEGF, and then a medium supplemented with 10ng/ml VEGF and 5ng/ml bFGF on day 8. Our results show that both of these hESC line-specific protocols reproducibly direct approximately 50% of hESCs towards the cardiac lineage.

Figure 21. Combinatorial effect of Activin A and BMP4 in CM fate. The induction of KDR+/PDGFR-α+ cells with varying concentrations of Activin A and BMP4 from day 1 to day 4 in (A) H7 and (B) H9 EBs. The CMs were examined at day 14 for cTn I expression in (C) H7 and (D) H9 hESCs and expression of NKx2.5 in (E) H7 and (F) H9 hESCs.  

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Figure 22. Dot plots of KDR/PDGFR-α expression of (A) H7 and (B) H9 EB with varying concentrations of Activin A and BMP4 from day 1 to day 4.68
Figure 23. Percentage of cardiac cells from H7 hESCs. The histograms represent the expression of cardiac markers (A) cTnI and (B) Nkx2.5 expression in H7 at day 14 with isotype controls for IgG1 both (C) Nkx2.5 and (D) cTnI.68
Figure 24. Percentage of cardiac cells from H9 hESCs. The histograms represent the expression of (A) cTnI and (B) Nkx2.5 expression in H9 at day 14.

Figure 25. Immunostaining for cTnI at day 14. The H7 (A) and H9 (B) cells were imaged under immunofluorescently stained with cTnI (green) and DAPI (blue).
3.4 Conclusions and Discussion

Using the optimization strategy outlined in Kattman et al.\textsuperscript{58}, we have demonstrated that the stage-specific optimization methodology for the efficient generation of cardiac cells also can be used on two more of the most prevalent and accessible hESC lines: H7 and H9. We also verify that coexpression of Flk-1/KDR and PDGFR-α can be used to effectively monitor the emergence of cardiovascular mesoderm without cell sorting, and that the kinetics of differentiation varies between cell lines. Lastly, the results also support the finding that different cell lines require different concentrations of Activin A and BMP4 in order to generate the high numbers of both KDR\textsuperscript{+}/PDGFR-α\textsuperscript{+} cardiovascular mesoderm populations and CMs, reinforcing the importance of quantification of Activin A/BMP4 signaling for cardiac differentiation for individual cell lines.

Interestingly, the optimal yields of KDR\textsuperscript{+}/PDGFR-α\textsuperscript{+} cells in H7 and H9 (24\% and 34\%, respectively, Figure 21 and 22) were lower than those of H1 and HES2 (~60\%) reported by Kattman et al., even though the day 14 differentiated H7 and H9 cells exhibited similar (> 50\%) cTnI expression as H1 and HES2 differentiated cells.\textsuperscript{58} However, the Keller group also noted that although the KDR+/PDGFR+ cells highlight cardiac mesoderm, their expression levels do not always predict efficient differentiation to CMs, but the absence a KDR+/PDGFR+ cell population indicates a lack of CM potential.\textsuperscript{58}

The differentiation of hESCs into CMs has been reported by a number of researchers.\textsuperscript{8, 27, 33, 69} Although several groups have demonstrated the roles for Activin/Nodal/TGFβ, Wnt, and BMP pathways in cardiovascular development\textsuperscript{64, 70-73}, Activin A and BMP4 signaling are the most efficient combination for the generation of cardiac cells from embryonic stem cells\textsuperscript{8, 58, 66}. The first group to identify the value of high density monolayer cultures of hESCs for efficient differentiation of CMs\textsuperscript{8} has recently also published updated protocols using sequential treatment with 100 ng/ml Activin A on day 0 and 10 ng/ml BMP4 on day 1 in H7 hESCs.\textsuperscript{59} This method does not utilize EBs, but rather, plates hESCs at high density on Matrigel from day 0, resulting in 35–40\% Nkx2.5+ cells.

Together with findings from other groups, our results show that CMs differentiation from hESCs can be enhanced using stage-specific culture conditions that involve precise growth factors, most importantly Activin A and BMP4. The differentiation methods described here, and those reported by other groups\textsuperscript{58}, should provide a valuable model system for further investigation of the detailed mechanisms of action by which growth factors act in promoting cardiogenesis in vitro.
Chapter 4. Precise Levels of Fibronectin and Laminin Signaling Promote Highly Efficient Cardiac Differentiation of hESCs through Integrin/FAK/ERK Signaling

4.1 Introduction

CMs differentiated from hESCs are a promising and potentially unlimited cell source for myocardial repair and regeneration. Recently, multiple methodologies – primarily based on the optimization of growth factors - have been described for efficient cardiac differentiation of hESCs. In addition to Wnt, Activin/Nodal, and BMP soluble signaling levels, another important signal mimicking the ‘niche’ of the developing embryo is the extracellular matrix (ECM). However, the role of ECM signaling in CM differentiation has not yet been explored fully. This study examined the role of ECM signaling in the efficient generation of CMs from hESCs.

