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Molecular Mechanisms That Govern Human Cardiac Stem Cell Age Disparity

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Molecular Mechanisms That Govern Human Cardiac Stem Cell Age Disparity

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree of
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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

αMHC	Cardiac Myosin Heavy Chain Alpha
αSA	Smooth Muscle Alpha Actin
AKT	Protein Kinase B
ATP	Adenosine Tri Phosphate
bFGF	Fibroblast Growth Factor (Basic)
BMMCs	Bone Marrow Mononuclear Cells
CDCs	Cardiosphere-Derived Cells
CKIT or CD117	Tyrosine Protein Kinase Kit
COX-2	Cyclooxygenase 2
CPCs	Cardiac Progenitor Cells
CSCs	Cardiac Stem Cells
cTnI	Cardiac Troponin I
CXCR4	Chemokine (C-X-C motif) Receptor 4
CXCR7	Chemokine (C-X-C motif) Receptor 7
EGFR	Epidermal Growth factor Receptor
EP1	Prostaglandin E Receptor 1
EP2	Prostaglandin E Receptor 2
EP3	Prostaglandin E Receptor 3
EP4	Prostaglandin E Receptor 4
EPO	Erythropoietin
ERK	Extracellular Signal-Regulated Kinase
E2F1	E2F Transcription Factor 1

FGF	Fibroblast Growth Factor
Gata-4	Gata Binding Protein 4
G-CSF	Granulocyte Colony-Stimulating Factor
hESC	Human Embryonic Stem Cells
HGF	Hepatocyte Growth Factor
HOXA5	Homeo Box A5
IGF-1	Insulin-like Growth Factor 1
IGF-2	Insulin-Like Growth Factor 2
IGF-1R	Insulin-Like Growth Factor 1R
iPSCs	Induced Pluripotent Stem Cells
ISL1	Islet-1
LVEF	Left Ventricular Ejection Fraction
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
miRNA	MicroRNA
MSCs	Mesenchymal Stem Cells
MDR-1	ATP-Binding Cassette, Sub-Family B, Member 1
MESP1	Mesoderm Posterior Homolog 1
Mlc2v	Cardiac Myosin Light Chain 2
Nkx2.5	NK2 Homeobox 5
OCT4	Octamer-Binding Protein 4
PDGFR or CD140a	Platelet-Derived Growth Factor Receptor

pAKT	Phosphorylated Protein Kinase B
pERK	Phosphorylated Extracellular Signal-Regulated Kinase
PGE_2	Prostaglandin E_2
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3’ Kinase
RAD50	DNA Repair Protein RAD50
SDF-1α	Stromal Cell-Derived Factor 1α
SSEA-4	Stage Specific Embryonic Antigen 4
Tbx1	T-Box 1
Tbx20	T-Box 20
TGF-β1	Transforming Growth Factor, Beta Receptor 1
TIMP-3	TIMP Metallopeptidase Inhibitor 3
VEGFA	Vascular Endothelial Growth Factor A
VEGFR1	Vascular Endothelial Growth Factor Receptor 1
VEGFR2 or KDR	Vascular Endothelial Growth Factor Receptor 2
VEGFR3	Vascular Endothelial Growth Factor Receptor 3
vWF	Von Willebrand Factor

ABSTRACT OF THE DISSERTATION

Molecular Mechanisms That Govern Human Cardiac Stem Cell Age Disparity

by

Tania Fuentes

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
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Dr. Mary Kearns-Jonker, Chairperson

Transplantation of adult endogenous cardiovascular progenitor cells for heart repair results in some clinical benefit, however these stem cells lack the regenerative capacity unique to neonatal cardiovascular stem cells. The goal of this work was to identify mechanisms that contribute to the decline of cardiac stem cell regenerative ability with age and investigate novel therapeutic strategies to improve cardiac stem cell function. When comparing neonatal and adult cardiovascular stem cell clones, both cell types were capable of cardiomyogenic differentiation. However, the expression levels of forty-one microRNAs were significantly altered with age. Expression differences were correlated with reduced proliferation and a limited capacity to invade in response to growth factor stimulation in adult progenitors. This information can be utilized to develop novel therapeutics and improve outcomes after stem cell-based treatment for heart failure.

CHAPTER ONE

INTRODUCTION

The ability of stem cells to stimulate repair of the heart, an organ once thought to be static and unable to regenerate, has the potential to transform modern medicine. Each year, an estimated 620,000 Americans have a new coronary attack and 295,000 have a recurrent attack (Go et al., 2014). On average it takes over an hour for a patient to receive medical attention (Fosbøl et al., 2013; Rasmussen et al., 2014), and once a patient is treated, there is significant heart muscle damage. Various types of stem cells capable of cardiac differentiation have been advanced to human clinical trials in an effort to repair the injured heart (R. Bolli et al., 2011; Delewi et al., 2014; Karantalis et al., 2014).

Bone Marrow Mononuclear Cells

More than 50 clinical trials using bone marrow mononuclear cells (BMMCs) as a cell-based therapy for the infarcted heart have been completed (Delewi et al., 2014). Unfractionated BMMCs are a heterogeneous mixture of cell types containing monocytes, lymphocytes, haematopoietic progenitor and precursor cells and an occasional haematopoietic stem cell. Thus far, the transplantation of unfractionated bone marrow mononuclear cells for heart injury has an excellent safety profile (Xu, Ding, Zhao, Pu, & He, 2014). The functional outcomes after transplantation, however, tend to be wide-ranging. Some have attributed heterogeneity in functional outcomes to differences in protocols for BMMC harvest, isolation, storage, and delivery (Wollert, 2015). Others have attributed the inconsistency of functional outcome to data integrity (Nowbar et al.,

2014). An analysis of 49 clinical trials, including both fully randomized and not fully randomized trials, revealed that there is a positive correlation in number of discrepancies in published work and the reported percentage improvement in LVEF (Nowbar et al., 2014). Meta-analyses that only include fully randomized clinical trials have determined that the benefit in left ventricular ejection fraction after BMMC transplantation either for myocardial infarction or chronic heart failure falls somewhere between 2.55 – 3.54% (Delewi et al., 2014; Xu et al., 2014). This modest benefit, although promising is not sufficient to completely ameliorate loss of function after heart injury.

