DIRECTION OF INDUCED PLURIPOTENT STEM CELL DIFFERENTIATION BY ENDOTHELIAL CELL SECRETOME

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by

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We love you and miss you every day.
CHAPTER 1

INTRODUCTION

Ischemic heart disease (IHD) is the leading cause of death worldwide accounting for approximately 7,249,000 deaths representing 12.7% of the total global mortality in a given year [1]. Established as a leading cause of mortality and morbidity in high income, industrialized countries, IHD is fast becoming the leading cause of death in middle and low-income countries as they approach a similar lifestyle [1]. According to the American Heart Association’s annual report on heart disease and stroke, heart disease has a higher prevalence in the aging population with African American males having the highest risk; followed by a similar risk for African American females and white males; and white females having the lowest risk [2]. Risk factors for heart disease famously outlined in the Framingham heart study include but are not limited to: age, obesity, smoking, a sedentary lifestyle, total cholesterol level, unhealthy diet and systolic blood pressure [3, 4]. A majority of these risk factors are considered modifiable in that modifying lifestyle can decrease the risk of a cardiovascular event [4]. The large-scale impact of heart disease on various regions and populations makes it a large consumer of healthcare funds, time and resources at a total cost of $320.1 billion dollars.
This, in combination with the associated high mortality and morbidity, makes IHD a worthy subject for continued research.

Ischemic heart disease

Coronary artery disease

Ischemic Heart Disease is a condition affecting the cardiac muscle in which there exists an imbalance between oxygen and nutrient demand and supply. For example, the cardiomyocytes that comprise the contractile unit of the heart demand oxygen and nutrients to maintain homeostasis and contractile function. Ischemic heart disease ensues when this demand goes unmet due to an obstruction in the coronary vessels feeding the myocardium. The tissue becomes ischemic and if oxygen demand continues to go unmet, a region of dead tissue develops as a myocardial infarct. This imbalance can be caused by a narrowing of the coronary lumen which limits delivery to a downstream site. This narrowing, or stenosis, occurs due to the atherosclerotic process of plaque formations within the vessel wall referred to as Coronary Artery Disease (CAD). Briefly, CAD is caused by a chronic inflammation of the coronary endothelial layer in conjunction with increased circulating low-density lipoprotein (LDL). This insult makes the generally tight, impermeable endothelial layer permeable to the LDL facilitating migration through the layer where it becomes oxidized (ox-LDL). In addition, this insult causes the endothelial expression of adhesion molecules that facilitate the attachment and rolling of monocytes through the layer. Once inside the vessel wall, monocytes differentiate to macrophages and become laden with ox-LDL effectively becoming foam cells. In conjunction with smooth muscle cells that migrate
from the medial layer and modify the extracellular matrix, these foam cells form a plaque with a fibrous cap that protrudes into the lumen. With the fibrous cap intact, a patient experiences stable angina: chest pain and discomfort during moments of exertion when metabolic demand of the myocardium increases beyond the baseline demand at rest. In contrast, unstable angina is more severe and occurs when a vessel is so narrow that pain can occur during rest when metabolic demand of the myocardium is at its lowest. Stenosis of the coronary vessel must be greater than 90% for an ischemic event to occur [5]. This level of stenosis occurs when the fibrous cap that provides a barrier between the atheromatous plaque and the vessel lumen ruptures and comes in contact with pro-thrombotic molecules of the blood forming a clot. This will significantly narrow the vessel at the sight of rupture and can cause complete occlusion of the vessel if the thrombus develops sufficiently. This cascade of CAD is the leading cause of IHD resulting in myocardial infarction (MI) and ischemic cardiomyopathy.

Myocardial infarction and ischemic cardiomyopathy

The majority of myocardial infarctions are localized to the subendocardial layer of the left ventricle (LV) [5]. The myocyte death and damage that occurs in the infarct and border zone disrupts the electrical impulse that propagates from atria to apex. The loss of electrical conduction and syncytial contraction of the LV has varying repercussions with the most severe being ventricular fibrillation and sudden cardiac death. Chronically, ischemic cardiomyopathy and heart failure develop as the myocardium weakens hindering the ability to function as a pump. A healthy individual will have a LV end diastolic volume of ~120mL with a stroke volume of ~70mL. In the ischemic heart,
this ejection fraction drops from 50-65% to below 40% effectively increasing the volume of blood and pressure in the LV. Concurrently, the increased LV end diastolic volume and pressure triggers high pre-load and pressures in previously low-pressure systems resulting in pulmonary and peripheral edema.

**Endogenous cardiac regeneration post MI**

Following myocardial infarction, the heart is capable of limited endogenous preservation and regeneration. Though the infarct scar impedes proper function of the myocardium, it serves as a preservation mechanism. Differentiated myofibroblasts migrate to the area of injury and secrete extracellular matrix in the place of dead cardiomyocytes [6]. This acute response strengthens the weakened myocardium to prevent ventricular collapse and rupture but does not regenerate the lost cardiomyocytes necessary to restore cardiac output [7]. In the healthy human heart 0.0014% of cardiomyocytes (out of 1 million) show markers consistent with mitosis and thus a proliferative capacity [8]. In zones distant and adjacent to an infarct, the percentage of cells undergoing mitosis increases to 0.03% and 0.08% respectively [9]. It is unclear whether the increased proliferation arises from mature cardiomyocytes or differentiating cardiac progenitor cells and whether or not the means of determining proliferation were accurate. In addition, what appears to be cardiomyocyte proliferation may actually be myocyte DNA replication without cytokinesis resulting in myocyte hypertrophy [10]. What does remain clear is that with approximately 1 billion cardiomyocytes lost in only a few hours, endogenous regeneration by means of cardiomyocyte proliferation is not sufficient to replace the injured/lost myocardium [11].
Although the goal of regenerating myocardium is laudatory, it is complicated by the necessity to regenerate a conductile system and a vascular supply in addition to muscle. These issues make the idea of regenerative therapies even more distant. A more attainable goal would be to regenerate coronary collateral vessels (CCV), which would serve to prevent myocardial infarction and sudden cardiac death. Referred to as ‘nature’s bypass’, coronary collateral vessels serve as connections that enable blood flow to bypass an occlusion. CCVs are largely unused in a healthy individual with no coronary obstruction meaning that they are present but not serving as a conduit for blood flow [12]. When a coronary becomes occluded, there is a pressure drop across the CCV. As the CCV undergoes outward remodeling it enables flow down a pressure gradient providing nutrient and oxygen rich blood to the ischemic myocardium beyond the occlusion [12]. Though a brilliant endogenous mechanism of ischemic protection, CCV remodeling is not always efficient. In a study of 74 patients with complete coronary occlusion 54% were found to have inadequate CCV development [13]. These individuals may have had a lesser number of coronary collaterals prior to occlusion or their existing collaterals failed to remodel upon onset of occlusion as compared to the 46% with adequate CCV development [12, 13]. The endogenous mechanisms the heart invokes to “combat” ischemic injury fall far short of rehabilitating the injured myocardium.
Exogenous stem cell therapies

Embryonic stem cells

In the absence of an effective endogenous repair mechanism, stem cells have become the focus of cell-based therapies for regeneration. There are three major types of stem cells that are used in cardiac regeneration: the embryonic stem cell (ES), the induced pluripotent stem cell (iPSC) and the adult stem cell. ES cells are pluripotent cells capable of self-renewal, prolonged undifferentiated proliferation and, as the definition of pluripotency entails, the ability to specialize function and give rise to any cell type in the body [14]. After an egg is fertilized, successive divisions occur and the embryo is termed a blastocyst. The blastocyst consists of an outer cell mass called the trophoblast that gives rise to the placenta and an inner cell mass (ICM) that gives rise to the three germ layers [15]. It is from the ICM that ES cells are harvested for culture. The use of ES cells in regenerative therapy is not as attractive as using iPSC cells or adult stem cells due to their allogeneic nature and subsequent need for immunosuppressive therapy. For the most part in cardiac regeneration, ES cells are primarily used to differentiate into cardiomyocytes—the idea being that they serve as a ready-to-use reservoir [16]. Although the embryos used for EC harvest are extra from in vitro fertilization, there is still a great deal of ethical controversy surrounding their isolation and use in research. In 2006 this ethical debate was lessened after the discovery of the iPSC cell (induced pluripotent stem cell).
**Induced pluripotent stem cells**

iPS cells start as somatic cells that are reprogramed back to a pluripotent state using the four embryonic transcription factors SOX2, OCT3/4, KLF4 and c-MYC [16]. This discovery was crucial to the advancement of regenerative medicine because it yielded a pluripotent, embryonic-like cell while circumventing the controversial need to harvest embryos. The method of isolation and creating iPS cells also means that treatments may be autologous unlike that of ES cells. However, like ES cells the pluripotent characteristics of iPS cells make them tumorigenic. If not controlled, the very pluripotency that makes ES and iPS cells ideal for regenerative therapy can result in tumors and unwanted cell types in target tissues. iPS cells serve a similar purpose as ES cells in regenerative therapy in that they are used to generate cardiomyocytes for intramyocardial injection or intracoronary infusion. iPS cell-derived cardiomyocytes are shown to improve cardiac function post MI through direct engraftment and electrically integrating with the host myocardium contributing to force generation [17].

