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Los Angeles

Generation of Cardiomyocytes from Human Endogenous and Pluripotent Stem-Cell Derived Endothelial Cells

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Raymond Truong

2013

ABSTRACT OF THE THESIS

Generation of Cardiomyocytes from

Human Endogenous and Pluripotent Stem Cell-Derived Endothelial Cells

by

Raymond Truong

Master of Science in Physiological Science

University of California, Los Angeles, 2013

Professor Alan Garfinkel, Chair

Ischemic damage from a myocardial infarction can have dramatic negative effects on the heart's ability to adequately perfuse the body, due to the irreversible loss of over a billion cardiomyocytes responsible for ventricular contraction. Regenerative therapies utilizing multipotent cardiac progenitor cells (CPCs) derived from either human embryonic (hESCs) or induced pluripotent stem cells have gained popularity as potential alternatives to someday replace expensive, high-risk medical procedures. Previous studies identified multipotent progenitors localized in the endocardial cushions of the developing mouse heart that co-expressed CD31 and PDGFRα. Additionally, recent data in mice have shown that the loss of a single transcription factor, Scl, enabled robust cardiomyogenic differentiation from endothelium

in the heart, resulting in the ectopic appearance of these progenitor cells. However, no studies have shown similar findings in humans.

The purpose of this study is to identify these $CD31^+/PDGFR\alpha^+$ cells in the human heart and generate cardiomyocyte-like cells from hESC-derived endothelium. It is hypothesized that human endogenous and embryonic stem cell-derived endothelial cells possess unrealized cardiomyogenic potential. Stained sagittal sections of week 9, 15, and 17 human fetal hearts showed the co-expression of the cell surface markers PDGFRα and CD31 on cells surrounding the ventricular vasculature. In addition, a 5-step protocol was used to differentiate hESCs into cardiomyocyte-like cells through an endothelial cell intermediate. Yield of isolated mesodermal progenitor cells was approximately 10.5%. Under the modified sorting protocol, yield of the mesodermal progenitor cells was approximately 18.6%. The subsequent cell sort yield for $CD31^+$ endothelial cells was approximately 4.8%. Under the modified protocol with and without the addition of lithium chloride, endothelial cell yields were 21.3% and 26.6% respectively. The hESC-derived endothelial cells showed the formation of blood tubes *in vitro* and, once in cardiac growth medium, expressed Troponin T under fluorescence microscopy. Based on these results, this population of $CD31^+/PDGFR\alpha^+$ cells could represent a latent population of cardiac progenitors capable of differentiating into cardiomyocytes.

The thesis of Raymond Truong is approved.

Patricia Emory Phelps

James N. Weiss

Alan Garfinkel, Committee Chair

University of California, Los Angeles

2013

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List of Abbreviations

bFGF = Basic Fibroblast Growth Factor 2

BSA = Bovine Serum Albumin

BVF-A medium = Differentiation medium containing BMP4, VEGF, bFGF, and Activin A

CPCs = Cardiac Progenitor Cells

DMEM = Dulbecco's Modified Eagle Medium

EDTA = Ethylenediaminetetraacetic acid

EGM = Endothelial Growth Medium

EMT = Endothelial-to-Mesenchymal Transition

FACS = Fluorescence Activated Cell Sort

FBS = Fetal Bovine Serum

hESCs = Human Embryonic Stem Cells

hiPSCs = Human Induced Pluripotent Stem Cells

HRP = Horseradish Peroxidase

MPCs = Mesodermal Progenitor Cells

MEFs = Mouse Embryonic Fibroblasts

MEM-α = Minimum Essential Medium, Alpha Modifications

PBS = Phosphate Buffered Saline

PDGFRα = Platelet-Derived Growth Factor Receptor Alpha

PFA = Paraformaldehyde

TGF-β = Transforming Growth Factor Beta

VEGF = Vascular Endothelial Growth Factor

VEGFR = Vascular Endothelial Growth Factor Receptor

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Chapter 1: Introduction

Heart Failure: A Public Health Concern

Heart failure is a growing problem in industrialized nations all over the world. According to the Centers for Disease Control and Prevention in 2011, approximately 26.5 million adults have diagnosed heart disease in the United States alone. In addition, cardiovascular diseases account for approximately one-third of all deaths worldwide (Hong, 2009). Despite the large amount of funds spent on treatments, some predict that heart failure, or the inability of the heart to properly perfuse the tissues and organs of the body, could become a global epidemic.

Most causes of heart failure are a result of damaged or defective cardiomyocytes, the cells of the heart responsible for ventricular contraction. Myocardial infarctions, for example, can potentially destroy a billion of these cardiomyocytes, drastically reducing the pumping power of the heart (Laflamme and Murry, 2011). Thus, various research groups are attempting to develop therapies that mitigate or replace these lost or dysfunctional cardiomyocytes. Cell regenerative therapies, utilizing stem cells for example, have gained popularity as potential treatments for a wide variety of cardiac diseases.

Human Embryonic Stem Cells: Endless Possibilities

As a result of the growing rate of heart failure, various scientists have aimed their research at maintaining or increasing the number of these cardiomyocytes through cell replacement therapies. One strategy is through the use of pluripotent human embryonic stem cells (hESCs), which have the innate ability to differentiate into all three embryonic germ layers

of the human body (Thomson et al., 1998). In effect, the discovery of hESCs has opened new possibilities for medical treatments in a wide variety of illnesses. Because hESCs have the potential to become any tissue or organ, their manipulation in biomedical research has become very popular. Specifically, scientists from various fields of research are attempting to differentiate hESCs into specific cell types, such as cardiomyocytes, with the goal of engrafting these differentiated cells into damaged tissue. Ideally, these engrafted cells would restore proper function to damaged organs by integrating into the host tissue and function together with their endogenous counterparts.

Several labs have had success in differentiating hESCs into cell types of all three embryonic germ layers. For example, human oligodendrocyte-like progenitor cells were differentiated from hESCs and shown to remyelinate injured spinal cords of adult rats. In addition, treated rats showed significantly improved motor function compared with control rats (Keirstead et al., 2005). As another example, hepatocytes were differentiated from hESCs and injected into damaged livers of immunosuppressed mice. After four weeks, harvested livers showed integration of the hESC-derived cells into host liver tissue. These results were shown through immunohistochemistry analysis for the expression of donor-specific hepatocyte markers (Agarwal et al., 2008).

Although hESCs have shown a potent innate potential to differentiate into different cell types of the human body, the derivation and clinical use of hESCs are ethically and medically complicated. To isolate these cells, the inner cell mass of a human embryo is removed, which effectively destroys the embryo and any potential for it to form a mature human being (Shand et al., 2012). Political debates within the last decade have focused on whether or not embryonic stem cell research is ethically justified. These discussions have had large negative impacts on

federal funding for hESC-related projects. The most dramatic impact was when President George W. Bush imposed strict limitations on federal funding for research using newly-derived hESCs (Parker, 2006). Although the current Obama administration has relaxed Bush-era funding limitations, ethical debates still continue as to whether embryonic stem cell research is justified.