4.1.1 Extracellular Matrix and Integrin

Differentiation is a continuously regulated process and interactions between the cell and its environment which play a major role in maintaining stable expression of differentiation specific genes. An important component of the cellular environment is the ECM—a network of secreted proteins and carbohydrates that fills the intercellular spaces. It is formed by macromolecules, locally secreted by resident cells. The two main classes of macromolecules are polysaccharide glycosaminoglycans, usually covalently linked to proteins in the form of proteoglycans, and fibrous proteins of two functional types, structural (collagen, elastin) and adhesive (fibronectin, laminin, collagen IV, vitronectin, etc.) (Figure 26).

![Figure 26. An overview of the macromolecular organization of the ECM.](http://219.221.200.61/ywwy/zbsw(E)/edetail4.htm)
In certain connective tissues, such as tendons, cartilage, and bone, the ECM has a mechanical function. During embryogenesis, the ECM influences cell proliferation, differentiation, and migration. Much of the cellular effects of ECM are mediated by the integrin family of cell surface receptors. Once extracellular molecules bind to integrins, changes in the cytoplasmic domain of integrins are triggered, which in turn causes appropriate assembly of the focal contact proteins, for example talin, paxillin, actin and so on to rearrange the cytoskeletal network.

Besides serving as a transmembrane linker between ECM and cytoskeleton, integrins have been shown to transduce biochemical signals across the plasma membrane to regulate various cellular functions.

4.1.2 Integrin Structure

Integrins are a diverse family of integral membrane glycoproteins that function as non-covalently associated αβ heterodimers to mediate cell-cell and cell-matrix interactions in multicellular organisms. The single subunits are formed by a long N-terminal extracellular domain, a transmembrane domain, and a short C-terminal intracellular domain. Sometimes a subunit is formed by two peptides linked extracellularly by a disulfide bond. Different integrins are formed by different permutations of α and β subunits and bind one or more ECM macromolecule(s). On the exterior side of the cell, integrins link an ECM, whereas in the cytosol, they bind the cytoskeleton, thereby forming a membrane bridge between extracellular and intracellular fibers (Figure 27).

In addition to their ability to link cells to their extracellular environment, integrins possess cytoplasmic domains that function in cellular signaling via their ability to associate with and activate signal transduction pathways. Extracellularly, integrins bind to proteins in the matrix as well as to counter receptors on adjacent cells. The binding specificity of an integrin extracellular domain is determined largely by the conformation adopted by the individual α and β chains. To date, 18 α chains and 8 β chains (Figure 28) have been characterized at the molecular level in vertebrates, and many of these proteins exist in multiple isoforms generated by alternative mRNA splicing. The diversity of different combinations of αβ heterodimers supplies a means to direct varied functional roles for the integrins.

4.1.3 Integrin/FAK/ERK Signaling

Three signaling pathways activated by integrin receptors are cytoskeletal organization, cell proliferation and cell survival pathways. Integrins do not themselves possess a kinase domain or enzymatic activity, but rely on specific ECM ligands, such as fibronectin (FN), laminins (LN), various collagens, tenascin, vitronectin and thrombospondin which interact with the actin cytoskeleton at focal adhesions on the cell surface containing localized concentrations of integrins, signaling molecules, and cytoskeletal elements. Focal adhesion kinase (FAK) is a major nonreceptor tyrosine kinase activated after integrin-mediated adhesion to ECM proteins such as FN and LN. Interaction between FAK and integrins results in
autophosphorylation of FAK tyrosine 397 (FAK pY397) that can lead to stimulation of a cell-signaling cascade that ultimately activates the Ras/MAPK/ERK pathway which in turn elicit a cascade of events that lead to cell proliferation, migration, differentiation or survival (Figure 29).  

Figure 27. Integrin, is an αβ heterodimer, in which each subunit is formed by a C-terminal cytosolic domain, a transmembrane domain, and an extracellular domain. The extracellular domain displays the high-affinity binding site for ECM proteins.
Figure 28. Integrin family of adhesion receptors and their main ligands. There are 18 alpha and 8 beta mammalian subunits which assemble to form 24 different heterodimers.  

Figure 29. Signaling pathways initiated by integrins at focal contacts. The 4 major signaling pathways activated by integrin engagement in adhesion complexes are shown. The key element in all these pathways is FAK which becomes activated through autophosphorylation at Y397 and thereby allow binding of Src and Fyn for further phosphorylation and full activation. Plain lines, direct activation or inhibition; dashed line, indirect functional interaction; red lines, FAK-mediated events mediated by specific phosphorylation events.  