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) have not yet advanced to clinical trials but are a cell type that is capable of cardiomyogenic differentiation and holds promise for regenerating the heart. Induced pluripotent stem cells originate as differentiated cells that, through the introduction of defined factors, gain a state of pluripotency functionally similar to embryonic stem cells. iPSC can be generated through viral-mediated overexpression of proteins (Giorgetti et al., 2009; Haase et al., 2009; Martinez-Fernandez et al., 2009; Takahashi & Yamanaka, 2006) or small RNAs (Anokye-Danso et al., 2011; S.-L. Lin et al., 2008; Miyoshi et al., 2011). However, due to cancer concerns, non-viral methods such as exposure to protein extracts (Park et al., 2014) or defined chemicals (Hou et al., 2013) have also been developed to reprogram cells.

iPSC-derived Cardiac Progenitors

Induced pluripotent stem cells can be utilized to form cardiovascular progenitors

by partially differentiating iPSCs into cardiomyocytes, these cardiovascular progenitors can be stably maintained in culture once differentiation-inducing signals are eliminated (Cao et al., 2013). iPSC-derived multipotent cardiovascular progenitors have the ability to form all three cardiovascular lineages (Carpenter et al., 2011; B. Lin et al., 2012; Lu et al., 2013; Alessandra Moretti et al., 2010; Nsair et al., 2012), and once differentiated, cardiomyocytes derived from iPSC multipotent cardiac progenitors can form electrically and mechanically coupled large-scale 2D cell cultures with mature electrophysiological properties (Christoforou et al., 2013). Human iPSC-derived multipotent cardiovascular progenitors, when seeded into a decellularized heart, migrate, proliferate, and differentiate into cardiovascular lineages. After 20 days, seeded hearts exhibited spontaneous contractions and generated mechanical force (Lu et al., 2013). *In vivo*, when transplanted into the rat myocardium, human iPSC-derived multipotent cardiac progenitors were retained long term (10 weeks) with evidence of differentiation into cells of cardiac lineage (Carpenter et al., 2011).

Not all partially differentiated pluripotent cells, have an equal propensity for cardiac lineage differentiation. In mice, sorting for the cell surface marker combination of VEGFR1 and VEGFR3 can enrich for an iPSC-derived $Isl1^{+}/Nkx2.5^{+}$ cardiac progenitors with trilineage cardiovascular potential *in vitro*. Twenty-eight days post-transplantation *in vivo*, there was evidence of cardiovascular progenitor cell engraftment and differentiation into mature cardiomyocytes *in vivo* (Nsair et al., 2012). Similarly, expression of VEGFR2 (KDR) in mouse iPSC-derived cardiac progenitors can separate multipotent progenitor functional ability. Transplantation of KDR^{+} cells results in

superior functional benefits and a greater number of vascular structures when compared with KDR^{-} cells (Mauritz et al., 2011).

Human iPSC in Large Animal Models

To date, the majority of studies completed in large animal models with iPSCs have utilized transplantation of human iPSCs once the cells have been successfully differentiated into relevant cardiovascular lineages.

Transplantation of cardiomyocyte cell sheets derived from human iPSC after ischemic cardiomyopathy significantly improved cardiac performance and attenuated left ventricular remodeling with no teratoma formation in pigs (Kawamura et al., 2012). After 8 weeks, however, few cells survived within the myocardium. Improved cell survival can be achieved with transplantation of highly vascularized omentum flap tissue along with iPSC-derived cardiomyocyte sheets (Kawamura et al., 2013). Transplantation of human iPSC-derived endothelial cells and smooth muscle cells into swine following ischemia-reperfusion injury can improve c-kit+ cell recruitment, vascular density, and ATP turnover rate after 4 weeks. This treatment also improves blood flow and contractile function in the border zone (Xiong et al., 2013). Combination of iPSC-derived cardiomyocytes, endothelial cells, and smooth muscle cells, when administered in pigs with a fibrin patch containing IGF-1 significantly improves left ventricular function, decreases infarct size, and reduces apoptosis after myocardial infarction (L. Ye et al., 2014).

Considerations for iPSC Clinical Use

In spite of promising results after application of iPSC (both differentiated and

undifferentiated) as a cell-based therapy for cardiac injury in large animals, there are several roadblocks that must be overcome before iPSC transplantation can feasibly be considered for use in a clinical setting. The whole process of reprogramming tends to be mutagenic resulting in chromosomal alterations and a significant number of copy number variants (Gore et al., 2011; Hussein et al., 2011; Ji et al., 2012; Park et al., 2014). Furthermore, induced pluripotent stem cells, although genetically reprogrammed, retain an epigenetic memory (including unique DNA methylation patterns) of their cell type of origin (Ohi et al., 2011). This epigenetic memory can result in an inherent predisposition towards differentiation into a given cell type (Rizzi et al., 2012). Therefore, induced pluripotent stem cells derived from easily accessible tissues such as the skin, may not be as ideal for cardiac transplantation as other cell types which are closer to the cardiac lineage. Optimization of reprogramming and differentiation methods is necessary to ensure that the iPSCs and iPSCs-derived cells fully mimic the desired cell type

Although induced pluripotent stem cells are an innovative and readily accessible source of cardiovascular progenitors, the possibility of cancerous transformation and low efficiency of reprogramming with non-viral methods present a significant barrier to their use in clinical practice.

Human Endogenous Cardiac Stem Cells

Of the cell types investigated for their ability to regenerate the heart after injury, resident cardiac progenitor cells (CPCs) are a preferred cell type because of their high propensity for cardiomyogenic differentiation, relative safety, and lack of cancerous transformation when transplanted *in vivo* (Konstantinos Malliaras et al., 2014b). When compared with stem cells that reside outside of the heart such as bone marrow-derived

mesenchymal stem cells, adipose-tissue-derived mesenchymal stem cells, or bone marrow mononuclear cells, resident cardiac progenitors have a higher capacity for cardiomyogenic differentiation and paracrine signaling resulting in greater improvements in cardiac function after transplantation *in vivo* (Koninckx et al., 2011; Li et al., 2012; Takehara et al., 2008). In human clinical trials, utilization of resident cardiac stem cells as a cell-based treatment after myocardial infarction have shown that these cells can reduce infarct size and result in improvements in patient outcomes lasting at least 1-2 years (R. Bolli et al., 2011; Makkar et al., 2012; Konstantinos Malliaras et al., 2014b).