**Adult stem cells**

Unlike ES and iPS cells, the adult stem cells are not pluripotent, but are instead multipotent meaning that the capacity to differentiate is restricted to specific lineages. Also termed 'somatic stem cells', adult stem cells are capable of self-renewal and function to replenish cells within their lineage and tissue. These cells can be found in the bone marrow (BM), heart, skin, gut, blood vessels and nervous system as well as other areas [15]. They are ideal for use in regeneration because they are non-tumorigenic, can be isolated from a biopsy with no ethical concerns and serve as a
autologous reservoir for patients [18]. With regards to cardiac regeneration, the most important adult stem cells to discuss are the bone marrow derived stem cells (BMDSC), cardiac stem cells (CSC) and endothelial progenitor cells (EPC).

**Adult stem cells: bone marrow derived stem cells**

BMDSC are predominantly isolated from the iliac crest and separated from the bone marrow aspirate into a mononuclear portion. Unfractionated bone marrow mononuclear cells (BMMC) are largely hematopoietic cells such as monocytes and lymphocytes while only 0.1% of BMMCs are actually hematopoietic and mensenchymal stem cells (MSC) [17]. These cells have the ability to improve cardiac function via direct autologous intracoronary infusion to the infarct-related artery, and may migrate from the bone marrow homing to areas of disease and injury [19]. In three separate trials (BOOST trial, REPAIR-AMI and FOCUS-CCTRN) examining the safety and efficacy of using BMMCs in cardiac regeneration, ejection fraction was shown to increase significantly up to 6 months after BMMCs intracoronary infusion to the infarct-related artery [17]. In 2001, Anversa et al hypothesized that using the cytokines stem cell factor (SCF) and granulocyte-colony-stimulating- factor (G-CSF) would mobilize a sufficient number of BMDSC to home to the injured myocardium and promote cardiac repair. The study reported significant tissue regeneration with $15 \times 10^6$ new myocytes vascularized by capillary connections with the unaffected ventricle [20]. The study also showed a decrease in mortality, infarct size, ventricular cavity dilation, diastolic stress and a progressive increase in ejection fraction [20]. While these studies focus on the
unfractionated BMDSC, specific components of the BM such as MSCs, CD133+ and CD34+ cells have also been shown to improve cardiac function post MI [21].

**Adult stem cells: cardiac stem cells**

In contrast to multilineage BMDSCs such as the MSCs that give rise to connective tissue cells, CSCs favor a cardiomyogenic fate allowing them to differentiate into the three main cardiac lineages: cardiomyocytes, endothelial cells and smooth muscle cells [22, 23]. CSCs are resident cardiac cells found in the interstitial space in areas of low wall tension such as the apex or atrial appendages [22]. The CSC contribution to endogenous regeneration is controversial and likely negligible with only 1 CSC for every 10,000 cardiomyocytes; however, with ex vivo proliferation CSCs may have a meaningful impact on regeneration [22]. After initial isolation from a biopsy, CSCs can be grown in suspension where they form 3D spheroids called cardiospheres that are said to be enriched for stemness and cardiogenic pathways [22]. To avoid thrombosis, cardiospheres may be plated in a 2D culture to yield a single cell monolayer termed cardiosphere derived cells (CDCs). This allows for the autologous injection or intracoronary infusion of CDCs; however, these cells are enzymatically isolated from myocardium that is diseased or stressed from injury, which creates the concern that these cells may not be capable of optimal regeneration. Regardless of this concern, the cardiosphere-derived autologous stem cells to reverse ventricular dysfunction post MI (CADUCEUS) trial reported a significant reduction in scar mass, an increase in viable heart mass, an increase in regional contractility and an increase in regional wall thickening [24]. While the CADUCEUS trial does not show any significant change
between the ejection fraction of CDC treated and control groups, the autologous human cardiac-derived stem cell to treat ischemic cardiomyopathy (ALCADIA) trial showed an improvement in LV function as measured by LV ejection fraction [24].

**Adult stem cells: endothelial progenitor cells**

Of the cells discussed (ES cells, iPSCs, BMDSCs and CSCs), progenitor cells are considered to be at the furthest stage of differentiation committed to a specific lineage. These cells are more lineage determined with no self-renewal and limited proliferation [21]. EPCs garner particular interest in regenerating ischemic myocardium due to their endothelial lineage differentiation stimulating neovascularization and paracrine secretion of proangiogenic factors [25]. This interest is rooted in the idea that these EPCs may be mobilized from the BM to the peripheral blood using cytokines or isolated from the BM and/or peripheral circulation and targeted to re-vascularize the ischemic myocardium [25]. In the transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI) study, 20 patients were randomly assigned to either receive intracoronary BM-derived progenitor cells or blood-derived progenitor cells. Progenitor cell transplantation resulted in increased ejection fraction, reduced end-systolic ventricular volume and improved wall motion in the infarct zone [26]. In a study by Kawamoto et al, 10^6 labeled EPCs were infused in athymic nude rats 3 hours after left anterior descending coronary artery ligation. Seven days later, fluorescence microscopy revealed that EPCs had incorporated into myocardial neovascularization [27]. In a second EPC group, necropsy performed 28 days after inducing ischemia revealed increased capillary density as compared to the control
group [27]. Both studies indicate that EPCs are modestly capable of regenerating cardiac function, as well as restoring blood flow to the ischemic zone.

**Stem cell mechanism of cardiac regeneration**

Each stem cell type—ES cell, iPS cell and adult stem cell—has been observed to modestly improve cardiac function post MI; the question that remains is how: are these exogenous cells engrafting and differentiating into the cells needed to rebuild injured myocardium; or are these exogenous cells acting through a paracrine effect recruiting resident progenitors? Four theories have been proposed: 1) bone marrow cells (BMC) transdifferentiate into cardiomyocytes; 2) cell fusion occurs between BMCs and myocytes which has been used to explain what is being observed and termed transdifferentiation; 3) BMC cytokines (vascular endothelial growth factor [VEGF], insulin like growth factor and platelet derived growth factor) induce myocyte growth and viable myocytes along the border zone of infarction; and 4) exogenous cells are stimulating resident CSCs to differentiate [28]. Injection of male Lin−/c-kit+ GFP enriched bone marrow cells into the peri-infarct zone of female mice resulted in newly formed myocardium characterized by GFP fluorescence, the presence of a Y chromosome, myocyte enhancer factor-2 (MEF2) and cardiac specific transcription factor GATA-4 with a smaller population also expressing the early markers of myocyte development Csx/Nkx2.5 [29]. This study supports the theory of engraftment and differentiation/transdifferentiation. Conversely, Lin−/c-kit+ BMCs were found to not undergo transdifferentiation to a cardiomyogenic fate, and did not stimulate the overt recruitment of resident CSC that may then assume a cardiomyogenic fate [30]. If we are
to amplify the current benefits observed using stem cell therapies, we must understand their mechanism of action within the ischemic microenvironment. As is evident in the given examples, current understanding of the stem cell mechanism of cardiac regeneration is inconsistent and contradictory indicating a clear need for continued research in the area.