Human Induced Pluripotent Stem Cells: A Potential Clinical Alternative

In 2007, the lab of Shinya Yamanaka was able to reprogram adult human fibroblasts back into a pluripotent state. These embryonic-like human induced pluripotent stem cells (hiPSCs) had similar characteristics, morphologies, and behaviors as hESCs (Takahashi et al., 2007). One advantage to the use of hiPSCs over hESCs is the potential clinical application through the development of autologous, patient-specific cell replacement therapies. The risks of a patient mounting an immune response against hiPSC treatments are thought to be very low or near zero due to the manipulation of the patient's own cells.

However, previous research has suggested that although hiPSCs may resemble hESCs, there are several epigenetic differences between the two. Generalized DNA methylation patterns between all pluripotent stem cells showed similar CG DNA methylation patterns. However, comprehensive analyses by Lister et al. (2011) revealed approximately 1,175 differentially methylated regions in comparisons with at least one hiPSC or hESC cell line. These results are thought to be due to various somatic DNA methylation patterns being resistant to reprogramming (Lister et al., 2011). Despite exhibiting embryonic-like qualities, hiPSCs may still retain somatic characteristics that could interfere with differentiation studies and hamper the effectiveness of future cell replacement therapies.

Thus, before hiPSCs are used in clinical treatments, current reprogramming techniques need to be refined and perfected to decrease the number of leftover epigenetic markers.

Cardiac Progenitor Cells: Resident Multipotent Cell Populations

Besides hESCs and hiPSCs, regenerative therapy research has also focused on manipulating endogenous populations of multipotent stem cells, or stem cells with limited differentiation potential, into desirable cell types. One such specific population is found in the heart called cardiac progenitor cells (CPCs). These CPCs typically are only able to differentiate into the specific cell types found in the heart: cardiomyocytes, smooth muscle cells, and endothelial cells. Although unable to fully differentiate into cells of all three embryonic germ layers, these CPCs, once differentiated into cardiomyocyte-like cells, are thought to be better candidates to integrate into heart tissue and function like endogenous cardiomyocytes, due to the CPCs' developmental commitment to the cardiac lineage.

Recent research has attempted to identify CPC-specific markers to potentially isolate and culture them *in vitro*. The transcription factor islet-1 (Isl1), for example, has been suggested to be an intracellular marker of CPCs in several animal species ranging from mouse to human. These $Isl1^+$ cardiac progenitor cells have the ability to differentiate into specific cardiac cell types, such as pacemaker cells, smooth muscle, and endothelial cells and may represent one of the earliest identified cells to diversify into the cardiac lineage (Moretti et al., 2006). Another marker, the homeobox protein Nkx2-5, has also been recognized as an early marker of precardiac cells (Durocher et al., 1997). Together, these two markers have been shown to be important in the early development of the heart.

However, despite the amount of research identifying CPCs that express the cardiac markers Isl1 and Nkx2-5, different isolation techniques are needed to translate the research to the clinic. Both Isl1 and Nkx2-5 are intracellular markers, which require the disruption of the cell membrane to analyze their expression. Thus, the identification of cardiac-specific cell surface markers has become vital in advancing research of translational therapies.

CD31 and PDGFRα: Candidate Surface Markers

Several labs have published research on various combinations of cell surface markers that are believed to label cardiac progenitor cells. For example, the cell surface markers VEGFR1 and VEGFR3 (Flt1 and Flt4, respectively) were found to label mouse cell populations that were also enriched for Isl1 and Nkx2-5, the intracellular markers previously shown to label CPCs. These $Flt1^{+}$/ $Flt4^{+}$ cells were also shown to differentiate into the three cell lineages of the heart, specifically cardiomyocytes, smooth muscle cells, and endothelial cells (Nsair et al., 2012). Other examples include the cell surface markers vascular cell adhesion molecule 1 (VCAM1) and signal receptor protein α (SIRPA), which have been shown to label cardiomyocytes (Elliott et al., 2011).

Recent research in mice has also identified a set of new CPC cell surface markers that emerge from cardiac endothelium. During cardiac development, a subset of the endothelium called the endocardial cushions undergo a process called endothelial-to-mesenchymal transition (EMT), in which the cushion cells lose their stationary epithelial characteristics and gain mobile mesenchymal characteristics (von Gise and Pu, 2012). These mesenchymal cells migrate towards the interior of the heart to eventually form the cardiac valves and septa of the heart (de Lange et al., 2004).

Previous studies have shown that the embryonic endothelium is a source for the outgrowth of hematopoietic progenitor cells, which are multipotent stem cells that contribute to the development of blood cells, in a process very similar to EMT (Bertrand et al., 2004). The development of this hemogenic endothelium, or endothelium with the ability to generate hematopoietic cells, was shown to be under the control of the transcription factor Scl. In addition, Scl was shown to direct mesoderm to differentiate into hemogenic endothelium (Lancrin et al., 2009). Interestingly, Scl was also found to silence cardiomyogenesis, as transgenic mice without Scl showed the development of cardiogenic cells expressing CD31 and PDGFRα (Van Handel et al., 2012), an endothelial cell surface marker (Newman, 1994) and a cardiogenic mesoderm marker (Kattman et al., 2011) respectively. These $CD31^{+}$/$PDGFR\alpha^{+}$ cells, based on their gene expression profile and location in the developing heart, were believed to also be precursors to the cardiac cushion mesenchymal cells (Van Handel et al., 2012).

These $CD31^{+}$/$PDGFR\alpha^{+}$ cells represent a potentially new type of CPC that could be manipulated and used for regenerative therapies. However, these cells were only identified in mouse and have not been identified in humans. In addition, previous studies have not shown the development of cardiomyocytes from endothelium. Because of this, the goal of the current study is to identify whether these cells exist in the human heart and whether stem cells could be manipulated to differentiate into cardiomyocytes through an endothelial cell intermediate.

Chapter 2: Materials and Methods

Tissue Immunofluorescent Labeling

Week 13 and week 15 fetal human hearts are collected, embedded in paraffin, sectioned, and mounted by the UCLA Translational Pathology Core Laboratory. Samples were treated with increasing concentrations of xylene to dissolve the paraffin and rehydrated with decreasing concentrations of ethanol in water. Antigen retrieval was then performed by exposing samples to a 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% Tween-20 solution, followed by a 10 mM citrate solution at 95° C for 10 minutes each. The samples were immersed in a 1% hydrogen peroxide/PBS solution to quench endogenous peroxidases for 20 minutes at room temperature in the dark.

Sample sections were incubated in a tyramide blocking buffer (Life Technologies, Carlsbad, CA) for one hour at room temperature to block nonspecific binding of the antibodies. Mouse anti-human CD31 primary antibody (Santa Cruz Biotechnology, Dallas, TX) was added to tyramide blocking buffer at a 1:250 dilution and the samples were incubated in the buffer at 4° C overnight. The samples were washed with PBS the next day and subsequently incubated in rabbit anti-mouse biotinylated secondary antibody (Vector Labs, Burlingame, CA) diluted in tyramide blocking buffer for 30 minutes and then in streptavidin-HRP (Life Technologies) for another 30 minutes. To amplify the labeling, the samples were incubated in biotin-XX tyramide (Life Technologies) for 10 minutes followed by incubating the samples in a fluorescent Alexa Fluor 488 tyramide (Life Technologies).