Several stem cell types have been isolated from the heart and defined on the basis of surface phenotype including ckit+ cells, cardiospheres, cardiosphere-derived cells, and ISL1+ cells (Table 1). Which of these cells types is most optimal for cardiovascular regeneration is unknown. In this chapter we will summarize the current literature on heart-derived cardiac stem cells.

Ckit+ Cardiac Stem Cells

Ckit+ cardiac stem cells (CSCs) are isolated from the heart by enzymatic digestion and sorted on the basis of tyrosine protein kinase, ckit, expression and absence of leukocyte common antigen, CD45. Ckit antigen expression can be found in a variety of tissues within the body (Lammie et al., 1994). Ckit+ cells isolated from the heart have the ability to form all three cardiac lineages (Claudia Bearzi et al., 2007). According to Bearzi et al. (2007), these cells typically do not express CD34, CD31 (platelet/endothelial cell adhesion molecule), or KDR (kinase insert domain receptor) and a small percentage (0.5%) of ckit+ cardiac stem cells express GATA4 (gata binding protein 4) and Nkx2.5

Table 1. Markers that define human heart-derived stem cells*

Ckit+ Cardiac Stem Cells (Bearzi et al., 2007)	**Negative for:** CD45, CD34, CD31, KDR **Positive for:** Ckit
└ Vascular Ckit+ Stem Cells (Bearzi et al., 2009)	**Negative for:** CD45, CD34, CD133, αSA **Positive for:** Ckit, KDR, $CD31^{low}$, TGF-$\beta1^{low}$
└ Myogenic Ckit+ Stem Cells (Bearzi et al., 2009)	**Negative for:** CD45, CD34, CD133, CD31, TGF-β1, KDR **Positive for:** Ckit, αSA^{low}
└ Endothelial Ckit+ Stem Cells (Sandstedt et al., 2010)	**Negative for:** CD45 **Positive For:** Ckit, KDR, CD31, CD34, CXCR4
└ Ckit+ cardiac stem cells (Koninckx et al., 2011)	**Negative for:** CD140, CD34, CD133, CD45, FGF **Positive for:** CD13, CD29, CD44, CD73, CD90, EGFR (lost Ckit expression with passaging)
└ Lin Depleted Ckit+ CSCs (He et al., 2011)	**Negative for:** CD45, CD34, CD133, CD31, KDR **Positive for:** Ckit
└ non-Lin depleted Ckit+ CSCs (He et al., 2011)	**Negative for:** CD45, CD34, CD133 **Positive for:** Ckit, KDR, CD31
Cardiospheres (Davis et al., 2009)	**Negative for:** CD45, CD34 **Positive for:** Core (Ckit, Nkx2.5, connexin 43, Desmin) Periphery (CD31, CD105, αMHC, cTnI, CD133, MDR-1, CD90)
Cardiosphere-derived Cells (Li et al., 2012)	**Negative for:** CD45, CD34, CD31, CD133 **Positive for:** $Ckit^{low}$, CD105, CD29, $CD90^{low}$
ISL1+ Cardiac Progenitors (Serradilfalco et al., 2011)	**Positive for:** ISL1, Ckit (only identified in fetus)
└ Ckit+ ISL1+ CPCs (T. Fuentes et al., 2013)	**Negative for:** HLADr **Positive for:** ISL1**,** Ckit, CD73, CD105, CD44, CD13, IGF1R, CXCR4, CXCR7, HLA class I, CD140a
└ hESC-derived ISL1+ CSCs (A. Moretti et al., 2006)	**Negative for:** Tbx5 **Positive for:** ISL1, KDR, Nkx2.5, Gata-4, Tbx20, Tbx1

└ hESC-derived ISL1+ CSCs (Bu et al., 2009)	**Negative for:** KDR, Nkx2.5 **Positive for:** ISL1
└ hESC-derived ISL1+ CSCs (K. O. Lui et al., 2013)	**Negative for:** Neurofilament **Positive for:** ISL1, VEGFR1, KDR^{low}

*Modified from (Tania Fuentes & Kearns-Jonker, 2013)

(cardiac-specific homeo box) (Claudia Bearzi et al., 2007). Sandstedt et al. (2014) using single cell PCR analysis found that 81% of these cell express vWF, 72% express KDR, and 1.1% express Nkx2.5 and suggested that instead of being truly multipotent, this population consists of some already lineage committed progenitors, more specifically endothelial progenitors (Sandstedt et al., 2014; Sandstedt, Jonsson, Lindahl, Jeppsson, & Asp, 2010).

The role of ckit+ cardiovascular progenitors in myocardial regeneration is highly debated (Molkentin, 2014; Nadal-Ginard, Ellison, & Torella, 2014a, 2014b; Torella, Ellison, & Nadal-Ginard, 2014; van Berlo et al., 2014). Ellison et al found that ablation of endogenous cardiac stem cells abolishes regeneration after myocardial damage. Recovery of myocardial regeneration can be achieved by exogenous administration of the progeny of one ckit+ cardiac stem cell (Ellison et al., 2013). After regeneration, selective suicide of transplanted cells and their progeny abolishes regeneration, severely impairing ventricular performance (Ellison et al., 2013). This suggests that the ckit+ cardiac stem cell is mainly responsible for the regenerative capacity of the heart.

Critics of this idea include van Berlo et al (2014) who, using a genetic lineage tracing, reported that endogenous c-kit+ cells produce new cardiomyocytes within the heart but at a very low percentage of approximately 0.03 or less which may be functionally insignificant to myocardial regeneration. Consistent with this, Senyo et al found, using pulse-chase and genetic fate mapping, that the genesis of cardiomyocytes with age occurs mainly through the division of pre-existing cardiomyocytes, and that this process increases after myocardial injury (Senyo et al., 2013). Whether or not ckit+ cells contribute significantly to the cardiomyocytes within the heart after injury, although

highly debated, may not be as important as their paracrine role in regeneration. A recent study by Hong et al found that despite rapid disappearance of ckit+ cardiac stem cells following transplantation, intracoronary delivery of stem cells significantly improved left ventricular function at 35 days post-transplant. Therefore, functional improvement may be not be dependent on survival and differentiation of transplanted cells (Hong et al., 2014).