**Hypotheses and specific aims**

iPS cells are an excellent stem cell source for regeneration as their pluripotency allows them to yield any of the cell types necessary for cardiac regeneration. Although ES cells have the same capacity, iPS cells are autologous and have minimal to no ethical concerns surrounding their isolation and use in research. CSCs also have the capacity to give rise to the lineage specific cells necessary for cardiac regeneration, however, these cells must first be harvested from a biopsy, an extremely invasive procedure compared to the isolation of dermal fibroblasts for reprogramming. Despite this evidence in support of the use of iPS cells over other cell types, the pluripotent tumorigenic nature of iPS cells makes their use concerning. The purpose of this study is to direct iPS cell differentiation into a desired cell type using naturally occurring queues from the microenvironment and thereby avoiding tumorigenesis. This study helps us to address two areas: 1) the ability to direct iPS cell differentiation in a desired direction; and 2) the fate of iPS cells infused/injected into the ischemic myocardium i.e. does the microenvironment facilitate differentiation. We hypothesize that iPS cell differentiation can be directed to a desired cell type and that the queues in the
microenvironment serve as the physiological method of control. We will examine the following specific aims:

1) Determine the ability of the microenvironment to direct iPS cell differentiation to a desired function and phenotype.
2) Determine if changes in the microenvironment influence the direction of iPS cell differentiation.
3) Determine if the exosomal fraction of the microenvironment has the greatest affect on iPS cell differentiation.

**Specific 1. Determine the ability of the microenvironment to differentiate iPS cells into a desired function and phenotype.** In order to examine this aim we had to establish an *in vitro* microenvironment. Conditioned medium (CM) collected from rat aortic endothelial cells (EC) served as the cell secretome and established the microenvironment used to direct iPS cell differentiation. We hypothesize that queues within the endothelial cell conditioned medium (EC-CM) will direct iPS cell differentiation into an endothelial function and phenotype. Differentiation from a rat iPS cell to an EC is measured using real time PCR (qPCR) analyses of endothelial markers platelet endothelial cell adhesion molecule (PECAM) and vascular endothelial cadherin (Cdh5); and analyses of iPSC markers NANOG and OCT4. Fluorescence microscopy is used to examine the endothelial glycoprotein binding of Isolectin GS-IB4 and immunostaining is used to detect stage specific embryonic antigen-1 (SSEA-1), which is used to denote endothelial and iPS cell phenotypes, respectively. A tube formation assay is used to
examine the functional capacity of EC-CM treated iPS cells (iPSC+CM) to form a lattice-like network. An acetylated-LDL (a-LDL) uptake assay analyzed by flow cytometry is used to determine if iPSCs+CM express the scavenger receptor for a-LDL uptake.

**Specific 2. Determine if changes in the microenvironment influence the direction of iPSC differentiation.** CM is collected from EC under normoxic (NCM) and hypoxic conditions (HCM) to represent a change in the microenvironment that will direct iPS cell differentiation. We hypothesize that this change in the microenvironment will change the differentiation of an iPS cell to an EC function and phenotype so that the iPS cells treated with EC derived hypoxic CM (iPSC+HCM) will have a more robust EC differentiation as compared to the iPS cells treated with EC derived normoxic CM (iPSC+NCM). Differentiation from an iPS cell to an EC is measured using qPCR analyses of endothelial markers PECAM, cdh5 and the angiopoietin receptor Tie-2. A tube formation assay is used to examine the functional capacity of iPSCs+HCM and iPSCs+NCM to form a lattice-like network. An a-LDL uptake assay analyzed by flow cytometry is used to determine if iPSCs+HCM and iPSCs+NCM express the EC scavenger receptor for LDL uptake.

**Specific 3. Determine if the exosomal fraction of the microenvironment has the greatest affect on iPS cell differentiation.** Portions of the normoxic and hypoxic EC-CM representing the microenvironment are removed and suspended in a separate media to determine if one fraction of the CM has a greater affect on iPS cell differentiation than another. Extracellular vesicles called exosomes are known to carry
mRNA and miRNA capable of influencing a target cell [31]. It is for this reason that we choose to focus on the removal of exosomes from CM and suspension of exosomes in a treatment media (TM). iPSCs treated with hypoxic exosomes are referred to as hypoxic conditioned media exosomes (HCMEXO); iPSCs treated with the portion of HCM that has had exosomes removed are referred to as HCM-EXO. iPSCs treated with normoxic exosomes are referred to as normoxic conditioned media exosomes (NCMEXO); iPSCs treated with the portion of NCM that has had exosomes removed are referred to as NCM-EXO. We hypothesize that removal of the exosomal fraction from the endothelial cell secretome will cause a decrease in iPS cell differentiation to an endothelial function and phenotype; whereas addition of endothelial secretome derived exosomes will increase iPS cell differentiation to an endothelial function and phenotype; these effects will be more apparent using exosomes derived under hypoxic conditions. The exosome specific antibody CD9 is used to confirm whether or not exosomes have been removed from the EC-CM. A qPCR analysis of endothelial markers PECAM, Cdh5 and Tie-2 is used to examine expression of endothelial markers. A tube formation assay is used to determine the functional capacity of iPSC treated cells to form a lattice-like network. An a-LDL uptake assay is used to examine if treated iPS cells express the scavenger receptor for LDL uptake.
CHAPTER TWO

METHODS

Cell culture

*Rat aortic ECs*

Rat aortic ECs isolated from Sprague Dawley rats were purchased from Cell Applications Inc and cultured in rat aortic endothelial cell growth media (Cell Applications Inc, San Diego, CA). ECs were cultured on plates coated for at least 30 minutes with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) in PBS. Cells were incubated in 37°C, 5% CO₂ and 95% air unless otherwise noted.

*Mouse embryonic fibroblasts*

MEFs were cultured in MEF media (500 mL DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, Utah), 1% Antibiotic-Antimycotic (Anti-Anti) (100X, Gibco, Grand Island, NY), 1% NEAA (100X, Gibco, Grand Island, NY) and 1% 200mM L-Glutamine (100X, Gibco, Grand Island, NY)). Cells were plated on a 100 mm plate on 0.1% gelatin in PBS and incubated in 37°C, 5% CO₂ and 95% air until 80-100% confluent. MEFs were inactivated using 0.4 mL of 2 mg mitomycin-C (Roche,
Mannheim, Germany) in fresh MEF media. Cells were incubated in mitomycin-C for 2.5 hours at 37° C, 5% CO₂ and 95% air and then counted and suspended at 7x10⁵ cells/1ml freezing media (10% DMSO (Sigma-Aldrich, St. Louis, MO), 50% FBS, 40% MEF media). iMEFs were frozen and then later used as an inactivated feeder layer for iPSCs.

*Induced pluripotent stem cells*

iPS cells were fully reprogrammed from rat fibroblasts at the Shanghai Institute for Biological Sciences and generously donated by Dr. Lei Xiao. A 6-well dish was coated with 0.1% gelatin in PBS and incubated for at least 30 min in 37° C, 5% CO₂ and 95% air. iMEF were then plated at 7x10⁵ cells/ 6-well plate and allowed to attach for at least 6 hours before MEF media was removed and iPSC media (500 mL KO DMEM (Invitrogen, Grand Island, NY), 5% ES cell qualified calf serum (Hyclone, Logan, UT), 5% KOSR (Hyclone, Logan, UT), 1% Anti-Anti, 1% NEAA, 1% 200mM L-Glutamine, 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and 112 μL Leukemia inhibitor factor (10⁷ units/mL, Millipore, Darmstadt, Germany)) was added. iMEF were allowed to incubate in iPSC media for at least 30 minutes before IPS cells were plated over the iMEF layer. Undifferentiated iPS cells were maintained on an iMEF layer unless being used for treatment in which case they were plated directly on 0.1% gelatin in PBS. iPSCs that were determined to be differentiating based on morphology were marked using bright field microscopy and picked out using a sterile Pasteur pipet.
**Conditioned media**

ECs were plated in a 100mm dish in rat endothelial cell culture media. After reaching ~80-90% confluency, ECs were washed 3x with PBS and then incubated in endothelial conditioning medium (500 mL DMEM, 1% Anti-Anti (100X), 1% NEAA (100X) and 1% 200mM L-Glutamine (100X) for 24 hrs in 37° C, 5% CO₂ and 95% air. After 24 hours, CM was collected and centrifuged at 3000 x g for 15 minutes to remove cells and cellular debris. CM was collected from EC between P6 and P7 and mixed to account for any differences in CM collected at different passages. CM was aliquoted into 1mL aliquots and stored at -80° C for no more than 6 months before use.