(http://www2.unil.ch/cepo_research/introduction.html)
4.1.4 ECM in the Heart

Cardiac ECM is defined as a network surrounding and supporting myocardial constitutive cells: CMs, cardiac fibroblasts, and blood vessels endothelial and vascular smooth muscle cells. The ability to synthesize ECM components differs among cells in the heart. Fibroblasts and smooth muscle cells (SMC) produce and release type I and III collagens and fibronectin (FN); CMs and endothelial cells produce collagen IV. Laminin (LN) is produced by SMC, CMs and endothelial cells (Figure 30). The cardiac ECM consists of three dimensional interstitial collagens—predominantly collagen types I and III—to which other matrix components are attached. CMs are surrounded by a basement membrane consisting collagen type IV, LN, FN, and several proteoglycans (Figure 31). Collagen types I and III maintain structural integrity of the CMs. Other components such as FN and LN also mediate important functions such as healing and remodeling. In the myocardium, ECM is linked to cellular cytoskeleton by integrins. These transmembrane heterodimers provide a physical connection between the cell surface and the surrounding ECM proteins. Integrins are essential for ECM interactions with CM and fibroblasts, and overall structure and cell communication in the heart. 

![Figure 30. Synthesis of principal components of the ECM in different cell types of the myocardium.](image)
Figure 31. Cardiac ECM. (A) Longitudinal cross section of an individual CMs. (B) basement membrane structure surrounding CMs.
(http://herkules.oulu.fi/isbn9514267214/html/x1329.html )

4.1.5 FN and LN in CM Differentiation

The ECM displays an array of macromolecular cues that, not only provide the physical support for cell adhesion, but can also direct cell proliferation, migration and differentiation. Many groups have suggested the role of various ECM molecules in cardiovascular differentiation of hESCs. For example, the presence of FN in 3D collagen constructs stimulated endothelial cell differentiation and vascularization of differentiating EBs, while LN increased the ability of ESCs to differentiate into beating CM clusters. Another group showed that hydrogels, in the absence of supplemental growth factors, containing higher percentages of native cardiac ECM (75%) increased the fraction of cells expressing cardiac marker troponin T compared with lower percentages (25%) of native cardiac ECM. Matigel ECM components have also been implicated in efficient differentiation of CMs by promoting the epithelial-to-mesenchymal transition. However, the 3D collagen gel, cardiac ECM hydrogel, cardiogel, and Matrigel™ matrix sandwich described to promote in vitro cardiac differentiation are mixed biomaterials and can vary from batch-to-batch. Therefore, it is not clear which specific molecules are critical in cardiac fate. We set out to explore combinatorial signaling from the most likely ECM molecules in CM commitment from embryonic stem cells. The FN and LN were chosen, as they are native cardiac ECM components derived from fibroblasts known to contribute to enhanced numbers of spontaneous beating of CMs from mESC.

FN is a major component of the ECM and the cellular basement membrane. It occurs as a thin layer of intercellular ground substance and surrounds blood vessels, together with the collagens and other matrix proteins, separating CMs from each other. It acts as an adhesion protein, promotes cellular migration, influences the organization of tissue in embryogenesis and is required for cardiovascular
development in fish and mice. FN mediates a wide variety of cellular interactions and plays important roles in cell adhesion, migration, growth and differentiation. Costa-Silva, et al., demonstrated that FN promotes neural crest differentiation towards vascular SMCs (Figure 32). Moreover, FN is essential for heart and blood vessel morphogenesis - the absence of which leads to defects in mesoderm and vascular development.

LN, another major ECM component of basement membranes in striated muscle, is critically important for myodifferentiation. It is believed to play a prominent role in promoting myoblast adhesion, migration, proliferation and differentiation. Suzuki, S. et al. showed that hMSCs cultured on LN-coated dishes yielded significantly upregulated genes and protein expression of SMC-specific contractile proteins. Van Dijk et al. showed that differentiation of human adipose-derived stem cells towards CMs is facilitated by LN, especially during late differentiation. In addition, Battista, et al., reported that the presence of LN in 3D collagen constructs stimulated a cardiac pathway and increased the ability of mESCs to differentiate into beating CMs.

Our study examined the role of ECM signaling in the efficient generation of CM from both H7 and H9 hESCs. The hESCs were differentiated on ECM substrates composed of a range of FN and LN ratios and gelatin and evaluated by the FACS analysis on day 14. Of the ECM substrates examined, the 70:30 FN:LN reproducibly generated the greatest numbers of CMs from both hESC lines. Moreover, the LN receptor integrin β4 (ITGB4) and FN receptor integrin β5 (ITGB5) genes, jointly with increased phosphorylated-focal adhesion kinase (p-FAK) and phosphorylated-extracellular signal-regulated kinases (p-ERK), were up-regulated over 13-fold in H7 and H9 cultured on 70:30 FN:LN compared with gelatin. Blocking studies confirmed the role of all these molecules in CM specification, suggesting that the 70:30 FN:LN ECM promotes highly efficient differentiation of CMs through the integrin/FAK/ERK signaling pathway.