Because of the inherent heterogeneity of stem cells sorted on the basis of ckit and absence of CD45 expression, subpopulations of ckit+ cardiovascular progenitors have differing functional properties. Ckit+ cardiovascular progenitors that also express KDR tend to be found in epicardial coronary arteries, arterioles, and capillaries and are vascular progenitors whereas KDR- Ckit+ cardiovascular stem cells are found in the myocardial interstitium and are myogenic progenitors (C. Bearzi et al., 2009). Insulin-like growth factor-1 receptor expression can also separate functionally distinct ckit+ progenitors. Transplanted IGF1R+ ckit+ cardiac stem cells can significantly increase infarcted tissue regeneration (12mm) when compared with IGF1R- cardiac stem cells (7mm). Pretreatment of these cells with IGF-2 can further improve tissue regeneration (15mm) and ejection fraction (D'Amario et al., 2011). Cardiac stem cells (because they use asymmetric chromatid segregation when dividing) can also be subdivided by the integrity of their DNA. Kajstura et al found that transplantation of cells that retained the template DNA when compared with ckit+ cardiac stem cells with the synthesized copy of DNA resulted in a greater restoration of infarct thickness ($60mm^3$ vs $25mm^3$) and a greater attenuation of diastolic wall stress (Kajstura et al., 2012).

The percentage and functional properties of ckit+ cardiac stem cells within the heart is not consistent from person to person (Gambini et al., 2012; Song et al., 2013). Females have a greater number of ckit+ cardiac progenitors in their heart than males (Itzhaki-Alfia et al., 2009) and failing hearts can have as many as 4 times the number of ckit+ cardiac progenitors (Kubo et al., 2008). Aging can also play a role in stem cell distribution and function. The total number of ckit+ cardiac stem cells in the heart decreases dramatically after the first month of life (Mishra et al., 2011). Even well into adulthood, age remains a factor in the yield of ckit+ cardiac stem cells that can be obtained from a tissue biopsy (Aghila Rani, Jayakumar, Sarma, & Kartha, 2009). With age, the percentage of senescent ckit+ stem cells in the heart increases, as demonstrated by telomere shortening, attenuated telomerase activity, telomere dysfunction- induced foci, and the expression of cyclin-dependent kinase inhibitor 1A and 2A (Cesselli et al., 2011). In young stem cells, pretreatment with Ephrin A1 prior to transplantation resulted in enhanced speed of CSC migration, a 37% decrease in infarct size (2 fold greater than CSC alone), and a significant increase in ejection fraction and left ventricular developed pressure (Goichberg et al., 2011). In aged stem cells, on the other hand, elevated levels of reactive oxygen species interfered with receptor auto-phosphorylation and downstream signaling resulting in inhibition of migratory ability *in vitro* and impaired translocation *in vivo* in the damaged heart. Overexpression of functionally intact Ephrin type-A receptor 2 in old CPCs corrected the defects in signaling and enhanced cell motility (Goichberg et al., 2013).

In spite of evidence to the contrary, the old heart is not devoid of functional cardiac stem cells, both senescent CSCs with extremely short telomeres and

phenotypically young CSCs carrying long, intact telomeres co-exist within the aged heart. The CSCs with long telomeres, however, are quiescent and do not re-enter the cell cycle or form differentiated progeny (Sanada et al., 2014). Therefore strategies to isolate or activate these phenotypically young stem cells within the aged heart are necessary to enhance regeneration.

Ckit+ Cardiac Stem Cells in Large Animals

Few studies have utilized ckit+ cardiac stem cells in large animal models. Bolli et al delivered ckit+ cardiac stem cells into the infarct-related artery three months after ischemia-reperfusion injury in pigs (n=11). This resulted in significantly greater ejection fraction (51.7±2.0% versus 42.9±2.3%, $P<0.01$ by echo) when compared with controls (n=10) at one month post infusion. Green fluorescent protein-labeling of CSCs allowed for identification of CSC differentiation into cells that expressed cardiac troponin I, troponin T, myosin heavy chain, and connexin 43. There was also evidence of labeled-CSCs forming vascular structures that expressed alpha-smooth muscle actin (R. Bolli et al., 2013).

Welt et al injected 1.6 million ckit+ CSCs 6 weeks after coronary ligation in the canine heart, and measured long-term changes in outcomes after 30 weeks. In the cell-treated group (n=13), the ejection fraction remained stable from 6 weeks to 24 weeks post-infarct (30.5% to 32.9%) when compared with the control group (n=6) which declined during the same time frame (35.2% to 26.4%). This suggest that the favorable outcomes after cell therapy can potentially last long-term (Welt et al., 2013).

A combination treatment of CSCs along with mesenchymal stem cells (MSCs) results in additive improvements in heart regeneration. Human cardiac stem cells at a dose of 1 million cells and MSCs at a dose of 200 million cells were administered 14 days after myocardial infarction in Yorkshire swine. After 4 weeks, there was a 2-fold greater reduction in infarct size (21.1% combo, n=5 vs 10.4% ckit+ CSCs alone, n=5 and 9.9% MSCs alone, n=5) and a 7-fold enhanced engraftment in the combination cell therapy group when compared to either cell therapy alone. In all cell treated groups, ejection fraction was restored to baseline whereas in control (n=5) it remained persistently depressed (Williams et al., 2013).