**Normoxic and hypoxic conditioned media**

Normoxic conditioned media (NCM) was made and collected as described above. To make hypoxic conditioned media (HCM), serum depleted media ((500 mL DMEM, 1% Anti-Anti (100X), 1% NEAA (100X) and 1% 200mM L-Glutamine (100X)) was incubated in a hypoxia chamber at 37° C, 5% CO₂ and 1% O₂ for 2 hours before being used. ~80-90% confluent endothelial cells were washed 3x with PBS before being incubated with the serum depleted media at 37° C, 5% CO₂ and 1% O₂ for 24 hours.
iPSC treatment

iPSC treatment with CM: Normoxic

iPS cells were plated in a 6-well plate on 0.1% gelatin in PBS and allowed 24 hrs to attach before treatment with CM. NCM aliquots were removed from -80°C and allowed to thaw at room temperature. iPS cells were washed 1x with PBS before adding 2mL of treatment medium (500 mL DMEM supplemented with exosome depleted FBS (System Biosciences, Mountain View, CA), 1% Anti-Anti (100X), 1% NEAA (100X) and 1% 200mM L-Glutamine (100X)). 1mL of NCM was then added in so that the ratio of treatment media to NCM was 2:1. iPS cells were treated every 72-96 hours with fresh treatment media and freshly thawed NCM. The course of treatment totaled 2 weeks. iPS cells that needed passaged during the course of treatment were allowed 24 hours to attach before resuming treatment.

iPSC treatment with CM: hypoxic

The protocol for treating iPS cells with HCM was adapted from above using HCM in place of NCM.

Exosome removal and treatment media supplement

Exosomes were removed from both NCM and HCM using ExoQuick-TC™ (System Biosciences, Mountain View, CA). NCM and HCM were removed from -80°C and allowed to thaw at room temperature. ExoQuick-TC™ was added to NCM and HCM at a 1:5 ratio; for every 4mL of NCM or HCM used, 0.8mL of ExoQuick-TC™ was
added. CM and ExoQuick-TC\textsuperscript{TM} were mixed using a pipette and refrigerated overnight (at least 12 hrs.). After refrigeration, CM + ExoQuick-TC\textsuperscript{TM} was centrifuged at 4°C for 30 minutes at 1500 x g. An exosomal pellet was sometimes observed. In the absence of a pellet, the lower 150μL of the CM + ExoQuick-TC\textsuperscript{TM} mixture was protected. The supernatant was removed and labeled as the fraction of CM without exosomes (NCM-EXO or HCM-EXO). The remaining pellet, or remaining 150μL, was then suspended in a volume of treatment media equal to the volume of CM initially used for exosome removal i.e. if 4mL of NCM or HCM was mixed with ExoQuick-TC\textsuperscript{TM}, than the remaining pellet was suspended in 4mL of treatment media. This fraction was labeled as the portion of treatment media supplemented with normoxic or hypoxic exosomes (NCMEXO or HCMEXO).

\textit{iPSC treatment with NCM or HCM depleted or supplemented exosomes}

Exosome depleted or supplemented solutions were prepared fresh before every treatment. iPS cells were washed 1x with PBS before adding 2mL of treatment medium. 1mL of NCM-EXO or HCM-EXO was then added in so that the ratio of treatment media to exosome depleted media was 2:1. In a separate well, 1mL of NCMDEXO or HCMDEXO was then added so that the ratio of treatment media to NCMDEXO and HCMDEXO was 2:1. iPS cells were treated every 72-96 hours with fresh treatment media and freshly made exosome depleted or supplemented media. The course of treatment totaled 2 weeks. iPS cells that needed passaged during the course of treatment were allowed 24 hours to attach before resuming treatment.
Collection of CM, removal and supplementation of exosomes and subsequent treatment of iPS cells

Normoxic Conditioned Media

Hypoxic Conditioned Media

Figure 1. Schematic of iPSC treatment protocol. Conditioned media is first collected from EC under normoxic or hypoxic conditions. CM is then separated into three different treatment groups: 1) whole normoxic or hypoxic CM; 2) exosome depleted normoxic or hypoxic CM; and (3) treatment media supplemented with normoxic or hypoxic derived exosomes.
Verification of Exosome removal and supplement

Verification of exosome removal from CM was achieved using exosome-human CD9 antigen flow detection (Invitrogen, Oslo, Norway). The human CD9 antibody was tested with rat derived exosomes and was not successful. Human derived pericardial fluid (PF, Summa Akron City Hospital, Akron, OH) was used instead to prove that the protocol for exosome removal works. Exosomes were first removed from PF (PF-EXO) in the same manner described above for exosome removal from NCM and HCM. Exosomes that were removed were then suspended in treatment media (PFEXO) to represent the treatment groups that were supplemented with NCM and HCM exosomes. Dynabeads coated with human CD9 antigen were prepared using 1mL of assay buffer (0.1% BSA in PBS) for washing steps and a magnetic separator. 40μL of the CD9 coated dynabeads were then added to 100μL of PF-EXO or PFEXO and incubated overnight in a cold room with end-over-end mixing. Beads were again washed with assay buffer, separated using a magnetic separator and suspended in 200μL of assay buffer. For flow detection, 100μL of the bead-bound exosome sample was incubated for 45 minutes at room temperature with either 20μL of mouse anti-human CD9 (BD Biosciences, Bedford, MA) or 20μL of the mouse IgG1 isotype control (BD Biosciences, Bedford, MA). Samples were again washed with assay buffer, separated using a magnetic separator and suspended in 200μL of assay buffer. Flow cytometry was then completed using the AccuriC6 Flow Cytometer. Bead count was set at 20,000 events for each sample not to exceed 150μL of the sample. Samples were gated to their isotype control and analyzed using BD accuri software.
Determining iPSC differentiation to an endothelial cell phenotype

Real time polymerase chain reaction

qPCR was done to analyze the mRNA expression of endothelial markers PECAM, Cdh5 and Tie-2; and iPS cell markers NANOG and OCT4. RNA was isolated from cultured cells using 0.8mL of trizol (Life Technologies Corporation, Carlsbad, CA) per treatment well. Cells were scraped from the culture dish and transferred to a 15mL conical tube. 0.5mL of chloroform was added for every 0.8mL of trizol used to isolate RNA. Tubes were shaken and allowed to incubate at RT for 10 minutes and then centrifuged at 4,000 x g for 2.5 minutes at 4°C. The upper aqueous layer was removed and transferred to an RNAse free microcentrifuge tube. The above steps were repeated with an additional 0.3mL of chloroform to increase the aqueous layer. 1mL of 100% isopropanol was added to the aqueous phase for every 0.8mL of trizol used. The sample was then incubated at RT for 10 minutes and centrifuged at 4,000 x g for 15 minutes at 4°C. The supernatant was removed and the RNA was washed with 75% ethanol and centrifuged at 12000 x g for 5 minutes at 4°C. After ethanol removal, the RNA pellet was dried and resuspended in RNase-free water. The RNA sample was then incubated in a water bath for 10-15 minutes at 55°C. RNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). RNA was then DNase treated (Life Technologies Corporation, Carlsbad, CA) to remove possible DNA contamination. Complementary DNA (cDNA) was then synthesized with reverse transcriptase enzyme using sample RNA as a template. Reverse transcriptase polymerase chain reaction (RT-PCR) was completed using TaqMan® Reverse
Transcription reagents (Life Technologies Corporation, Branchburg, NJ) in a BioRad T100 Thermal Cycler (Hercules, CA). qPCR was then performed using Power SYBER® Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA). Primer sequences for qPCR were made using longer exon sequences separated by shorter intron sequences to avoid genomic DNA contamination. Sequences were then copied into the Integrated DNA Technologies Database and the primer was designed. Primer sequences are detailed in table 1. A master mix was made for each primer used and 4μL of cDNA template was added for a 20μL reaction. Loaded MicroAmp Optical 96-well reaction plates were analyzed using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA)).
<table>
<thead>
<tr>
<th>TARGET</th>
<th>PRIMER SET</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>SET 1</td>
<td>sense 5’-TCC AGT ATG ACT CTA CCC ACG-3’, &lt;br&gt; antisense 5’-CAC GAC ATA CTC AGC ACC AG-3’</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Tie2</td>
<td>SET 1</td>
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</tr>
<tr>
<td>NANOG</td>
<td>SET 1</td>
<td>sense 5’-TTC TGA ACC TGA GCT ATA AGC AG-3’, &lt;br&gt; antisense 5’-CAA TGG ATG CTG GGA TAC TCC-3’</td>
</tr>
<tr>
<td>OCT 4</td>
<td>SET 1</td>
<td>sense 5’-AGA ACA TGT GTA AGC TGC GG-3’,  &lt;br&gt; antisense 5’-TCA CAC GGT TCT CAA TGC TAG-3’</td>
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Table 1. qPCR primers used to examine iPS cell expression of endothelial and stem cell markers.