To identify PDGFRα antigen expression, rabbit anti-human PDGFRα primary antibody (Santa Cruz Biotechnology) was added to tyramide blocking buffer at a 1:4000 dilution and the

samples were incubated in the buffer at 4° C overnight. The samples were then washed with PBS and subsequently incubated in mouse anti-rabbit biotinylated secondary antibody (Vector Labs) diluted in tyramide blocking buffer for 30 minutes and then in streptavidin-HRP for another 30 minutes. The samples were incubated in a fluorescent Alexa Fluor 594 tyramide, washed, and then immersed in a 0.02N HCl/water solution at pH 2.0 for 20 minutes to quench peroxidases.

Sample sections were also labeled for intracellular Troponin T. This protocol required cell membrane permeabilization by incubating the sections in a 1% Triton X-100/PBS solution for 10 minutes at room temperature. Nonspecific binding of the antibody was blocked by incubating the samples in a 1% BSA/0.25% Triton X-100/PBS buffer for 30 minutes. Mouse anti-human Troponin T primary antibody (Sigma-Aldrich) was added to the blocking buffer at a 1:400 dilution and the samples were incubated in the buffer overnight at 4° C. Sections were then incubated in a mixture of goat anti-mouse Alexa Fluor 647 secondary antibody (1:400, Life Technologies) in blocking buffer for 1 hour at room temperature. The sample slides were counterstained with DAPI (1 μg/mL, Life Technologies) for 1 minute, washed three times with PBS, mounted with a drop of Prolong Gold antifade reagent (Life Technologies), coverslipped, and sealed with nail polish. The triple-labeled slides were visualized under a Zeiss Axio Imager A1 microscope.

Because both CD31 and Troponin T primary antibodies were derived from mouse, we performed control experiments to test the effectiveness of the peroxidase quenching. The quenching step inactivates the mouse anti-human CD31 primary antibody. After samples were processed for CD31 and PDGFRα expression, the experimental groups received the mouse anti-human Troponin T primary antibody, followed by the goat anti-mouse Alexa Fluor 647

secondary antibody. The control group only received the secondary antibody. Specific reactivity was seen in the experimental group while no evidence of reactivity was seen in the control group.

hESC Culture and Maintenance

The H9 line of hESCs was received from the UCLA Broad Stem Cell Research Center Stem Cell Core. hESC medium containing DMEM/F12 (Life Technologies), 20% knockout serum replacement (Life Technologies), 1% Glutamax (Life Technologies), 0.0007% 2-mercaptoethanol (Sigma-Aldrich), 10 ng/mL bFGF (Peprotech, Rocky Hill, NJ), and 1% non-essential amino acids (Life Technologies) was changed daily. After 4 days when hESC colonies were approximately 60-70% confluent, two new gelatin-coated 6-well plates were prepared for passaging by plating a feeder layer of MEFs (2 million MEFs per 6-well plate). The hESCs were then passaged the next day at a 1:2 ratio of 6-well plate. The ROCK inhibitor Y-27632 (Millipore, Billerica, MA) was added to hESC medium during the passage to promote cell attachment. The passaging method involved manually cutting cell colonies using a flat-bottom glass pipette (Fisher Scientific, Waltham, MA) into smaller sizes consisting of anywhere between 100 to 500 hESCs. Cells are scraped, centrifuged, and redistributed to new MEF feeder plates and left undisturbed overnight at 37° C.

hESC-Derived $CD56^+$ Mesodermal Progenitor Cell Differentiation

This protocol was developed and published by Evseenko et al (2010). In general, once H9 hESCs reached approximately 70% confluency, 6-well plates were coated with a 1:30 dilution of Matrigel (Fisher Scientific) in ice-cold PBS to prepare for plating hESCs in feeder

free conditions. The hESCs were manually passaged as previously stated and transferred to the Matrigel-coated 6-well plates undiluted in mTeSR medium supplemented with the included 5x supplement (StemCell Technologies, Vancouver, Canada). After approximately 2-3 days in feeder free conditions, the H9 hESCs underwent the MPC differentiation protocol, which involves exposure of the hESCs day 1 to A-BVF medium, which consisted of X-Vivo 15 (Lonza, Basel, Switzerland), 10 ng/mL Activin A (R&D Systems), 10 ng/mL BMP4 (R&D Systems, Minneapolis, MN), 10 ng/mL VEGF (R&D Systems), and 10 ng/mL bFGF (Peprotech). During days 2-3.5, A-BVF medium was replaced with BVF medium, which is the same as A-BVF without Activin A. On day 3.5, the differentiated cells are sorted by FACS.

Fluorescence Activated Cell Sorting of $CD56^+$ MPCs

Cells are lifted from culture plates by incubation in 0.05% Trypsin-EDTA (Life Technologies) at 37°C for approximately 5-10 minutes. The cell suspension is filtered through a 70 micron cell strainer to dissociate cell colonies, counted using a hemocytometer, and then centrifuged at 300 *g* for 5 minutes. The supernatant is discarded while the resultant cell pellet is resuspended in approximately 1 mL of a 1% FBS in PBS solution (Life Technologies), which nourishes cells during the staining preparation. Three small aliquots containing 500,000 cells were removed for the unstained and single-color controls.

The cell solutions were then stained by addition of fluorophore-conjugated antibodies – 40 µL of PerCP Cy5.5-conjugated CD326 and 40 µL FITC-conjugated CD56 antibodies (BioLegend, San Diego, CA) – to the cell sort sample, as well as to each single-color control aliquots. The cell suspension was allowed to remain for at least 20 minutes on ice in the dark. Afterwards, 1% FBS in PBS was added to the cell solution to dilute and remove excess antibody.

The cells were again centrifuged at 300 *g* for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in approximately 1.5 mL 1% FBS in PBS. The cell suspension was again filtered through a 45 micron cell strainer-capped FACS tube (Fisher Scientific). Finally, the cells were sorted for $CD56^{+}/CD326^{-}$ using the BD FACSAria cell sorter at the Broad Stem Cell Research Center Flow Cytometry Core Laboratory.

Modified Preparation for the Fluorescence Activated Cell Sort of $CD56^{+}$ MPCs

Approximately 1 hour before differentiated cells are passaged, the BVF medium is replaced with 1 mL/well of BVF medium supplemented with 10 ng/mL Y-27632 ROCK inhibitor. After 1 hour, cells are lifted from culture plates by incubation in 0.05% Trypsin-EDTA (Life Technologies) at 37°C for approximately 5-10 minutes. The cell suspension is filtered through a 70 micron cell strainer to dissociate cell colonies, counted using a hemocytometer, and then centrifuged at 300 *g* for 5 minutes. The supernatant is discarded while the resultant cell pellet is resuspended in approximately 100 µL/10 million cells of a 1x IgG blocking buffer, which prevents non-specific antibody binding. Three small aliquots containing 500,000 cells were removed for the unstained and single-color controls.