Cardiospheres and Cardiosphere-derived Cells

Cardiospheres are exclusively heart-derived stem cells (White et al., 2013) that are treated with specific growth medium to form non-adherent sphere-like structures (Smith et al., 2007). The core of cells within the cardiosphere contains cells that express ckit, nkx2.5, and desmin (Davis et al., 2009). Cardiospheres also have a percentage of cells that are CD45+, these cells do not play a role in cardiosphere formation and their function in regeneration is unclear (J. Ye et al., 2013). Cardiospheres when plated and grown as a monolayer are called cardiosphere-derived cells (CDCs). When compared with other cell types such as bone marrow-derived mesenchymal stem cells, adipose tissue-derived mesenchymal stem cells, and bone marrow-derived mononuclear cells, CDCs have superior angiogenic potential and secrete higher levels of angiogenic and antiapoptotic factors. *In vivo* transplantation of CDCs, when compared with these other cell types, results in the highest improvements of cardiac function and highest levels of

cell engraftment after myocardial infarction (Li et al., 2012). The mechanism for the regeneration of viable myocardium after CDCs transplantation is mediated mainly through upregulation of host cardiomyocyte cycling and recruitment of endogenous progenitors (K. Malliaras et al., 2013).

Cardiosphere-derived cells retain their ability to form sphere-like structures (secondary cardiosphers) when exposed to epidermal growth factor (Chimenti et al., 2010). Exposure of CDCs to epidermal growth factor can also significantly improve functional parameters such as migration, proliferation, and wound healing of CDCs (Aghila Rani & Kartha, 2010). Secondary sphere formation can enhance the functionality of cardiosphere-derived cells. The 3-D formation of the cardiospheres when compared with the monolayer of cardiosphere derived cells has higher levels of Troponin I (Machida, Takagaki, Matsuoka, & Kawaguchi, 2011). When transplanted into the infarcted myocardium, secondary cardiospheres result in higher levels of engraftment, greater improvements in left ventricular dysfunction and reduced infarct size when compared with transplantation of CDCs alone (Cho et al., 2012).

Cardiospheres have the unique advantage of diverse cell types within a natural 3-D formation which mimics the stem cell niche. This periphery of supporting cell populations have a paracrine role that enhances regeneration (Davis et al., 2009). In spite of the superior regenerative effects after cardiosphere transplantation when compared with CDCs, their relatively large size is a disadvantage and presents safety concerns. Cardiospheres exert a dose-dependent functional benefit, with higher doses resulting in greater benefits (Shen, Cheng, & Marban, 2012), however transplantation of large

numbers of cardiospheres via intracoronary infusion may result in adverse events such as microembolism therefore cardiosphere-derived cells are more widely used.

The majority of the benefit after CDC transplantation is mediated through secreted factors and cell-to-cell signaling. Direct differentiation of CDCs after transplantation only accounts for 20-50% of increases in myocardial viability and capillary density (Chimenti et al., 2010). This was measured by injecting human cells into SCID mice and measuring the origin of regenerated tissues. Only 9.6% of the total capillaries were found to be of human origin which amounts to approximately 20% of enhanced angiogenesis, and only 11.8% of myosin heavy chain expressing cells in the myocardium were of human origin accounting for half of the 20% increase in relative tissue viability (Chimenti et al., 2010).

Transplantation of both syngeneic and allogeneic CDCs significantly improves infarct size, infarcted wall thickness, and ejection fraction after transplantation, compared to controls even though the number of retained cells significantly drops at 3 weeks (K. Malliaras et al., 2012). This suggests that paracrine secretions rather than direct differentiation are mainly responsible for functional improvement as cells do not engraft long-term. When transplanted *in vivo*, CDCs secrete VEGF, HGF, and IGF1, and *in vitro* CDC-conditioned media exerts an antiapoptotic effect on neonatal rat ventricular myocytes, a proangiogenic effect on human umbilical vein endothelial cells, and can reverse angiotensin II-mediated contractile dysfunction in adult cardiomyocytes (Chimenti et al., 2010; Maxeiner et al., 2010). The paracrine activity of CDCs is regulated by the ERK pathway. In ERK stimulating conditions growth factors such as VEGF have increased secretion, whereas in ERK inhibitory conditions cells are more

prone to cardiomyogenic differentiation (H. J. Lee, Cho, Kwon, Park, & Kim, 2013). CDCs also secrete exosomes which play a large role in their regenerative ability. Exosomes isolated from CDC conditioned medium inhibits cardiomyocyte apoptosis, promotes cardiomyocyte proliferation, and enhances angiogenesis of endothelial cells. Injection of exosomes into the heart of injured mice can recapitulate the benefit of CDC transplantation (Ibrahim, Cheng, & Marban, 2014).

Paracrine secretions can also influence the plasticity of surrounding cell types. Mesenchymal stem cells derived from the mediastinal fat when exposed to cardiosphere conditioned medium partially differentiate towards the cardiac lineage (Siciliano et al., 2015). Although the benefit of CDC transplantation is mainly paracrine mediated, *in vivo*, transplantation of CDC conditioned media alone does not result in equal improvements in left ventricular ejection fraction and number of cycling cardiomyocytes when compared with transplantation of CDCs. *In vitro*, direct cell to cell contact results in the greatest enhancement of cardiomyocyte proliferation compared with CDCs grown in a transwell contact-free system (Xie et al., 2014).

Although the heterogeneity of cell types within cardiosphere-derived cells contribute to their therapeutic efficacy, some cell types are not required for their benefits. A cell type commonly within the CDC population is the CD90 population. CD90 expression usually identifies a mesenchymal/myofibroblast cell. This cell type when isolated from cardiospheres displayed a reduced cardiac differentiation potential when compared with CD90- cells (Gago-Lopez et al., 2014). *In vivo*, depletion of cells that express CD90 prior to transplantation in a mouse model of myocardial infarction augments the functional potency of CDCs resulting in a ~7% increase in LVEF above

transplantation of CDCs alone (Cheng, Ibrahim, et al., 2014). Most interestingly, depletion of another common cell type within CDCs, the ckit+ cardiac stem cell, does not compromise the structural and functional benefits of CDCs in a mouse model of myocardial infarction. In the CADUCEUS clinical trial, there was no correlation between percentage of ckit+ cells and the benefit of CDC transplantation in humans (Cheng, Ibrahim, et al., 2014). Therefore, the cell type responsible for the therapeutic benefits after CDC transplantation are distinct from ckit+ cardiac stem cells.