Controls

Four control groups were used for comparison of treatment groups after qPCR analysis: EC, iPSC, VEGF and TM. Untreated ECs and iPS cells were used as positive and negative controls respectively. Rat aortic endothelial growth media was supplemented with 50ng/μL of VEGF (R&D Systems, Minneapolis, MN). EC media +
VEGF treatment of iPS cells was used as a second positive control as VEGF and has been previously used as a pharmacological method of differentiating iPS cells to endothelial cells [32]. Treatment of iPS cells with TM alone was used to make sure that the observed effects resulted from the exosomes and not the media they were suspended in.

**Fluorescence microscopy**

Fluorescence microscopy was done to observe an endothelial phenotype using Isolectin GS-IB4 (Life Technologies Corporation, Carlsbad, CA) or a stem cell phenotype using SSEA-1 (Stemgent, Cambridge, MA). Cells were fixed in 4% PFA (ChemCruz, Dallas, TX) and incubated at RT for 20 min. PFA was aspirated and cells were washed in cold PBS then allowed to sit in cold 5% BSA (Sigma Aldrich, St. Louis, MO) in 0.1% PBSTween\(^{20}\) (Sigma Aldrich, St. Louis, MO) for 60 minutes in the refrigerator. Isolectin GS and the SSEA primary antibody were prepared at 1:100μL and 1:200μL dilutions respectively in 2.5% BSA in PBSTween\(^{20}\), added to their respective wells and kept overnight in the refrigerator. Secondary antibody anti-mouse SSEA-1 was diluted at 1:300 in 2.5% BSA in PBSTween\(^{20}\). After incubating overnight cells were washed three times in 0.1% PBSTween\(^{20}\) on an orbital shaker for 5 min. The secondary antibody was added to the appropriate wells and PBS was added to wells who’s primary was already conjugated. Plates were wrapped in foil and allowed to incubate at RT for 1-2 hours on the orbital shaker. Cells were washed 3x as described above. Enough Prolong Gold Antifade reagent (Life Technologies Corporation, Eugene,
OR) was then added to cover each well. Cells were then examined using an Olympus IX71 microscope.

**Determining iPSC differentiation to an endothelial cell function**

*Tube Formation*

Matrigel assay was completed to determine if treated iPS cells could form a tube-like network indicative of endothelial function. Aliquoted growth factor depleted Matrigel (BD Biosciences, Bedford, MA) was removed from the -80° C and allowed to thaw in the refrigerator overnight. 48 well dishes and pipette tips for Matrigel assays were kept in the -20° C to ensure that Matrigel stay cooled throughout the experiment. When adequately thawed, 150-200μL of Matrigel was loaded into individual wells of a 48 well plate kept on ice. Matrigel was loaded slowly to avoid bubbles. The Matrigel loaded plate was allowed to incubate at 37° C, 5% CO₂ and 95% air for 30 minutes. 0.5mL of appropriate media (control media or treatment media) supplemented with 30ng/μL of VEGF was added to each well and incubated for an additional 2 hours. The media was drained and cells were seeded at 60K per well with appropriate media supplemented with 30ng/μL VEGF. Cells were imaged for tube-like networks at 2, 4 and 6 hours using an Olympus IX51 microscope.

*Acetylated-LDL uptake*

Acetylated LDL uptake assay was done to determine if treated iPSCs expressed the scavenger receptor indicative of endothelial function. α-LDL (Life Technologies Corporation, Eugene, OR) was mixed with 1mL of culture medium at a 1:100μL dilution and incubated with cells at 37° C, 5% CO₂ and 95% for 4 hours. Cells were then
washed in PBS, trysinized and suspended in 200μL of FACS buffer (PBS, 5%BSA and 0.5M EDTA (Sigma Aldrich, St. Louis, MO). α-LDL uptake was then measured by the AccuriC6 Flow Cytometer and BD accuri software. Samples were compared to their isotype control.
CHAPTER THREE

RESULTS

Specific 1. Determine the ability of the microenvironment to differentiate iPS cells into a desired phenotype and function.

Determination of optimal time course of treatment

To test the hypothesis that EC-CM could influence the differentiation of iPS cells into an endothelial function and phenotype, we had to determine the optimal time course for treatment. iPS cells were treated with endothelial derived CM every 72-96 hours for a total of 7 weeks to determine the optimal time course for treatment. Figure 2 shows the 7 week period broken up into 5 times based on visible changes in cell morphology: T1 (D1-D14), T2 (D15-D23), T3 (D24-D33), T4 (D34-D40) and T5 (D41-49). iPS cell differentiation to an endothelial phenotype was analyzed by qPCR analyses of endothelial markers PECAM and Cdh5; and stem cell markers NANOG and OCT4 over a 7 week period. PECAM expression was elevated at T1 (14 days of treatment) and increased at T2 (23 days of treatment), but showed a decrease in PECAM expression after 23 days of treatment (Figure 3A). Similarly, Cdh5 expression was increased at T1, but showed a decrease in expression after two weeks of treatment.
(Figure 3B). Relative NANOG (Figure 4A) and OCT4 (Figure 4B) levels showed a decrease in expression over the 7-week course of treatment, which is consistent with a loss of pluripotency. Based on the increased expression of endothelial markers at 2 weeks of treatment and the decrease in stem cell markers, a time course of 2 weeks was chosen for the duration of the experiments.

Figure 2. Schematic depicting the time course for the days of iPSC treatment.
Figure 3. Seven weeks of iPS cell treatment EC-CM. (A) PECAM expression of iPS cells after 7 weeks of EC-CM treatment. (B) Cdh5 expression of iPS cells after 7 weeks of EC-CM treatment. PECAM and Cdh5 levels are expressed as a fold change over untreated iPS cells.
Figure 4. Seven weeks of iPS cell treatment with EC-CM. (A) NANOG expression of iPS cells after 7 weeks of EC-CM treatment. (B) OCT4 expression of iPS cells after 7 weeks of EC-CM treatment. NANOG and OCT4 levels are expressed as a fold change over native EC.
EC-CM directs iPS cell differentiation to an endothelial phenotype