The cell solutions were then stained by addition of 10 µL PerCP Cy5.5-conjugated CD326 and 60 µL FITC-conjugated CD56 antibodies (BioLegend) to the cell sort sample, as well as to each single-color control aliquot. The cell suspension was allowed to incubate for at least 20 minutes on ice and in the dark. Afterwards, 1% FBS in PBS (Life Technologies) was added to the cell solution to dilute and wash the cells of excess antibody. The cells were again centrifuged at 300 *g* for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in approximately 1.5 mL 1% FBS in PBS. The cell suspension was again filtered

through a 45 micron cell strainer-capped FACS tube (Fisher Scientific). DAPI was added before the cells were sorted for CD56$^+$/CD326$^-$ using the BD FACSAria cell sorter at the Broad Stem Cell Research Center Flow Cytometry Core Laboratory.

hESC-Derived CD31$^+$ Endothelial Cell Differentiation

In preparation for endothelial cell differentiation, OP9 mouse stromal cells were plated on a 6-well plate at a density of 100,000 cells/well approximately one day before the CD56$^+$ MPC cell sort. The sorted CD56$^+$/CD326$^-$ cells were then plated at a concentration of 100,000-200,000 cells/well on the OP9 feeder layer in EGM-2 medium plus the supplied growth factors (Lonza). The EGM-2 was also supplemented with an additional 100 ng/mL VEGF and 10 μM of the TGF-β inhibitor SB-431542 (Sigma Aldrich). Sorted cells were cultured for a total of 7 days with medium changes every other day.

Fluorescence Activated Cell Sort of CD31$^+$ Immature Endothelial Cells

The cell sort for CD31$^+$ cells was similar to the previously described modified FACS protocol. Differentiated cells from the endothelial cell differentiation protocol were trypsinized and blocked as previously stated. However, this staining protocol involved the addition of APC/Cy7-conjugated CD31 and PE-conjugated PDGFRα antibodies (BioLegend). CD31$^+$ cells were again sorted using the BD FACSAria cell sorter at the Broad Stem Cell Research Center Flow Cytometry Core Laboratory.

hESC-Derived Troponin T$^+$ Cardiomyocyte Differentiation

The sorted CD31$^+$ cells were collected from the Core Laboratory and plated on fibronectin-coated 8-well chamber slides (BD Biosciences) at a density of approximately 50,000 cells/chamber. The sorted cells were cultured in cardiac medium, which contained MEM-α (Life Technologies), 20% FBS (Life Technologies), 1 μM of the Wnt inhibitor XAV939 (Sigma Aldrich), 10μM of another Wnt inhibitor KY02111 (Tocris Bioscience, Bristol, United Kingdom), 5 ng/mL VEGF, 30 ng/mL bFGF, 10 ng/mL BMP4, 1% Penicillin/Streptomycin (Life Technologies), and 1% Fungizone (Life Technologies). Medium was changed every other day for a total culture time of 3 weeks. The cells were then fixed with 4% PFA.

Chapter 3: Results

Localization of CD31$^+$/PDGFRα$^+$ Cells in Human Fetal Heart Ventricles

As previously mentioned, recent studies have shown the potential of CD31$^+$/PDGFRα$^+$ cells to differentiate into the cells of the cardiac lineage in mice (Van Handel et al., 2012). To determine whether CD31$^+$/PDGFRα$^+$ cells are also located in the human heart as well as their specific location in the heart, immunofluorescent labeling was performed on sagittal sections of human fetal hearts fixed at weeks 9, 15, and 17. Reportedly, CD31 is expressed on the surface of endothelial cells and typically seen around the inside lining of blood vessels in the heart (Albelda et al., 1991). Slides of human fetal hearts from all three time points showed distinct CD31 localization (green label) in the endothelial cells lining blood vessel lumens, as expected (Figure 1B, F, J).

The PDGFRα cell surface marker is typically highly expressed in epicardial and smooth muscle cells (Figure 1M, P, red label; Kang et al., 2008). Epicardial cells were also stained for CD31 to verify the low expression in the epicardium (Figure 1N, O). Slides of all fetal heart time points show low PDGFRα expression in the myocardium and endothelial cells, but high expression in smooth muscle cells surrounding the blood vessels. There are a few areas of the fetal human heart ventricle, however, where CD31 and PDGFRα appear colocalized in cells (Figure 1A, E, I). The small population of cells that express both CD31 and PDGFRα represent the similar cell type seen in mice (Van Handel et al., 2012). The DAPI counterstain marks individual nuclei of cells identified with CD31 and PDGFRα. Merged images indicate a small endogenous population of CD31$^+$/PDGFRα$^+$ cells in the human fetal heart (Figure 1D, H, L).

Generation of Troponin T$^+$ Cardiomyocyte-Like Cells from Human Stem-Cell Derived Endothelial Cells

The protocol to culture hESCs into cardiomyocyte-like cells involved a 5-step protocol (Figure 2A) that required approximately 5-6 weeks. Pluripotent H9 hESCs are maintained and cultured until about 75% confluency (Figure 2Ba). At this stage they exhibit a low cytoplasm-to-nucleus ratio, tight cell-to-cell contacts, and colonies with defined borders (Amit and Itskovitz-Eldor, 2012). Once confluent, the H9 hESCs are transferred to feeder-free conditions in which hESC colonies continue to exhibit similar morphological characteristics as when grown on MEFs (Figure 2Bb). After 2-3 days on feeder-free conditions, the cells are exposed to BVF-A medium for 1 day and then BVF medium for 2.5 days. The differentiating colonies now show changes in their morphology, such as the flattening of cells around the borders of each colony, increased amounts of cell cytoplasm, and loose cell-to-cell contacts (Figure 2Bc).

Approximately 3.5 days after exposure to differentiation conditions, hESCs begin to express CD56 and stop expressing CD326, a process that is indicative of hESCs undergoing EMT (Evseenko et al., 2010). CD56$^+$/CD326$^-$ mesodermal progenitor cells are sorted from the differentiated hESCs by FACS. The percent of sorted CD56$^+$/CD326$^-$ cells was approximately 10.5% using the preparation protocol as previously described (Figure 3Aa). These MPCs are plated on OP9 mouse stromal cells under endothelial growth conditions. Approximately 5 days later, cells exhibit endothelial cell morphology in which cells organize into blood tube formations and express CD31 (Figure 2Bd; Folkman and Haudenschild, 1980).

CD31$^+$ cells are then isolated by FACS. The percent of CD31$^+$ cells sorted was approximately 4.84% (Figure 3Ab). These isolated cells were plated on fibronectin 8-well chamber slides under feeder-free and cardiac growth conditions over a period of approximately 2

weeks. $CD31^+$ endothelial cells begin to proliferate and elongate, initially exhibiting fibroblast-like morphology (Figure 2Be). After 2 weeks of exposure to cardiac growth conditions, the endothelial cells were fixed and identified with the cardiomyocyte marker Troponin T (red; Figure 2Bf).