With disease, the characteristics of cardiosphere-derived cells changes. Some of these alterations can negatively influence CDC function, CDCs cultured from diabetic patients had 1.6-fold more CD90+ cells than those from non-diabetic patients (Chan et al., 2012). Other disease-induced modifications can surprisingly improve CDC function. CDCs from end-stage heart failure patients demonstrated a significantly higher restoration of ventricular function, prevented adverse remodeling, and enhanced angiogenesis after transplantation in an infarcted rodent model when compared to control heart disease patients (Cheng, Malliaras, et al., 2014; Sharma et al., 2015). These positive changes were found to be due to an enhanced secretome activity of end stage heart failure-derived CDCs.

Cardiospheres and Cardiosphere-derived Cells in Large Animals

There have been several studies that have utilized large animal models to study the therapeutic efficacy of cardiosphere-derived cell transplantation. Some have used human cells in large animals. In a cyclosporine A immunosuppressed pig, Takehara et al (2008) transplanted human CDCs or bone marrow-derived mesenchymal stem cells with basic fibroblast growth factor in a slow release hydrogel after myocardial infarction.

Four weeks after transplantation, mesenchymal stem cells with bFGF (n=6) did not significantly increase LVEF above bFGF alone (n=8). Whereas, transplantation of CDCs with bFGF (n=9) resulted in an additive effect over bFGF alone and significantly increased LVEF (38.4% vs 30.1% baseline) and regional wall motion (58.5% vs 46.2% baseline) which was significantly evident when compared with the change in cardiac function in the control group (n=6). In pigs that received CDCs, thirty-three percent of cardiomyocytes generated from the transplanted cells were the result of direct differentiation rather than cell fusion as confirmed by immunohistochemical colocalization of the human Y chromosome with sarcomeric alpha actin. In pigs that received mesenchymal stem cells only 10% of cardiomyocytes were the result of direct differentiation (Takehara et al., 2008).

Others have employed pig cardiosphere-derived cells delivered either syngeneically or allogeneically. Cardiosphere derived cells have a diameter of 21μm which when compared with the 7-10μm capillary luminal diameter is relatively large. Therefore infusion of high numbers can result in microvascular occlusion and infarct (Johnston et al., 2009). Regeneration of tissue without microvascular infarction can be achieved with transplantation of $\geq 10^7$ CDCs (300,000/kg) but less than 2.5×10^7 CDCs. Unfortunately, due to low levels of engraftment, transplantation of 10^7 CDCs by over-the-wire balloon catheter (n=7) did not significantly improve left ventricular mass, infarct size, end-diastolic volume, end-systolic volume, left ventricular ejection fraction or end-diastolic pressure. However, cardiac magnetic resonance revealed that CDC treatment did decrease relative infarct size (19.2% to 14.2% of left ventricle infarcted, P=0.01), whereas placebo did not (17.7% to 15.3%, P=0.22) after 8 weeks (Johnston et al., 2009).

To improve engraftment an open-chest surgery with twenty intramyocardial injections of either cardiospheres or cardiosphere-derived cells was used in pigs four weeks post myocardial infarction (S. T. Lee et al., 2011). After 24 hours engraftment regardless of dose (0.5 million, 2 million, or 10 million) was 8-9% in remote normal myocardium. In the peri-infarct zone, however, engraftment was inversely related to the number of injected cells with the lowest dose of cell (0.5million) resulting in the highest percentages of engraftment. By ventriculography, both cell-treated groups (cardiospheres n=8, CDCs n=10) had a significant increase in LVEF when compared with the placebo group ($n=11$, $p<0.01$). In 2 animals lacZ labeling was used to trace cell fate, after 8 weeks, the expression of lacZ was confirmed in cardiomyocytes, however whether these are due to direct differentiation or cell fusion is unknown. Cardiosphere and cardiosphere-derived cells had very similar outcomes with cardiosphere transplantation resulting in superior improvements in end-systolic elastance, delta end-diastolic pressure, and apical septal thickening (S. T. Lee et al., 2011).

Allogeneic transplantation of mismatched CDCs by intracoronary infusion in a porcine model 2-3 weeks after myocardial infarction revealed that in large animals allogeneic transplantation (n=5) can attenuate remodeling, improve global and regional function, decreased scar size, and increase viable myocardium when compared with vehicle (n=5) 2 months post-treatment (Konstantinos Malliaras et al., 2013). Left ventricular ejection fraction was preserved in cell-treated (Δ -0.5, $p=0.73$) when compared with control animals (Δ -9.9, $p<0.001$). By MRI, scar size was unchanged in controls (Δ 0.4%, $p=0.33$) but decreased in CDC-treated animals (Δ -3.6%, $p=0.026$). There was also a significant increase in viable myocardial mass in CDC-treated animals

(Δ 10.7 g) when compared with controls (Δ 2.7 g, p=0.003) after 2 months. Although allogeneic transplantation results in mild local mononuclear infiltration, there is no systemic immunogenicity associated with mismatched CDC transplantation. Cardiac retention after 24 hours was only 4.3% using luciferase labeled CDCs. At 2 weeks post infusion cells could still be detected within the recipient myocardium, however by 2 months, no allogeneic cells were detected (Konstantinos Malliaras et al., 2013).

CDCs also have beneficial effects when administered very early after myocardial infarction. When applied this early, they can be used as therapy to prevent cardiomyocyte death as opposed to a therapy to regenerate lost tissue. Kanazawa et al (2015) conducted a short-term (48 hours) study of CDC administration 30 minutes after reperfusion to investigate relative safety as an adjunctive therapy to reperfusion in porcine model of myocardial infarction. Comparison of doses revealed that higher doses of allogeneic CDCs (7.5 or 10 million cells, n=5 each) rather than low dose (5 million cells, n=5) resulted in cardioprotection. Transplantation of ~8.7 million cells (n=4) resulted in significant decreases in TUNEL positive cardiomyocytes within the border zone after 48 hours. There was also a significantly smaller infarct size in the CDC-treated group (59.7%) when compared with sham (81.0%, n=5) or placebo (80.3%, n=5) as well as a decrease in microvascular obstruction area when compared with controls (Kanazawa et al., 2015).

Current Clinical Trials

On the basis of the promising outcomes in large animal work, administration of endogenous cardiac stem cells as a therapy for cardiac injury has moved into human

clinical trials. Currently there are four phase I, safety and efficacy, clinical trials completed.