Figure 32. Model for the effect of FN on neural crest cell differentiation in vitro. FN stimulates the neural crest cell differentiation into smooth muscle cells and this effect is conserved in mammalian and avian neural crest cells.
4.2 Materials and Methods

4.2.1 hESC Culture and Differentiation

Native H7 hESCs (WA07, WISC Bank) and H9 hESCs - transfected with GFP expression linked to the α-MHC promoter (courtesy of Bernstein Lab, UCSF) - were maintained in MEF-conditioned medium supplemented with 5 ng/ml bFGF (Sigma) and plated on tissue cultured plates coated with Matrigel™ (BD) according to previously published protocols. For CM differentiation, we used our recently published stage-specific optimized protocol for H7 and H9 ESCs. In brief, hESCs were detached using Accutase (Invitrogen), and dissociated into small clumps (3-5 cells). The cells were then spun down and re-suspended in 2ml RPMI (Invitrogen) medium supplemented with 2% B27 (Invitrogen). To generate EBs, a 400-µm Honeycomb Microwell microchip was placed into each well of a standard 24-well plate and 6×10^5 cells were added to the Microwell. Ten µM of ROCK inhibitor (Y-27632; EMD) was added during EB formation to enhance cell survival. After 24 hours, uniform EB were formed in Microwell and then transferred to ultra-low attachment plates (Corning) in RPMI/B27 media. Then EBs were dissociated into small clusters (3-5 cells) by pipetting 1×10^5 cells into individual wells of a 96-well flat bottom plate (BD) coated with various ratios of FN (BD) and LN (BD) and compared against gelatin control (Table 5) for day 5 and 6 days for H7 and H9, respectively. Each 60mm dish was coated to a final concentration of 5µg/cm^2 of total ECM and incubated for one hour before use. The growth factors were added with the following sequence: day 0-1: 2 ng/ml BMP4; day 1-4: 5 ng/ml bFGF, 6 ng/ml Activin A, 30 ng/ml BMP4 for H7 or 5 ng/ml bFGF, 10 ng/ml Activin A, 60 ng/ml BMP4 for H9; day 4-8: 150ng/ml DKK1 and 10ng/ml VEGF; day 8-14, 5ng/ml bFGF and 10ng/ml VEGF. All growth factors were purchased from R&D Systems, Inc.

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Table 5. Composition of six different extracellular matrix substrates for CM differentiation.
4.2.2 Quantitative PCR Microarray

Total RNA was extracted using TRIzol® (Invitrogen), following the manufacturer's instructions. The concentration of RNA was determined using a Nanodrop spectrophotometer (Thermo Scientific). Two μg of RNA was processed with an RT² First Strand Kit (SA Biosciences) according to the manufacturer's specifications. Quantitative PCR analysis of ECM and cell adhesion molecules (CAM) were assessed using the Human Extracellular Matrix and Adhesion Molecules PCR Array (SA Biosciences), RT² SYBR® Green qPCR master mix (SA Biosciences) and an Applied Biosystems 7300 Real-Time PCR System. At least three RNA samples were analyzed per sample.

4.2.3 PCR Microarray Data Analysis

PCR array data were analyzed by the web based RT² Profiler PCR Array Data Analysis from SA Biosciences in the ΔΔCt method with five different housekeeping genes. The array data were plotted on volcano plots in which the x-axis indicates the fold change and the y-axis indicates the statistical reliability of the fold change. Genes significantly up-regulated (p<0.1) in both H7 and H9 differentiated cells were identified.

4.2.4 Functional Blocking Integrin β4/β5 Assays

In order to block the integrin β4 (ITGB4) and integrin β5 receptors (ITGB5), either 10 μg/ml of mouse monoclonal anti-integrin beta 4 antibody (Novus Biologicals) or 10 μg/ml mouse monoclonal anti-integrin beta 5 antibody (Millipore) were added into the differentiation media from day 5-14 for H7, or day 6-14 for H9.

4.2.5 MEK Inhibitor Studies

In order to inhibit the phosphorylation of ERK, 10 μg/ml the MEK inhibitor U0126 (Promega) were added into the differentiation media from day 5-14 for H7, or day 6-14 for H9.

4.2.6 Flow Cytometry and Fluorescent Microscopy

EBs and cardiac cells generated from hESC differentiation experiments were dissociated into single cells by incubation with cell dissociation buffer (enzyme free, PBS-based, Invitrogen). The cells were then fixed with 4% paraformaldehyde (Tousimis Research Corporation) in PBS and permeabilized with 0.7% Triton X100 for 15 minutes, and immunostained with the following antibodies: anti-cTnI (US Biological), anti-Nkx2.5 (R&D), anti-ITGB4 (Millipore), anti-ITGB5 (Novus Biologicals), anti-p-FAK (Santa Cruz Biotech), and anti-p-ERK (Santa Cruz Biotech). Mouse IgG1 and IgG2b (Sigma) were used as isotype controls. A LSRII flow cytometer (BD) was used for data acquisition and data analysis was performed using FlowJo software (Tree Star Inc).
4.2.7 Statistics

All experiments were repeated a minimum of 3 times (N=3). Statistical significance was measured using a Student’s T-test individually between all groups.