To determine whether changes in the FACS staining protocol could improve cell yield, a slightly modified protocol was used on an additional group of H9 hESCs. After the first MPC sort, the yield of $CD56^+/CD326^-$ cells was approximately 18.6% (Figure 3Ba). Based on previous studies, lithium chloride acts as an agonist to the Wnt signaling pathway (Li et al., 2012) and, when added to endothelial growth medium, suppresses cardiomyogenesis and stimulates continued endothelial cell proliferation (Van Handel et al., 2012). To test if lithium chloride has a similar effect on hESC-derived endothelial cell proliferation and differentiation, lithium chloride was added to endothelial growth medium at a concentration of 20 mM. One group of the sorted $CD56^+/CD326^-$ cells was exposed to regular endothelial growth medium and the other group was exposed to medium with lithium chloride. Both groups were separately sorted by FACS for the expression of CD31. The group without lithium chloride showed a yield of approximately 26.6% $CD31^+$ endothelial cells (Figure 3Bb), while the group with lithium chloride produced only 21.3% of these cells (Figure 3Bc).

Chapter 4: Discussion

The current study has provided preliminary support for the existence of human $CD31^+/PDGFR\alpha^+$ cardiac cells, previously described only in mice. These cells were located around the vasculature of the heart, near the endothelial cells lining of the blood vessels. In addition, this study also provided support for the idea that cardiomyocytes can be differentiated from hESC-derived endothelial cells. In the current study, endothelial cells expressing $CD31^+$ were differentiated from H9 hESCs. These endothelial cells, when put under cardiac growth conditions, were able to continue to differentiate and express the cardiomyocyte marker Troponin T. Overall, these results provide a foundation for further study on the potential of cardiac endothelial cells to become cardiomyocytes.

The location of these $CD31^+/PDGFR\alpha^+$ cells around the vasculature of the heart brings up questions as to their role in the heart (Van Handel et al., 2012). As previously mentioned, $CD31^+/PDGFR\alpha^+$ cells emerged from mouse endothelium after the loss of the transcription factor Scl and then were shown to have cardiomyogenic differentiation potential (Van Handel et al., 2012). In addition, these $CD31^+/PDGFR\alpha^+$ cells in mice could be linked to the development of the cardiac valves and septa from the endocardial cushions. The close proximity of these cells to the endothelial lining in both mouse and human cardiac vasculature suggests that they may have similar differentiation potentials.

More research is needed to characterize these human endogenous $CD31^+/PDGFR\alpha^+$ cells. Future research studies would remove $CD31^+/PDGFR\alpha^+$ cells from the human heart by FACS, culture the cells in cardiac growth medium for 3 weeks, and then identify the cells by their Troponin T expression. To further analyze their clinical potential, the Trop T^+ cells could be

isolated and injected into the hearts of immunosuppressed mice and large animals, such as pigs, to determine their ability to engraft and function *in vivo*.

In addition to generating cardiomyocytes from endogenous CPCs, the differentiation of pluripotent hESCs into cardiomyocytes also provides an alternative route towards a clinical application. In fact, hESC therapy could provide a better means to mass produce cardiomyocytes. Compared to isolating CPCs from the human heart, hESCs are easy to expand. Previous studies demonstrated the high plasticity of stem cells to be differentiated into specific cell types (Thomson et al., 1998). Endogenous multipotent CPCs, although able to differentiate towards a specific cardiac lineage, may be more resilient to differentiation when exposed to similar factors used to differentiate hESCs. Because of that, the study of differentiation potentials in hESCs could lead to faster clinical treatments utilizing hESC-derived cardiomyocytes.

The protocol to differentiate hESCs in this current study attempts to mimic *in vivo* human development. Specifically, the endothelial lining inside the heart, or the endocardial cushions, undergo EMT to eventually become the cardiac valves and septa seen during heart development. These endothelial cells, after becoming mobile and gaining mesenchymal characteristics, develop into cardiomyogenic cells (von Gise and Pu, 2012). Due to the flexibility of hESCs to differentiate into other cell types, differentiating hESCs into cardiomyocytes would be relatively easy to perform compared to differentiating adult stem cells or other multipotent stem cells. In addition, the differentiation protocol used in this current study could further instill the hESCs with a greater lasting potential to differentiate into cardiomyocytes.

Our results serve as a “proof-of-concept” that the differentiation protocol allows for the development of cardiomyocytes from hESCs through an endothelial cell intermediate. This

differentiation protocol would first need to be replicated to corroborate the current study. Additionally, further research is needed to improve the yield of desired cell types at each sort stage. Altering the concentration of various growth factors and the length of time the cells are exposed to the factors throughout the protocol could provide insight as to how to maximize the cell yield. Once the desired numbers of cardiomyocytes are differentiated from hESCs, these cardiomyocytes could be tested in immunosuppressed mice and other large animal models to determine their functionality in an *in vivo* setting.

The use of lithium chloride as an agonist of the Wnt-signaling pathway was shown to be effective at allowing endogenous endothelial cells to proliferate (Van Handel et al., 2012). In the current study, however, lithium chloride added to the cell medium did not improve endothelial cell yield after FACS and instead, decreased yield. Possible explanations could involve the inherent environmental differences between endogenous and stem cell populations. Endogenous cells, originally being exposed to the cardiac milieu, could have Wnt signaling pathways involving the protein glycogen synthase kinase 3β that are more receptive to the effects of lithium chloride compared with hESCs. The timing of exposure to lithium chloride could also have an effect on whether endothelial cells proliferate or differentiate. The greater differentiation potential of hESCs compared to more committed cells could also contribute to the effect of lithium chloride. More experiments that alter lithium chloride concentration or the exposure time need to be conducted to further elucidate the effect of Wnt agonists in this stem cell model.

Overall, the current study sought to explain the development of cardiomyocytes from endogenous and stem cell-derived endothelium. Future experiments would expand on the aforementioned results in hopes of moving towards a clinical treatment. Before human testing

can begin, the *in vitro* culture needs to be optimized and fully understood. Once large enough numbers of CPCs are generated from endothelium, *in vivo* large animal model testing can begin. These animal studies, combined with an understanding of the basic science of these CPCs, can lead to a clinical trial. Considering the urgent need to develop quick, cost-effective solutions to the growing heart disease problem, the preliminary data derived from this current project can direct new questions for study in cardiac regenerative research.