The clinical trial entitled, "Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction" or CADUCEUS trial was led by Eduardo Marban. This trial included 25 patients (17 cell-treated, 8 standard care) who received a dose of autologous CDCs. Cell therapy, after 6 months, resulted in reductions in scar mass ($p=0.001$), increases in viable heart mass ($p=0.01$) and regional contractility ($p=0.02$), and regional systolic wall thickening ($p=0.015$) by MRI. At this time point, changes in end-diastolic volume, end-systolic volume, and LVEF did not differ between groups (Makkar et al., 2012). At 12 months, transplantation of CDCs resulted in a significant reduction in scar mass (11.9g decrease) and significant increase in viable mass (22.6g increase). There was also improved regional contractility and regional systolic wall thickening. However, at 12 months, functional measurements of end-diastolic volume, end-systolic volume, and LVEF did not differ significantly between groups (Konstantinos Malliaras et al., 2014a). At 12 months the only serious adverse event determined by the data safety monitoring board to be possibly related to CDC therapy was a non-ST-segment elevation MI in 1 CDC-treated patient at 7 months after cell infusion.

One of the largest clinical trials completed to date, using endogenous cardiovascular stem cells, is the "Administration of cardiac stem cells in patients with ischemic cardiomyopathy" or SCIPIO trial. This trial led by Roberto Bolli, had a total of 33 patients (20 cell-treated, 13 control) and tested the safety and efficacy of ckit+ cardiac stem cells (Roberto Bolli et al., 2012; Roberto Bolli et al., 2011). Ckit+ stem cells were

isolated from the right atrial appendage, grown to sufficient numbers (maximum 10^6 CSCs) and injected during coronary artery bypass grafting surgery 4 months after myocardial infarction. After 2 years, there were significant improvements in left ventricular ejection fraction (41.7% at 2 years vs 29.7% at baseline). This was accompanied by an increase in left ventricular viable mass (12.2% increase) and decrease in infarct size (15.7% decrease). Other outcome measures such as the New York Heart Association classification and the Minnesota Living With Heart Failure Questionnaire score also improved significantly after 2 years. No CSC-related adverse effects were reported.

The clinical trial entitled, "Transcoronary Infusion of Cardiac Progenitor Cells in Patients With Single Ventricle Physiology" or TICAP is one of the only clinical trials in young patients with hypoplastic left heart syndrome and is led by Hidemasa Oh (Ishigami et al., 2014). Fourteen patents (average age 1.8+/- 1.5 years) were enrolled in this study and received autologous CDCs ~30 days after staged procedures. No serious adverse events were reported in the 18-month follow-up. CDC-treated patients had increased cardiac function as measured by left ventricular ejection fraction at 3 months (52.1% vs 46.9% at baseline) this increase persisted over the period of follow up (54.0% at 18 months). In contrast control patients had no significant increase in heart function at 3 months (47.7% vs 46.7% at baseline) which was not significantly changed during the follow-up period (48.7% at 18 months).

Cardiac stem cell transplantation, as evidenced by these three completed clinical trials, can result in improved patient outcomes, however full regeneration of the injured myocardium is likely to be achieved with a combination of cell types (Latham et al.,

2013) and growth factors delivered in an engraftment-promoting medium (Mayfield et al., 2014). Experiments in large animals and human clinical trials have highlighted the utility of administration of select growth factors (without cardiac stem cells) to ameliorate cardiac injury (Braitsch, Kanisicak, van Berlo, Molkentin, & Yutzey, 2013; Gao et al., 2010; Henry et al., 2003; Hibbert et al., 2014; Jabbour et al., 2011; Koudstaal et al., 2014; Silverberg et al., 2001; Simons et al., 2002; Tritos & Danias, 2008). A summary of results from human clinical trials with growth factors administered after cardiac injury can be found in table 2.

The last of the completed clinical trials, which was completed by Hiroaki Matsubara in Japan, is the only clinical trial that uses both cardiac stem cells and bFGF-infused biodegradable hydrogel. This clinical trial is entitled, "Autologous Human Cardiac-Derived Stem Cell to Treat Ischemic Cardiomyopathy" or ALCADIA (Nakata et al., 2012) and uses stem cells from endomyocardial biopsisies, which express CD105, CD90, CD29, Nanog, Oct4, Gata4 and are negative for CD45. These CSCs (5×10^5 cells/kg) were delivered using 20 intramyocardial injections which were covered with a biodegradable gelatin hydrogel which contained 200µg of infused bFGF. Six patients with ischemic cardiomyopathy were enrolled in this study. This study has not yet published final results. Preliminary data after 6 months indicate an improvement in LVEF in the cell-treated patients (from 26.7% to 35.8% by 3D Echo and 22.6% to 34.7%

Table 2 Summary of paracrine factors used in clinical trials for myocardial repair.*

Factor	Phase	Patient type and no.	Results	Ref
rhEPO	Randomized, controlled trials	Chronic heart failure, n=32	An increase of 5.5% and a decrease of 5.4% in LVEF in the treatment and control groups, respectively	(Silverberg et al., 2001)
rhFGF2	Phases I and II	Coronary artery disease, n=337	No improvement in exercise tolerance or myocardial perfusion at day 180. Significant reduction in angina was observed at day 90 but not at day 180 compared to that of the placebo group.	(Simons et al., 2002)
rhG-CSF	Randomized, controlled trials	Acute MI, n=560 in 14 trials (285 ctrl, 275 treated)	No statistically significantly difference in LVEF vs placebo, no significant adverse effects	(Hibbert et al., 2014)
rhGrowth hormone	A mixture of controlled and uncontrolled trials	Congestive heart failure, n=212 in 14 studies	LVEF +4.3% (above baseline, most benefit in uncontrolled trials). Increased left ventricular mass and wall thickness.	(Tritos & Danias, 2008)
rhNeuregulin-1	Phase I	Chronic heart failure, n=15	LVEF +4.1% (above baseline), $p<0.001$ at 12 weeks. Adverse side effects were reported.	(Jabbour et al., 2011)
	Phase II	Chronic heart failure, n=44 (11 ctrl, 33 treated)	No statistically significant difference in LVEF vs placebo, adverse effects with high (1.2μg/kg) dose.	(Gao et al., 2010)
rhVEGF	Phase I (VIVA trial)	Coronary artery disease, n=178 (63 ctrl, 115 treated)	High dose (50ng/kg) improved angina by day 120 compared to placebo.	(Henry et al., 2003)
	Phase I	Coronary artery disease, n=14	Improved myocardial perfusion at rest in a dose-dependant manner.	(Braitsch et al., 2013)