4.3 Results

4.3.1 Substrate Signaling in CM Differentiation

In order to investigate the effect of various proportions of FN and LN ECM proteins on CM differentiation, hESCs were cultured in a series of FN- and/or LN-coated plates and compared with a gelatin control (Table 5). Expression of cardiac markers cTnI and Nkx2.5 was assessed by quantitative flow cytometry analysis on day 14 of differentiation. As seen in Figure 33, H7 and H9 cells derived on 70:30 FN:LN substrate gave rise to the greatest percentage of CMs compared to other ratios of FN and LN. H7 monolayer on 70:30 FN:LN consisted of 76.5% ± 4.8% cTnI+ cells and 55.0% ± 4.2% Nkx2.5+ cells, whereas the control H7 monolayer on gelatin contained approximately 50% cTnI+ and 35% Nkx2.5+ cells (* P< 0.01, ** P<0.001, # P< 0.1, ## P<0.01) (Figure 33 A and B). H9 monolayer on 70:30 FN:LN contained over 60% CM, while H9 on gelatin only contained about 50% CM (^ P< 0.1, ^^ P<0.01, & P< 0.1, && P<0.01) (Figure 33 C and D). On day 14, the percentages of Nkx2.5+ cells are consistently lower than the percentage of cTnI+ cells.100 We expect that this is due to the Nkx2.5 expression peaking earlier on day 10, compared with the more mature cardiac marker, cTnI, that is expressed in a later stage.40,101,102 Compared with other ratios of FN and LN, the 70% FN + 30% LN was consistently superior in generating CM, suggesting that FN and LN are both contributing to the cardiogenesis.

4.3.2 Gene Expression of ECM and Adhesion Molecules

In order to further understand the molecules involved in FN/LN signaling, we evaluated the gene expression of using a quantitative RT-PCR microarray that simultaneously analyzes the gene expression of 84 ECM and adhesion molecules. Volcano plots H7 (Figure 34A) and H9 (Figure 34B) compared the human ECM and adhesion molecules gene expression fold change between cells on optimal substrate and cells on gelatin. Fold-Change is the normalized gene expression in the test sample - cells differentiated on the optimal substrate divided the normalized gene expression in the control sample - cells differentiated on gelatin. X-axis indicates the fold change, while the y-axis indicates the p-value of the fold change. Each dot represents the expression of one gene. Dots above the horizontal line show significant difference (P < 0.1). Dots in red represent up-regulated genes, dots in green represent down-regulated genes, while dots in black represent unchanged genes. Based on volcano plots of the array data, 12 genes were significantly up-regulated (P < 0.1) in H7 hESCs and 25 genes were significantly up-regulated in H9 hESCs (Figure 34 and Table 6). Based on the categorization of the gene products; 5
out of 12 of the up-regulated genes in H7-derived CMs and 8 out of 25 of the up-regulated genes in H9-derived CMs were transmembrane molecules. The remaining up-regulated genes were adhesion molecules (3 and 4); ECM proteases (1 and 5); and cell-cell adhesion molecules (1 and 4) in H7 hESCs and H9 hESCs, respectively. Based on these data, we deduced that the transmembrane molecules may play the most important roles for directing CM fate.
Figure 33. Percentage of cardiac cells from H7 and H9 hESC on different substrates at day 14. H7 (A and B) differentiated cells cultured on 70:30 FN:LN contained highest percentage of (A) cTnI+ (* P< 0.01, ** P<0.001) and (B) Nkx2.5+ cells (# P< 0.1, ## P<0.01). H9 (C and D) differentiated cells cultured on 70%:30% FN:LN contained highest percentage of (C) cTnI+ (^ P< 0.1, ^^ P<0.01) and (D) Nkx2.5+ cells (& P< 0.1, && P<0.01).
Figure 34. Human ECM and adhesion molecules gene expression of cells cultured on 70:30 FN:LN compared with gelatin. Volcano plots H7 (A) and H9 (B) compared
the human ECM and adhesion molecules gene expression fold change between cells on optimal substrate and cells on gelatin. X-axis indicates the fold change, while the y-axis indicates the p-value of the fold change. Each dot represents the expression of one gene. Dots above the horizontal line show significant difference (P < 0.1). Dots in red represent up-regulated genes, dots in green represent down-regulated genes, while dots in black represent unchanged genes.

Looking more closely at the specific genes up-regulated in both H7 and H9 derived-CMs cultured on 70:30 FN:LN compared with 100% gelatin, we find 7 genes (CNTN1, COL6A2, CTGF, ITGAM, ITGB4, ITGB5 and MMP8) in common when we set the p-value = 0.1, but only 4 genes (CNTN1, CTGF, ITGB4 and ITGB5) in common when we set the p-value = 0.01 (Table 6). Two of these are integrin transmembrane molecules: ITGB4, a LN receptor and ITGB5, a FN receptor. Because these integrins exhibited much higher fold changes than CNTN1 and CTGF in both H7 and H9 ESCs, the effects of ITGB4 and ITGB5 on CM differentiation were selected for further investigation.