Figures

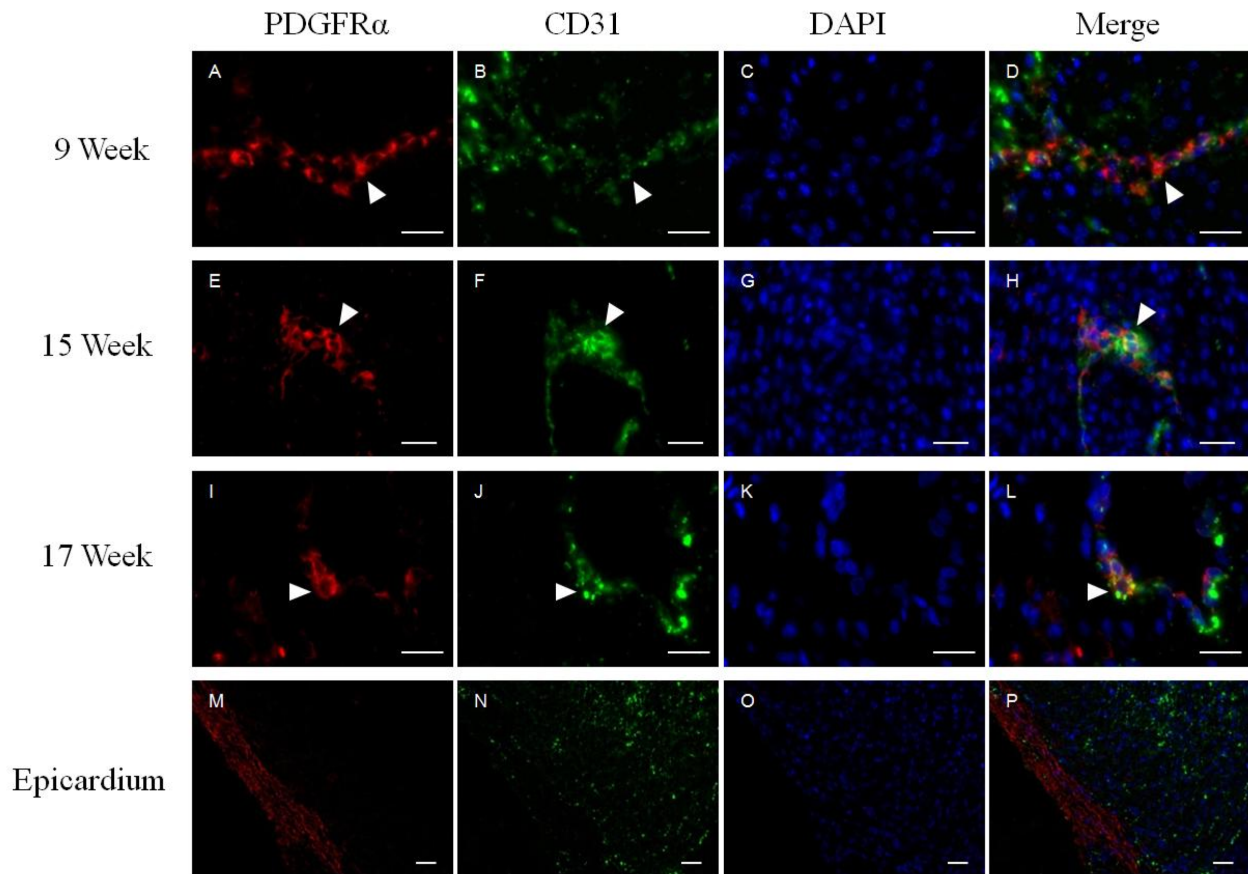
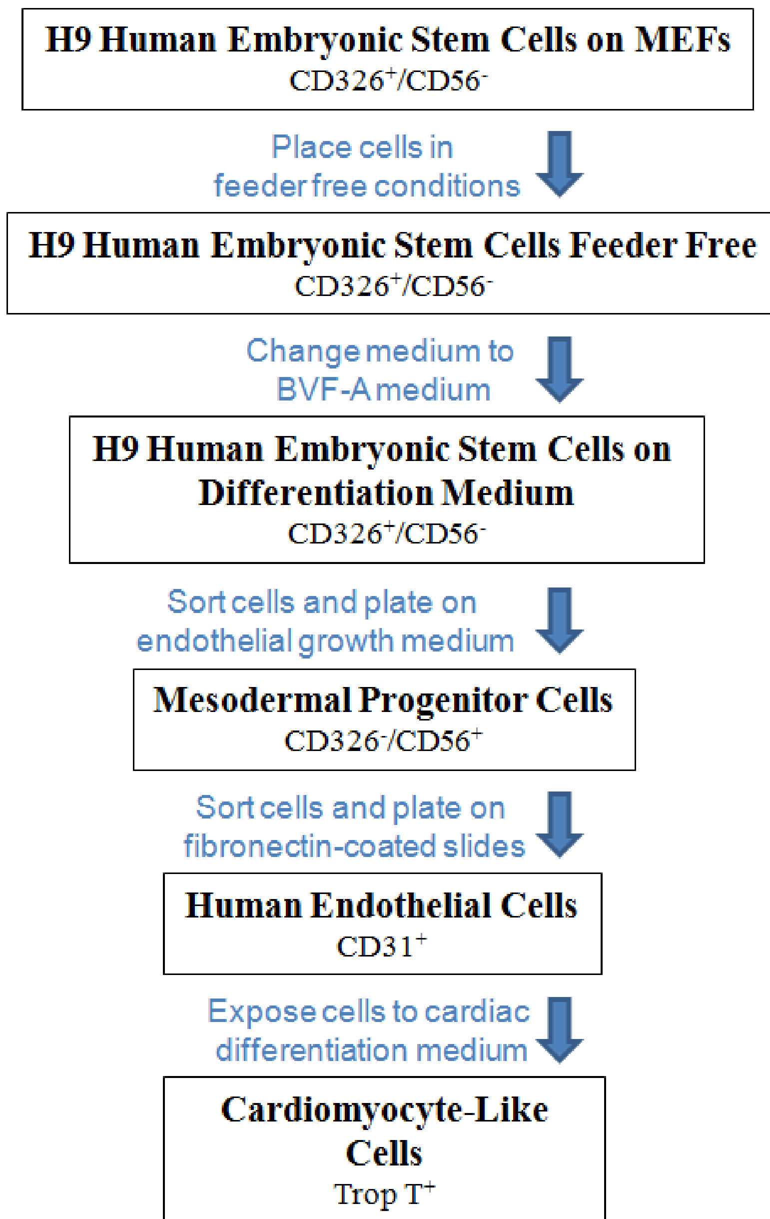


Figure 1. CD31$^+$/PDGFRα$^+$ cell populations are located around the vasculature of the fetal ventricular myocardium. Sections of 9 week human fetal hearts (A-C) were labeled for PDGFRα (red), CD31 (green), and stained for DAPI (blue). The three images were merged (D). CD31 endothelial cells line the inside of myocardial vasculature with areas of surrounding PDGFRα smooth muscle cells. Areas of co-localization (white arrowheads) indicate potential cardiac progenitor cells located nearby vasculature. Labeling of 15 week fetal human hearts (E-H) and 17 week fetal human hearts (I-L) also reveal similar cell locations surrounding the lumens of myocardial vasculature. Images of 17 week fetal heart (M-P) show delineated areas of strong PDGFRα-expressing epicardium lining the outside of the myocardium and CD31 endothelium in myocardial vasculature. Scale bars = 25 µm.