iPS cell expression of an endothelial phenotype was determined by expression of endothelial cell markers PECAM and Cdh5 through qPCR analyses; and fluorescence microscopy of Isolectin GS and SSEA-1. Cells treated for two weeks with EC-CM are labeled iPSC+CM. Relative PECAM expression of iPSC+CM is unchanged compared to untreated iPS cells (Figure 5A). Relative Cdh5 expression of iPSC+CM is significantly greater than untreated iPS cells (p<.001) (Figure 5B). Figure 6 depicts the nuclear Dapi stain in blue; the Isolectin GS signal in green; and the SSEA-1 signal in red. ECs have a positive green signal for Isolectin GS while iPS cells have a positive red signal for SSEA-1. Conversely, ECs stained negative for SSEA-1 while iPS cells show a slight positive signal for Isolectin likely due to the density of iPS cell colonies. Taken together, these positive and negative signals indicate that we can distinguish differences between iPS cells and endothelial cells using fluorescence microscopy and immunostaining. The iPSC+CM panels have been treated with EC-CM for 2 weeks. These cells have a positive signal for Isolectin GS and a negative signal for the stem cell marker SSEA-1 indicating that they no longer have a pluripotent phenotype consistent with an iPS cell, but rather are exhibiting a phenotype consistent with ECs. Collectively, the positive expression of PECAM, Cdh5 and the positive Isolectin signal; and the negative signal for the stem cell marker SSEA-1 indicate that iPSCs+CM assume an endothelial phenotype.
Figure 5. Relative PECAM and Cdh5 expression of native EC, untreated iPS cells and iPS+CM treated cells. (A) Relative PECAM expression of iPS+CM cells as compared to untreated iPS cells. (B) Relative Cdh5 expression iPS+CM cells as compared to untreated iPS cells. Relative expression of PECAM and Cdh5 are expressed as a fold change over native EC cells.
Figure 6. Isolectin binding and SSEA-1 staining for native EC, iPSC+CM cells and untreated iPSCs. Nuclear stain is indicated with blue Dapi. Isolectin binding is indicated with a green signal. A positive SSEA-1 stain is indicated with a red signal.
EC-CM directs iPS cell differentiation to an endothelial phenotype

iPS cell differentiation to an endothelial function was determined by analyses of iPSC+CM formation of a tube-like network on Matrigel and uptake of a-LDL. Figure 7 depicts 6 hours of tube formation on growth factor depleted Matrigel supplemented with 30ng/μL of VEGF. ECs formed a tube-like network consistent with their function while iPS cells stayed within colonies consistent with their phenotype on 0.1% gelatin. iPSC+CM formed a tube-like network consistent with an endothelial function. iPSC+CM were further analyzed for endothelial function by a-LDL uptake, a typical function of endothelial expression of the scavenger receptor. As expected, 97% of endothelial cells passing through the flow cytometer showed a positive uptake for a-LDL (Figure 8). Although only 6.4% of iPSC+CM showed a positive uptake for a-LDL, 0% of untreated iPS cells demonstrated uptake indicating a progression from an iPS cell to an endothelial phenotype (Figure 8). Taken together, these figures indicate the functional capacity of iPSC+CM to function as endothelial cells by forming a tube-like network on Matrigel and taking up a-LDL.
Figure 7. Tube formation assay on growth factor depleted Matrigel supplemented with 30ng/μL of VEGF. Native EC, iPSC+CM and untreated iPSC cells shown at 6 hours.
Figure 8. % of native EC, untreated iPSCs and iPSC+CM treated cells that take up a-LDL.
Specific 2. Determine if changes in the microenvironment influence the direction of iPS cell differentiation.

A hypoxic microenvironment induces greater directed differentiation of EC-CM treated iPS cells to an endothelial phenotype

Changes in the microenvironment were achieved by generating EC-CM under normoxic and hypoxic conditions. iPS cells were then treated with normoxic CM (NCM) and hypoxic CM (HCM). qPCR analyses were done to determine if EC derived HCM has a greater ability to direct iPS cell differentiation to an endothelial phenotype. Figure 9 depicts the relative PECAM, Cdh5 and Tie-2 expression of native EC; untreated iPS cells; iPSC+NCM and iPSC+HCM. PECAM expression of iPSC+HCM is greater than expression of iPSC+NCM; however, this expression still falls short of native EC (Figure 9A). Cdh5 expression of iPSC+HCM is 30 times greater than Cdh5 expression iPSC+NCM and is approaching Cdh5 expression of native EC (Figure 9B). iPSC+HCM had a significantly greater expression of Tie-2 as compared to both untreated iPSCs and iPSC+NCM (p<.05) and Tie-2 expression greater than that of native EC (Figure 9C). These data indicate that a change in the microenvironment by means of EC derived hypoxic CM induces a greater differentiation of iPS cells to an endothelial phenotype.
Figure 9. Relative PECAM, Cdh5 and Tie2 expression of native EC, untreated iPSCs, iPSC+NCM and iPSC+HCM. (A) Relative PECAM expression (B) Relative Cdh5 expression (C) Relative Tie2 expression of iPSC+HCM treated cells is significantly higher than untreated iPSCs (p<0.05) and significantly higher than iPSC+NCM (P<0.05). Relative expression is expressed as a fold change over native EC.
A hypoxic microenvironment induces greater directed differentiation of EC-CM treated iPS cells to an endothelial function

To determine if a change in the microenvironment by means of HCM induces a greater change in iPS cell directed differentiation to an EC function; tube formation on Matrigel was performed. Figure 10 depicts tube formation after 6 hours on growth factor depleted Matrigel supplemented with 30ng/μL of VEGF. ECs formed a tube-like network while untreated iPS cells remained in colonies (Figure 10). Interestingly, there was no appreciable difference between the tube-like network formed by iPSC+NCM and iPSC+HCM (Figure 10). In addition to a tube formation assay, α-LDL uptake was done to determine if iPSC+HCM cells showed a greater propensity for LDL uptake. iPSC+HCM showed an 11 fold greater uptake of α-LDL as compared to untreated iPS cells; and a nearly 2 fold greater uptake of α-LDL as compared to iPSC+NCM (Figure 11). Though iPSC+HCM don’t appear to have a greater endothelial function than iPSC+NCM with regards to tube formation on Matrigel; iPSC+HCM do show a greater endothelial function than iPSC+NCM with regards to their ability to take up α-LDL.
Figure 10: Tube formation assay on growth factor depleted Matrigel supplemented with 30ng/μL of VEGF. Native EC, iPSC+NCM, iPSC+HCM and untreated iPS cells shown at 6 hours.
Figure 11. % of native EC, untreated iPSCs, iPSC+NCM and iPSC+HCM treated cells that take up a-LDL.
Specific 3. Determine if the exosomal fraction of the microenvironment has the greatest affect on iPS cell differentiation.

An exosome depleted hypoxic microenvironment incudes greater directed differentiation of EC-CM treated iPS cells to an endothelial phenotype.

The normoxic and hypoxic microenvironment was divided into different fractions by removing and adding exosomes. Exosomes were removed from NCM and HCM (NCM-EXO and HCM-EXO) and transferred to treatment media. Treatment media (TM) supplemented with NCM and HCM exosomes are annotated as NCMEXO and HCMEXO respectively. CD9 coated dynabeads were used to verify that exosomal removal and TM supplementation was successful. Figure 12 shows that PF where exosomes were removed had a very low percentage of dynabeads staining positive while TM that was supplemented with PFEXO showed a high percentage of dynabeads staining positive for exosomes. Expression of an endothelial phenotype was analyzed by qPCR. However, we found that there was no appreciable difference in iPS cells treated with exosomes from NCM or HCM and so the data was combined so that CM encompasses both NCM and HCM; CM-EXO encompasses both NCM-EXO and HCM-EXO; and CMEXO encompasses both NCMEXO and HCMEXO. iPSCs treated with CM-EXO have an increased PECAM expression as compared to CM treated cells (Figure 13A). When iPSC cells were treated with CMEXO the previously observed increase in PECAM expression was blunted (Figure 13B).

A similar trend can be observed for Cdh5 expression. When iPSCs are treated with CM-EXO, Cdh5 expression increases to a level equal to that of native EC (Figure
14A). When the cells are treated with CMEXO, Cdh5 expression is blunted (Figure 14B).