4.3.3 Quantification of ITGB4, ITGB5, p-FAK, and p-ERK Positive Cells

The ITGB4 and ITGB5 protein expression in H7 and H9 differentiation cultures on the optimal substrate and gelatin were first analyzed by flow cytometry analysis. The H7 differentiated on the optimal substrate contained 21% ITGB4 and 31% ITGB5 on day 14; whereas the same cells on the gelatin contained 6% ITGB4 and 10% ITGB5 (Figure 35A). Similarly, H9 hESC differentiating cells on the FN/LN substrate contained higher numbers 27% of ITGB4 positive cells and 44% ITGB5 positive cells versus 12% and 13%, respectively, when derived on gelatin (Figure 35B). Evidence of downstream integrin signaling was also investigated by analyzing the phosphorylation of both FAK and ERK in cells differentiated on the 70:30 FN:LN compared with gelatin. The significantly increased numbers of p-FAK (25% vs. 9% in H7; 27% vs. 12% in H9) and p-ERK (23% vs. 15% in H7; 31% vs. 8% in H9) (* P<0.1, ** P<0.01, and *** P<0.001) were detected in hESCs differentiated on the optimal substrate compared with gelatin, respectively (Figure 35).

4.3.4 Blocking ITGB4/ITGB5 Signaling Reduces CM Differentiation

In order to verify the role of ITGB4 and ITGB5 signaling in CM differentiation, cells were treated with ITGB4 and/or ITGB5 blocking antibodies starting from day the cells were plated down on as a monolayer (optimized as day 5 and 6 for H7 and H9, respectively) through day 14. ITGB4 blocking antibody treatment reduced cTnI positive cells in H7 hESCs and H9 hESCs by 50% and 45% respectively, and reduced Nkx2.5 positive cells in H7 hESCs and H9 hESCs by 74% and 64% respectively (Figure 36). ITGB5 blocking antibody treatment reduced cTnI and Nkx2.5 expression in H7 and H9 by 83~89% (Figure 36). Blocking both ITGB4 and ITGB5 simultaneously almost completely eliminated the CM derivation in these
culture, reducing cTnI and Nkx2.5 expression in both H7 and H9 by 92~97% (Figure 36). Moreover, the 79%~91% reduction (#, *, &, ^ P<0.1, ** P<0.01, and *** P<0.001) of p-FAK expression in cells treated with ITGB4 and/or ITGB5 inhibitors suggests that blocking the integrin signaling leads to modulation of FAK signaling as well.

4.3.4 The Effects of MEK Inhibitor U0126 on CM Differentiation

To verify the significance of the ERK signaling pathway, a downstream effector of FAK, in regulating CM differentiation of hESCs, we cultured the cells in the presence of the MEK inhibitor U0126 to inhibit the phosphorylation of ERK starting on day the cells were plated down on as a monolayer (day 5 and 6 for H7 and H9, respectively) through day 14. The flow cytometry data confirm that the U0126 reduced the p-ERK positive cells from 23% to 4% in H7 ESCs and from 31% to 2% in H9 hESCs (Figure 37). In addition, direct suppression of ERK by U0126 treatment significantly decreased the cTnI positive cells to only 8% and 2% in H7 and H9, respectively (Figure 37). The Nkx2.5 expression were reduced to 4% and 2% (** P<0.001) in H7 and H9 treated with U0126 respectively as well (Figure 37), indicating that ERK also plays a critical role in integrin signaling during cardiac specification.

4.4 Conclusions and Discussion

Stem cell fate choices, including the regulation of cardiac structure and function of individual cells and tissues, have been shown to be critically impacted by cell-matrix interactions. Therefore, we hypothesized that the ECM signaling from niche-appropriate molecules, FN and LN, play a role in hESCs specification towards CMs. By investigating the effects of various ratios of FN and LN, we found that hESCs differentiating on 70:30 FN:LN generated a greatest percentage (~60-70%) of CMs compared with other ratios of FN:LN or gelatin (~50-60%). Because the activation of FAK/ERK-induced differentiation of a variety of non-CM cell types is also triggered by the binding of FN and LN, we used a PCR array to highlight the transmembrane molecules playing a dominant role in ECM-induced CM differentiation. Specifically, the expression of FN receptor, ITGB5, and LN receptor, ITGB4, were found to be significantly increased in both H7 and H9 hESCs, and blocking these 2 molecules confirmed their role in generating CMs. The larger impact of ITGB5 blocking compared with ITGB4 blocking (Figure 36) is also consistent with the greater amount of the ITGB5 ligand, FN, in the FN/LN combined substrate. Similar to the importance for optimization of the Activin A/BMP4 signaling levels in CM differentiation, we show here that a precise balance of FN and LN can also improve the differentiation efficiency of CM differentiation from hESCs.