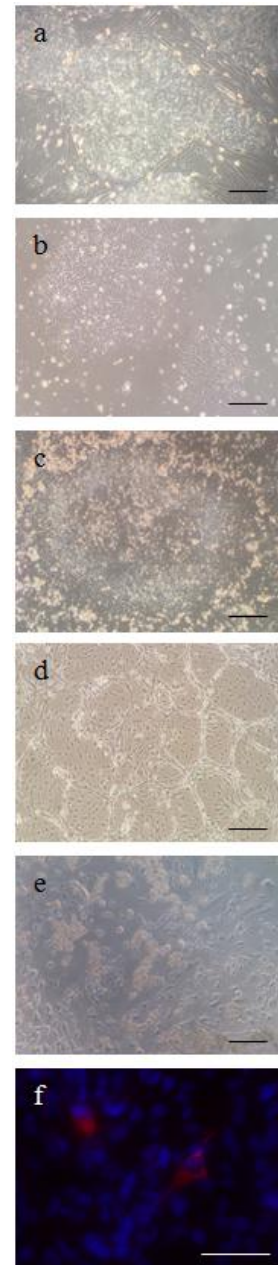


Figure 2. Differentiation of H9 hESCs into cardiomyocyte-like cells. The protocol to differentiate the H9 line of hESCs into Trop T-expressing cardiomyocyte-like cells involves a 5 step procedure with stage-specific differentiation media (A). Images of each cell stage are illustrated (B): H9 hESCs, day 5 post passage (a); H9 feeder free hESCs, day 3 post passage (b); H9 feeder free hESCs on BVF-A medium, day 2 post exposure (c); Mesodermal progenitors cells, day 5 post MPC sort (d); $CD31^+$ endothelial cells, day 5 post endothelial sort (e); Trop T^+ cardiomyocyte-like cells, day 14 post cardiac induction (f). Scale bars = 50 µm (a-e) and 25 µm (f).

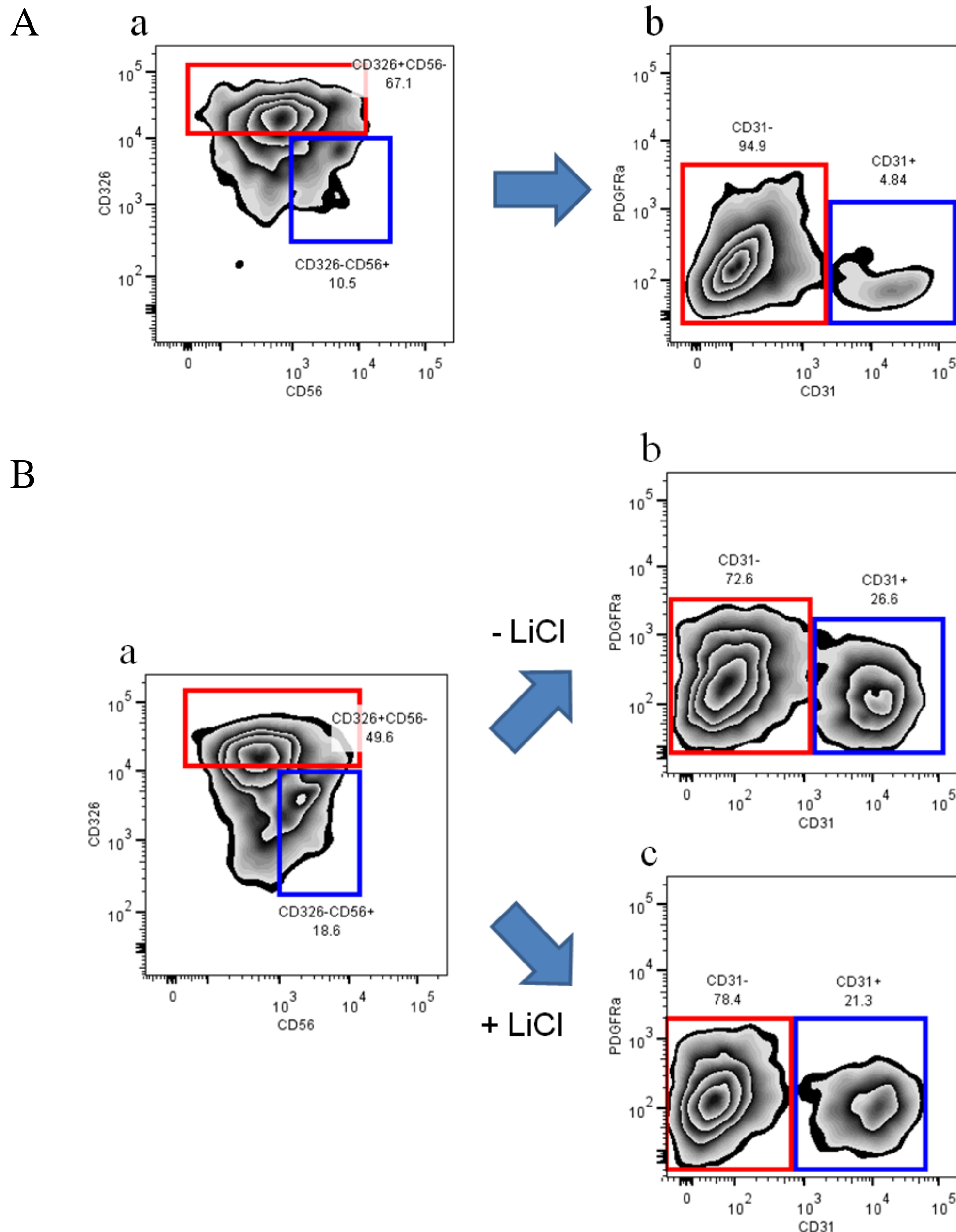


Figure 3. FACS zebra plots of MPC and endothelial cell sorts. H9 hESCs under differentiation medium are stained and sorted using the previously mentioned protocol (A). The MPC sort separated $CD56^+$ MPCs from $CD326^+$ cells, with the blue gate encircling the sorted desired population (Aa). After MPCs are plated on endothelial growth medium for 7 days, the $CD31^+$ endothelial cells are sorted, with the blue gate indicating the percentage of the sorted $CD31^+$ cell population (Ab). In a modified preparation protocol previously described, H9 hESCs are also stained and sorted (B). $CD56^+$ MPCs are again sorted from the $CD326^+$ cells, with the blue gate circling the sorted population (Ba). MPCs are then plated in two endothelial growth conditions, one without lithium chloride (Bb) and one with lithium chloride (Bc), and then sorted as previously, with the blue gates indicating the percent of sorted $CD31^+$ cells.

References

Abelda, S.M., Muller, W.A., Buck, C.A., Newman, P.J. (1991). Molecular and Cellular Properties of PECAM-1 (endoCAM/CD31): a Novel Vascular Cell-Cell Adhesion Molecule. J Cell Biol *114*, 1059-1068.