A similar, more robust trend was observed with Tie-2 expression of treated cells. As with the other endothelial markers, iPSCs treated with CM-EXO showed an increase in Tie-2 expression greater than that of native EC (Figure 15A) while treating iPSCs with CMEXO significantly decreased Tie-2 expression (p<0.05) (Figure 15B). These data on Tie2 expression—taken in conjunction with the data on PECAM and Cdh5 expression—strongly indicate that the depletion of exosomes from EC-CM has a greater impact on directing iPS cell differentiation to an endothelial phenotype than EC-CM alone; and that the addition of exosomes decreases directed iPS cell differentiation.
Figure 12. % of CD9 coated dynabeads showing a positive signal for exosomes. PF-EXO is exosome depleted pericardial fluid while PFEXO is treatment media supplemented with pericardial fluid derived exosomes.
Figure 13. Relative PECAM expression of (A) iPSCs treated with CM and CM-EXO and (B) cells that were then treated with CMEXO. PECAM expression is relative to native EC.
Figure 14. Relative Cdh5 expression of (A) iPSCs treated with CM and CM-EXO and (B) cells that were then treated with CMEXO. Cdh5 expression is relative to native EC.
Figure 15. Relative Tie-2 expression of (A) iPSCs treated with CM and CM-EXO and (B) cells that were then treated with CMEXO. iPSC treated with CM-EXO have a significantly higher expression of Tie-2 as compared to CMEXO treated cells (p<0.05). Tie-2 expression is relative to native EC.
An exosome depleted hypoxic microenvironment includes greater directed differentiation of EC-CM treated iPS cells to an endothelial function.

To determine if the depletion of exosomes from the hypoxic microenvironment induces a greater directed differentiation of EC-CM treated iPS cells; analyses of tube were formation performed. Figure 16 depicts tube formation after 6 hours on growth factor depleted Matrigel supplemented with 30ng/μL of VEGF. As previously observed in Figure 10, NCM and HCM treated iPS cells are capable of forming tube-like networks on Matrigel—the same can be observed in Figure 11. There is no appreciable difference in the tube-like networks of NCMEXO and HCMEXO. The NCMEXO appear to have formed a more robust network while the HCMEXO are not as defined. Though they appear to be trending toward a well-defined network (Figure 16). Interestingly, even though exosome depleted treatments elicit a high expression of endothelial markers they do not form well-defined tube-like networks on matrigel although with a later time stamp, well defined networks may have been observed. This lack of a defined tube-like network is similar between iPSC+NCM-EXO and iPSC+HCM-EXO, which is of further interest considering the robust impact HCM-EXO has on directing iPS cell differentiation to an endothelial phenotype.

Further analyses on the impact of exosome depletion on iPS cell directed differentiation to an endothelial function was performed by a-LDL uptake. Figure 17 shows a slightly higher percentage of iPSCs treated with CM-EXO taking up a-LDL while treating iPSCs with CMEXO results in a significant decrease in the percentage of cells taking up a-LDL (p<0.05). This supports previous data that removing exosomes from CM enhances directed differentiation while adding exosomes decreases directed
differentiation. The Matrigel assay data are not conclusive in indicating whether or not exosome depletion has an impact on directing iPS cell differentiation into an endothelial function. However, the a-LDL uptake data indicate that exosome depleted CM does slightly increase cellular uptake of a-LDL indicative of endothelial function.
Figure 16. Tube formation assay on growth factor depleted Matrigel supplemented with 30ng/μL of VEGF. Treated cells are shown at 6 hours.
Figure 17. % of cells positive for acetylated LDL uptake. A significantly higher % of iPSCs treated with CM-EXO take up a-LDL as compared to iPSCs treated with CMEXO (p<0.05).
CHAPTER FOUR

DISCUSSION

This study was completed with the intent of furthering our understanding of iPS cell differentiation for their use in cardiac regeneration. The purpose of this thesis was two fold: 1) determine the ability to direct iPS cell differentiation; and 2) determine if changes in the microenvironment facilitate differentiation. Three specific aims were developed to examine these two points:

1) Determine the ability of the microenvironment to direct iPS cell differentiation to a desired function and phenotype.

2) Determine if changes in the microenvironment influence the direction of iPS cell differentiation.

3) Determine if the exosomal fraction of the microenvironment has the greatest affect on directing iPS cell differentiation.

The microenvironment used to direct iPS cell differentiation was generated through collection of CM derived from ECs. The use of CM as a suitable microenvironment was used for directing iPS cell differentiation as opposed to other methods such as nuclear reprogramming and/or the addition of VEGF because the use
of CM is more indicative of the in vivo interaction between iPS cells and their microenvironment [33]. This concept of using CM as a means of directing stem cell differentiation was previously demonstrated in the use of bladder-cancer derived CM in the successful directed differentiation of amniotic stem cells to an urothelial specific lineage [33]. In addition, the use of CM has previously been used to represent in vivo microenvironment interactions of different cells. Pre-osteoblast mouse cells were treated with conditioned media derived from a mouse osteosarcoma line in an effort to better understand in vivo microenvironment interactions between osteosarcoma cells and osteoblasts that allowed the progression of osteosarcoma [34]. This ex vivo CM model was able to give insight to in vivo pathogenesis. EC-CM was chosen as opposed to cardiomyocyte-CM because we believe that revascularization of the injured myocardium is essential to rescuing cardiomyocytes in the border zone; facilitating the break down of the collagen rich matrix of the infarct zone; and creating a suitable oxygen and nutrient rich environment for the engraftment of new cardiomyocytes [35]. By inducing the differentiation of iPS cells to endothelial cells, we hope to promote neovascularization to not only support the coronary collateral network, but to also facilitate oxygen delivery and flow to the stressed myocardium with the goal of preventing progression to ischemic cardiomyopathy.

Establishing a time line for treatment

The decrease in relative PECAM and Cdh5 expression of treated iPS cells in Figures 3A and 3B served as a model for deciding on the 2 week time course for treatment. It was not altogether surprising to see this decrease in endothelial marker
expression after 2 weeks of treatment. In a study aimed at characterizing functional endothelium derived from human iPS cells (HiPSC), García-Cerdeña et al. showed a similar decrease in PECAM and Cdh5 expression with more than two weeks of treatment with differentiation medium [36]. This study shows that PECAM expression of treated HiPS cells peaks at 12 and 14 days and then declines with more than 15 days of treatment [36]. These data are consistent with our findings in Figure 3A which depicts peaks in PECAM expression at T1 (14 days of treatment) and T2 (just over 14 days of treatment) and then a decline in PECAM expression at T3. This study also depicts an abrupt decrease in expression of Cdh5 after 14 days of treatment with a very slight increase in expression at 15 days and a decrease in expression at more than 15 days of treatment [36]. These data are consistent with our findings in Figure 3B which depicts an increase in Cdh5 expression at 14 days of treatment that drops off with more than 15 days of treatment. This study, in addition to the decrease in stem cell markers NANOG and OCT4 in Figures 4A and 4B respectively, supports our finding that the optimal time course for iPS cell treatment is two weeks.

**EC-CM directs iPS cell differentiation to an endothelial phenotype and function**

EC-CM shows a modest capacity for differentiating iPS cells to an endothelial phenotype as evidenced by Figures 5 and 6. Figure 5A shows that iPSC+CM express higher levels of PECAM; however, this increase is minimal while Cdh5 expression of iPSC+CM is significantly higher than that of the untreated iPSCs (Figure 5B). Unfortunately, PECAM and Cdh5 expression of iPSC+CM cells remains far below that of native EC (Figures 5A and 5B). Figure 6 shows the ability of Isolectin GS-IB4 to bind
to the iPSC+CM. In comparison to untreated iPS cells, treated cells show a much greater ability to bind Isolectin GS-IB4 (Figure 6). Similarly, treated iPS cells did not stain positive for SSEA-1 while untreated iPS cells did (Figure 6). The ability to bind Isolectin GS-IB4 while not staining positive for SSEA-1 indicates a progression from a pluripotent state to an endothelial phenotype. As is similar to the data regarding PECAM and Cdh5 expression, the positive Isolectin signal in Figure 6 is present but faint. EC-CM shows a robust capacity for differentiating iPS cells to an endothelial function as compared to untreated iPS cells in Figures 7 and 8. iPSC+CM formed a tube-like network on Matrigel comparable to network formation by native EC (Figure 7). As expected, 6.4% of iPSC+CM cells took up a-LDL as compared to 0% of untreated iPS cells (Figure 8). While this attribute of endothelial function is greater in treated cells as opposed to untreated cells, it is still much lower than of native endothelial cells. Figures 3-8 indicate an EC-CM directed differentiation of treated iPS cells, however, this is not the robust differentiation we would have expected. A study completed by Rufaihah et al showed that iPS cells differentiated to an endothelial function displayed endothelial heterogeneity [32]. It is possible that this endothelial heterogeneity is responsible for the diluted positive data observed in Figures 3-8. In order to see a more robust signal, endothelial cells were selected from the overall population using a CD31 antibody (PECAM) [32]. Once isolated, these cells then showed a more robust positive signal comparable to that of native EC [32]. It is possible that the positive signals indicating an EC-CM directed iPS cell differentiation were diluted due to a mixed cell population. In addition, endothelial cells display functional heterogeneity i.e. PECAM expression is higher in arterial EC and they’re more likely to form a well-defined tube-
like network and bind Isolectin GS-IB4; Cdh5 levels are higher in lymphatic EC and they're not as likely to form well-defined tube-like networks; and capillary and aortic EC have a higher propensity for a-LDL uptake [32, 37, 38]. It is possible that the EC-CM treated iPS cells not only yielded a heterogeneous cell population but also yielded a heterogeneous endothelial population, which may have further washed out the positive data observed in Figures 3-8. Interestingly, stem cell differentiation with regards to in vivo signaling from endothelial cells may experience the same EC functional heterogeneity yielding endothelial cells that are not conducive to forming the capillary network necessary to vascularize the ischemic myocardium. For specific aim 1, we hypothesized that EC-CM treated iPS cells would differentiate to functional EC. Though iPSC differentiation to an endothelial phenotype and function is not as robust as we might have thought, there is still a clear trend towards directed differentiation.