One of the primary mechanisms for ECM signaling is the intracellular signal transduction pathway activated following the binding of ECM molecules to integrin receptors on the cell. The interplay between ECM components and integrin receptors
regulate signal transduction in cells by first associating with FAK, and subsequently initiating several downstream signaling events, including activation of ERK cascades that are known to regulate many developmentally relevant processes including cell fate. The ITGB4, ITGB5 and ERK functional blocking studies confirmed that activation of integrin/FAK/ERK signaling pathways are essential for the induction of CMs differentiation from hESCs. These combined data suggest that the 70:30 FN:LN ECM activate integrin signaling pathways resulting in phosphorylation of FAK and then elicit a MEK/ERK signaling pathway to promote CM (Figure 38).

Although this study focused on investigating the ITGB4 or ITGB5 integrin signaling, the PCR array results also suggest that adhesion molecules CNTN1 and CTGF are involved in cardiomyocyte differentiation. CNTN, a ligand for Notch, has been shown to promote the oligodendrocyte maturation. The Notch pathway is also a crucial cell-fate regulator for proper heart development, suggesting the CNTN 1 might have a functional role in promoting CM fate through Notch signaling pathway. Additionally, while CTGF has been described to induce the differentiation of many cells, a role in CM specification has not yet been suggested.

Table 6. Up-regulated human ECM and adhesion molecule genes in differentiation culture on the optimal substrates 70:30 FN:LN at Day 14. Twelve genes were up-regulated in H7 (A) and twenty-five genes were up-regulated in H9 (B) significantly. Fold-Change is the normalized gene expression in the test sample - cells differentiated on the optimal substrate divided the normalized gene expression in the control sample - cells differentiated on gelatin. The p-values of fold changes are lower than 0.1.

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A) Bar chart showing the expression levels of ITGB4, ITGB5, p-FAK, and p-ERK in 70:30 FN:LN and Gelatin conditions.

B) Bar chart showing the expression levels of ITGB4, ITGB5, p-FAK, and p-ERK in 70:30 FN:LN and Gelatin conditions.
Figure 35. Substrate signaling from 70:30 FN:LN compared with gelatin increased the number of p-FAK, p-ERK, ITGB4 and ITGB5 positive cells. Percentages of p-FAK, p-ERK, ITGB 4 and ITGB 5 expressing cells from (A) H7 and (B) H9 hESCs differentiating on 70% fibronectin+30% laminin were higher than those of cells on gelatin at day 14. (* P<0.1, ** P<0.01, and *** P<0.001).

Figure 36. Blocking ITGB 4 and/or ITGB 5 reduced cardiac markers and p-FAK expression in H7 (A) and H9 (B) differentiation culture on the optimal substrate. cTnI and Nkx2.5 and p-FAK expression of normal differentiation culture were much higher than differentiation culture treated with ITGB 4 and/or ITGB 5 blocking antibodies at day 14. (#, *, &, ^ P<0.1, ** P<0.01, and *** P<0.001).
Figure 37. Blocking p-ERK inhibited cardiomyocyte differentiation from H7 hESCs (A) and H9 hESCs (B). cTnI, Nkx2.5 and p-ERK expression of normal differentiation culture were much higher than differentiation culture treated with MEK inhibitor U0126 at day 14 (** P<0.001).

Figure 38. Schematic drawing summarizes the role of optimal ECM in mediating CMs differentiation from hESCs. The optimal ECM (70:30 FN:LN) activates integrin, FAK and ERK pathways and, in turn, leading to the specification of CM differentiation from hESCs.
Chapter 5. Conclusions

The heart is one of the least regenerative organs in the body. The prevalence of heart failure worldwide continues to increase. It has high rates of morbidity and mortality, imposing enormous human, social and economic costs.\(^3\) The death of CMs, which is irreversible, can often lead to the development of progressive heart failure. The average left ventricle contains approximately 4 billion CMs, but a heart with infarction-induced heart failure often has only one billion functioning CMs. For many researchers, the idea of addressing this deficit with a stem cell-based therapeutic approach is highly attractive because they can be isolated and maintained by well-established protocols, expanded in culture, and differentiated into definitive CMs. In addition, ESC derived CMs have robust proliferative capacity both in vitro and after implantation, implying that delivery of an initially therapeutic cell dose may suffice to obtain a functionally meaningful cardiac implant over time. Therefore, derivation of CMs from ESCs would be a boon for treatment of the many millions of people worldwide who suffer significant cardiac tissue damage. There is an intensive effort to develop stem cell–based strategies for cardiac repair and several lines of evidence support the concept that new endogenous or exogenous cells can incorporate and become functional within the heart.\(^2^1\) However, this treatment has been limited by inefficient differentiation of hESCs into CMs. The spontaneous differentiation efficiency is lower than 1% from hESCs, but approximately 1-5 million cells per injection are required for administration for each patient.\(^3^0, 3^1\) Therefore, increasing the yield of CMs differentiated from hESCs is an important step for stem-cell based therapy for cardiac repair. To direct the differentiation of hESCs into CMs, it is critical to identify the inductive effect of various cues. hESCs differentiation is strongly influenced by physical and chemical signals comprising the local extracellular microenvironment, including EB aggregation, soluble factor signals, and cell-extracellular ECM interactions and cell–cell interactions. The goal of the work in this thesis has focused on optimizing the homogeneous EB formation methods, followed by stage- and cell line-specific Activin A/BMP4 Signaling and ECM signaling for highly efficient cardiac differentiation.