Agarwal, S., Holton, K.L., Lanza, R. (2008). Efficient Differentiation of Functional Hepatocytes from Human Embryonic Stem Cells. Stem Cells *26*, 1117-1127.

Amit, M., Itskovitz-Eldor, J. (2012). Morphology of Human Embryonic and Induced Pluripotent Stem Cell Colonies Cultured with Feeders. *Atlas of Human Pluripotent Stem Cells: Derivation and Culturing*, 15-39.

Bertrand, J.Y., Giroux, S., Golub, R., Klaine, M., Jalil, A., Boucontet, L., Godin, I., Cumano, A. (2004). Characterization of Purified Intraembryonic Hematopoietic Stem Cells as a Tool to Define their Site of Origin. PNAS *102*, 134-139.

de Lange, F.J., Moorman, A.F.M., Anderson, R.H., Manner, J., Soufan, A.T., de Gier-de Vries, C., Schneider, M.D., Webb, S., van den Hoff, M.J.B., Christoffels, V.M. (2004). Lineage and Morphogenetic Analysis of the Cardiac Valves. Circ Res *95*, 645-654.

Durocher, D., Charron, F., Warren, R., Schwartz, R.J., Nemer, M. (1997). The Cardiac Transcription Factors Nkx2-5 and GATA-4 are Mutual Cofactors. EMBO J *16*, 5687-5696.

Elliott, D.A., Braam, S.R., Koutsis, K., Ng, E.S., Jenny, R., Lagerqvist, E.L., Biben, C., Hatzistavrou, T., Hirst, C.E., Yu, Q.C., Skelton, R.J.P., Ward-van Oostwaard, D., Lim, S.M., Khammy, O., Li, X., Hawes, S.M., Davis, R.P., Goulburn, A.L., Passier, R., Prall, O.W.J., Haynes, J.M., Pouton, C.W., Kaye, D.M., Mummery, C.L., Elefanty, A.G.,

Stanley, E.G. (2011). NKX2-5$^{eGFP/w}$ hESCs for Isolation of Human Cardiac Progenitors and Cardiomyocytes. Nat Methods *8*, 1037-1040.

Evseenko, D., Zhu, Y., Schenke-Layland, K., Kuo, J., Latour, B., Ge, S., Scholes, J., Dravid, G., Li, X., MacLellan, W.R., Crooks, G.M. (2010). Mapping the First Stages of Mesoderm Commitment during Differentiation of Human Embryonic Stem Cells. PNAS *107*, 13742-13747.

Folkman, J., Haudenschild, C. (1980). Angiogenesis in vitro. Nature *288*, 551-556.

Hong, Y. (2009). Burden of Cardiovascular Disease in Asia: Big Challenges and Ample Opportunities for Action and Making a Difference. Clin Chem *55*, 1450-1452.

Kang, J., Gu, Y., Li, P., Johnson, B.L., Sucov, H.M., Thomas, P.S. (2008). PDGF-A as an Epicardial Mitogen During Heart Development. Dev Dyn *237*, 692-701.

Kattman, S.J., Witty, A.D., Gagliardi, M., Dubois, N.C., Niapour, M., Hotta, A., Ellis, J., Keller, G. (2011). Stage-Specific Optimization of Activin/Nodal and BMP Signaling Promotes Cardiac Differentiation of Mouse and Human Pluripotent Stem Cell Lines. Cell Stem Cell *8*, 228-240.

Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., Steward, O. (2005). Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Remyelinate and Restore Locomotion after Spinal Cord Injury. J Neurosci *25*, 4694-4705.

Laflamme, M.A., Murry C.E. (2011). Heart Regeneration. Nature *473*, 326-335.

Lancrin, C., Sroczynska, P., Stephenson, C., Allen, T., Kouskoff, V., Lacaud, G. (2009). The Haemangioblast Generates Haematopoietic Cells through a Haemogenic Endothelium Stage. Nature *12*, 892-895.

Li, B., Jia, Z., Wang, T., Wang, W., Zhang, C., Chen, P., Ma, K., Zhou, C. (2012). Interaction of the Wnt/β-Catenin and Notch Signaling in the Early Stage of Cardiac Differentiation of P19CL6 Cells. J Cell Biochem *113*, 629-639.

Lister, R., Pelizzola, M., Kida, Y.S., Hawkins, R.D., Nery, J.R., Hon, G., Antosiewicz-Bourget, J., O'Malley, R., Castanon, R., Klugman, S., Downes, M., Yu, R., Stewart, T., Ren, B., Thomson, J.A., Evans, R.M., Ecker, J.R. (2011). Hotspots of Aberrant Epigenomic Reprogramming in Human Induced Pluripotent Stem Cells. Nature *471*, 68-73.

Moretti, A., Caron, L., Nakano, A., Lam, J.T., Bemshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M., Martin-Puig, S., Sun, Y., Evans, S.M., Laugwitz, K.L., Chien, K.R. (2006). Multipotent Embryonic $Isl1^{+}$ Progenitor Cells Lead to Cardiac, Smooth Muscle, and Endothelial Cell Diversification. Cell *127*, 1151-1165.

Newman, P.J. (1994). The Role of PECAM-1 in Vascular Cell Biology. Ann N Y Acad Sci *714*, 165-174.

Nsair, A., Schenke-Layland, K., Van Handel, B., Evseenko, D., Kahn, M., Zhao, P., Mendelis, J., Heydarkhan, S., Awaji, O., Vottler, M., Geist, S., Chyu, J., Gago-Lopez, N., Crooks, G.M., Plath, K., Goldhaber, J., Mikkola, H.K., MacLellan, W.R. (2012). Characterization and Therapeutic Potential of Induced Pluripotent Stem Cell-Derived Cardiovascular Progenitor Cells. PLoS One *7*, e45603.

Parker, G.C. (2006). Embryonic Stem Cell Research: The State of the Union of "Those Who Create the Lines" and "Those Who Draw the Lines". Stem Cells and Dev *15*, 623-629.

Shand, J., Berg, J., Bogue, C. (2012). Human Embryonic Stem Cell (hESC) and Human Embryo Research. Pediatrics *130*, 972-977.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell *131*, 861-872.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M. (1998). Embryonic Stem Cell Lines Derived from Human Blastocysts. Science *282*, 1145-1147.

Van Handel, B., Montel-Hagen, A., Sasidharan, R., Nakano, H., Ferrari, R., Boogerd, C.J., Schredelseker, J., Wang, Y., Hunter, S., Org, T., Zhou, J., Li, X., Pellegrini, M., Chen, J., Orkin, S.H., Kurdistani, S.K., Evans, S.M., Nakano, A., Mikkola, H.K.A. (2012). Scl Represses Cardiomyogenesis in Prospective Hemogenic Endothelium and Endocardium. Cell *150*, 590-605.

von Gise, A., Pu, W.T. (2012). Endocardial and Epicardial Epithelial to Mesenchymal Transitions in Heart Development and Disease. Circ Res *110*, 1628-1645.