A hypoxic microenvironment induces greater directed differentiation of EC-CM treated iPS cells

A hypoxic environment serves as a catalyst for the local release of growth factors cytokines and chemokines that influence the migration and proliferation of endothelial cells to form new blood vessels in the angiogenic pathway [39]. VEGF has been identified as one of the most predominant growth factors in initiating angiogenesis and, as previously mentioned, VEGF is also a leading component in directing stem cell differentiation to an endothelial function and phenotype [32, 39]. We believe that ECs cultured under hypoxic conditions secrete growth factors and chemokines that would not only facilitate angiogenesis, but also secrete growth factors that would influence the
differentiation of iPS cells to an endothelial function further aiding the angiogenic pathway. The data presented in figures 9-11 display the capacity for HCM to direct iPS cell differentiation to an endothelial function and phenotype as compared to NCM. Interestingly, iPSC+HCM displayed a greater expression of endothelial markers PECAM, Cdh5 and Tie-2 than iPSC+NCM (Figures 9A,B and C); the greatest difference being the significant increase in Tie-2 expression of iPSC+HCM as compared to untreated iPSCs and iPSC+NCM (p<0.05) (Figure 9C). What makes these data most interesting is that this significant increase in Tie-2 expression brings HCM treated cellular expression of Tie-2 equal to that of native EC (Figure 9C). The Tie-2 receptor is essential for the hypoxia driven angiogenesis of embryonic development indicating the role of hypoxia in inducing the angiogenic endothelial receptor [40]. Figure 9C indicates that CM derived under hypoxic conditions not only directs the differentiation of iPS cells to an endothelial phenotype, but drives the endothelial cells to express high levels of Tie-2 thus facilitating the angiogenic pathway by increasing the number of endothelial cells expressing Tie-2. Although Tie-2 expression is much higher in iPSC+HCM and these cells would be expected to form a more well defined tube like network than NCM treated cells, Figure 10 indicates comparable tube formation between HCM and NCM treated cells. It is possible that the lack of tube formation in the presence of high Tie-2 mRNA expression may be because the mRNA is not translated into a functional protein. If Tie-2 mRNA was translated into a functional protein, the lack of a more well defined network may be due to the high concentration of the competitive inhibitor angiopoietin-2 as compared to the Tie-2 agonist angiopoietin-1 [41]. The competitive inhibition of the Tie-2 agonist would have decreased cell contact and resulted in a diminished tube-like
network like the one seen in the iPSC+HCM panel of Figure 10. Consistent with the increased endothelial phenotype observed in iPSC+HCM, Figure 11 shows increased a-LDL uptake of HCM treated cells as compared to NCM treated cells. This increase in uptake likely has less to do with the hypoxic environment itself and more to do with hypoxia driving differentiation to an endothelial function with typical a-LDL uptake. iPSC+HCM differentiation towards high expression of Tie-2 perpetuates a more robust angiogenic response that may be indicative of a physiological compensation in the microenvironment to alleviate the hypoxic tissue. For specific aim 2, we hypothesized that HCM would induce a greater direction of iPS cell differentiation to an endothelial function and phenotype. The increased expression of endothelial markers PECAM, Tie2 and Cdh5 by iPS cells treated with HCM as compared to NCM supports our hypothesis. Figures 9-11 indicate a clear trend for greater HCM directed differentiation.

An exosome depleted hypoxic microenvironment induces greater directed differentiation of EC-CM treated iPS cells

Though the injection of stem cells into the injured myocardium improves cardiac function, the differentiation and engraftment of these cells is simply too low to account for the observed benefit [31]. The paracrine theory has been proposed as a means of explanation. ES cell CM alone was injected into an ischemic myocardium and was able to restore cardiac function [42]. Similarly, MSC derived CM injected into the injured myocardium showed a decrease in infarct size [43]. Exosomes involved in intercellular communication between different types of cells are being characterized as playing a central role in this paracrine effect [31]. It is for this reason that we hypothesized that
the addition of HCMEXO to the microenvironment would increase directed iPS cell differentiation while the depletion of exosomes from the microenvironment would decrease iPS cell differentiation. However, it was observed that there was no appreciable difference between normoxic and hypoxic treatment groups. The data presented in Figures 13-17 contradict our hypothesis, but show an interesting trend none-the-less. In Figures 13-15 the removal of exosomes increases marker expression, while the addition of exosomes decreases this expression. This decrease implies that exosomes are playing an inhibitory role in the directed differentiation of iPS cells. The increase in Tie-2 expression (Figure 15) does not translate to a well-defined tube-like network on Matrigel as would be expected of increased Tie-2 expression (Figure 16). Admittedly, Figure 16 is difficult to reconcile. At this time, it appears that the addition and removal of exosomes has no consistent impact on the ability of treated iPS cells to form a tube-like network on Matrigel. a-LDL uptake in Figure 17 is more consistent with the treated cellular expression of PECAM, Cdh5 and Tie-2 in Figures 12-15. The removal of exosomes shows an increased uptake of acetylated LDL as compared to iPS cells treated with exosomes. This uptake of a-LDL is consistent with typical endothelial function and is further consistent with the data presented in Figures 12-15. Although these data are not consistent with the typical positive effects observed with exosomes, it is possible that the endothelial cells were not incubated in conditions hypoxic enough to generate exosomes that would induce endothelial differentiation of iPS cells. In a more hypoxic microenvironment, these endothelial cells may generate exosomes with the intention of differentiating iPS cells to an endothelial function and phenotype as a rescue mechanism.
In this study we have demonstrated that EC-CM has the ability to direct iPS cell differentiation into an endothelial function and phenotype and that this directed differentiation is more robust when EC-CM is derived under hypoxic conditions. These data indicate that HCM may serve as a rescue function by increasing the differentiation of endothelial cells for vascularization of the ischemic tissue. iPC cell differentiation with the removal and addition of exosomes is inconsistent with our hypothesis and current literature on the therapeutic benefits of exosomes; however these data may be more indicative of the level of ischemia necessary for endothelial cells to secrete exosomes directed at iPS cell differentiation as a rescue mechanism.

**Future Studies**

To better understand the inconsistencies of the tube formation assays, analyses for angiopoietin 1 and 2 should be conducted to determine their presences in CM. Future Matrigel studies should include treatment groups without VEGF and with CM alone to determine if the growth factors necessary for endothelial tube formation on matrigel are present in the CM; this may help to address the role of growth factors that are not found in exosomes but are still present in the CM. In order to further observe differences in iPS cells treated with HCM as opposed to NCM, glucose deprivation should be done along with hypoxia as simulating an ischemic environment would also lead to a decrease in nutrients. Importantly, the contents of the exosomes should be analyzed to determine if it is a miRNA exerting this affect and if it is, which one.
REFERENCES