First, collaborator, Michelle Khine (UCI) designed and provided an ultra-rapid fabrication and culture method utilizing a laser-jet printer to generate closely arrayed Honeycomb Microwells of tunable sizes for the induction of uniform EBs from single cell suspension. By printing various microwell patterns onto pre-stressed polystyrene sheets, and through heat induced shrinking, high aspect micromolds are generated. Furthermore, by simply controlling the size of the microwells and the concentration of the cell suspension we can control the initial size of the cell aggregate, thus influencing lineage commitment. In addition, these microwells are easily adaptable and scalable to most standard well plates and easily integrated into commercial liquid handling systems to provide an inexpensive and easy high throughput compound screening platform.
I then used this technology to compare EBs generated using our Honeycomb Microwells with the commercially available AggreWell™ 400, and the more traditional Hanging Drop method. I compared the efficiency, viability, quality, and control of EB sizes. Results indicate that the Honeycomb Microwell and AggreWell™ 400 efficiently generate uniform EBs at approximately 500 cells per EB. However, the cone-bottomed AggreWell plate generates cone-shaped EBs at 1000-2000 cells per EB. Moreover, the cone-shape correlates with a reduction in the formation of the primitive endoderm GATA-4+ cells, but does not significantly affect mesoderm or ectoderm development. We have shown that the non-spherical EB shape correlates with a reduction in the development of primitive endoderm, and that these AggreWell plates should be avoided in deriving endoderm tissue products.

Most importantly, the Honeycomb Microwell and AggreWell plates are able to generate EBs from single hESCs with greater efficiency than conventional Hanging Drop Methods. This is a significant advancement in the field, as the generation of human EBs from single cells is notoriously challenging. We expect the success of these well plates in generating EBs from single cells is due to the physical aggregation of individual cells that bring the cells into closer contact with one another compared with a Hanging Drop.

Secondly, we verify a published methodology that required proper balance of the Activin A and BMP4 signaling for highly efficient cardiac specification and investigated the cardiac differentiation in the H7 and H9 hESC lines, two of the most utilized hESC lines that had not yet been optimized. Our studies examined a range of time points for the initial culture of hESCs as EBs formed via Honeycomb Microwell prior to transfer to monolayer culture, as well as, concentrations of Activin A and BMP4 in the medium formulations. The results indicate an efficient protocol for reproducibly generating cultures with approximately 50% CMs from H7 and H9 hESC lines.

To date, the most of the efficient cardiac differentiation methodologies have been primarily based on the optimization of growth factors. The role of ECM signaling in CMs differentiation has not yet been thoroughly explored. Therefore, we examined the role of ECM signaling in the efficient generation of CM from both H7 an H9 hESCs. The hESCs were differentiated on ECM substrates composed of a range of FN and LN ratios and gelatin and evaluated by the FACS analysis on day 14. Of the ECM substrates examined, the 70:30 FN:LN reproducibly generated the greatest numbers of CMs (70~80%) from both hESC lines. Moreover, the LN receptor ITGB4 and FN receptor ITGB5 genes, jointly with increased p-FAK and p-ERK, were up-regulated over 13-fold in H7 and H9 cultured on 70:30 FN:LN compared with gelatin. Blocking studies confirmed the role of all these molecules in CM specification, suggesting that the 70:30 FN:LN ECM promotes highly efficient differentiation of CMs through the through activation of ITGB4, ITGB5 and integrin/FAK/ERK signaling pathway.

As a summary, using Honeycomb Microwell to generate a large number of homogenous EB, precisely balanced Activin A/BMP4 concentration for each cell
line and a substrate containing a 70:30 FN:LN (Figure 39) promotes the highly efficient generation of CMs (70%~80%) for stem-cell therapy for cardiac repair. These hESC derived CMs will be enriched and tested for their ability to enhance cardiac function in preclinical animal models and for utility in drug discovery for future study.

Figure 39. Final scheme for highly efficient generation of CMs from H7 (B) and H9 (C) hESCs.
References

34. Takei, S. et al. Bone morphogenetic protein-4 promotes induction of cardiomyocytes from human embryonic stem cells in serum-based embryoid