Abbreviations - rh: recombinant human protein; LVEF: left ventricular ejection fraction

*Modified from (Kathy O Lui, Zangi, & Chien, 2014)

by MRI), as well as a decrease in infarct volume/left ventricular mass (%) by MRI (from 23% to 19.7%). There was also a concomitant decrease in wall motion score (from 17.2 to 6.6). Maximum aerobic exercise capacity (VO_2 peak) was increased by 4.5ml/kg/min. Of the 6 patients, one patient experienced an acute occlusion of a graft and was excluded from further evaluation and one experienced worsening heart failure during follow-up. No other adverse events were reported. Comparison with matched controls has not been reported.

There are two clinical trials using endogenous cardiac stem cells that are currently enrolling participants. The first is a phase I/II clinical trial led by Eduardo Marban and is the first trial to investigate the allogeneic use of endogenous cardiac stem cells in humans. This trial is entitled, "Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration" or ALLSTAR. The primary endpoint is to test the safety of allogeneic cardiosphere-derived cell product. This study expects an enrollment of 274 patients. Cells will be transplanted <3 months or 3-12 months after myocardial infarction to assess the ideal time frame for cell delivery. At present, there are no released outcomes from this trial.

The second is a phase II trial entitled PERSEUS and is a follow up trial on the phase I TICAP trial. This trial uses cardiac progenitor cell infusion to treat univentricular heart disease. This study has an estimated enrollment of 34 patients with hypoplastic left heart syndrome between the ages of 0 and 20 years of age. The primary endpoint is to measure cardiac functional improvement after 3 months. Cardiac function will also be measured after 12 months. This study is currently enrolling patients and is expected to be completed in 2018.

Considerations for Transplantation of Endogenous CSCs

There is growing evidence for the benefit of using allogeneic cardiac progenitors for cardiac transplantation (K. Malliaras et al., 2012; Tseliou et al., 2013). However, it is unclear whether an immune response to these cells would negate their benefits when transplanted in humans. The transplantation of allogeneic stem cells is likely to lead to T-cell mediated rejection by either indirect allorecognition, or through polymorphic variants of donor MHC being recognized by recipient T cells (Nehlin, Isa, & Barington, 2011). This T-cell mediated rejection is mediated by the expression of both MHC class I and co-stimulatory molecules including CD40, CD80, and CD86 (Nehlin et al., 2011). Without the presence of the co-stimulatory molecules, MHC class I binding to T cell receptors results in anergy, apoptosis of T cells, or tolerance-inducing immunoregulation (Nehlin et al., 2011).

Results of experiments using ckit+ cells *in vitro* suggest they actually help reduce inflammation by activating T-regulatory cells through the expression of programmed death ligand 1. They also lack the expression of CD40, CD80 or CD86 which are co-stimulatory molecules needed for conventional allogeneic T-helper 1 or T-helper 2 type responses (Lauden et al., 2013). However, further studies using allogeneic ckit+ stem cells *in vivo* are needed. Studies with other types of endogenous cardiac progenitors have similar results. Cardiosphere-derived cells express class I MHC antigens but not class II antigens or B7 co-stimulatory molecules (K. Malliaras et al., 2012). The absence of MHC class II gives cells the potential to escape recognition by alloreactive CD4+ lymphocytes (Tasso & Pennesi, 2009). In culture, they elicit negligible lymphocyte proliferation and inflammatory cytokine secretion and when transplanted *in vivo* into an

infarcted rat heart, they cause a mild local immune reaction, without histologically-evident rejection or systemic immunogenicity with similar sustained benefits to syngeneic transplantation at 6 months post injection (K. Malliaras et al., 2012). Cardiospheres *in vitro* inhibited proliferation of alloreactive T cells, and interestingly when transplanted *in vivo* attenuated the inflammatory response observed histologically in the peri-infarct area (Tseliou et al., 2013).

Although stem cells seem to have a low immunogenic profile, once transplanted they do not survive long-term (Konstantinos Malliaras et al., 2013). This may due to the fact that differentiated progeny of transplanted allogeneic pluripotent stem cells are rejected by the host immune system (Nussbaum et al., 2007). During differentiation, hESC express very low levels of surface MHC1 molecules but following implantation they differentiate and up-regulate MHC expression thus triggering effective immune responses (Calderon et al., 2012). Some would argue that although allogeneic CPCs are eventually rejected, much of the benefit of cell transplantation is due to indirect mechanisms such as paracrine secretion or activation and recruitment of existing endogenous stem cells. Therefore, allogeneic transplantation may still be beneficial if rejection is delayed long enough to allow cells to exert their protective and regenerative paracrine effects (K. Malliaras et al., 2012).

Overall, endogenous cardiac progenitors are a promising cell-based therapeutic for the heart. They have relative safety as there have been no adverse events such as cancer or arrhythmias. Thus far, they have demonstrated a relatively low immunologic profile and can result in functional benefits that last 1-2 years. Yet cells are rarely retained long-term. Malliaras et al demonstrated that 24 hours after intracoronary infusion

the percentage of cells that engrafted within the heart was 4.3% (Konstantinos Malliaras et al., 2013). It is likely that enhanced engraftment could result in improvements in existing outcomes. The greatest benefits will most likely come from transplantation of carefully selected cell types that have been pre-treated for optimal functioning, within an engraftment promoting medium.

ISL1+ Cardiac Progenitors

One of the endogenous cardiovascular progenitor types that has yet to be used in clinical trials is the ISL1+ cardiac progenitor. Islet-1 (ISL1) is a protein that is expressed by stem cells early during cardiovascular development and is essential for cardiogenesis; progenitors that express ISL1 are required for the formation of the right ventricle, atria and outflow tract (Cai et al., 2003). Addition of purified ISL1 protein to human embryonic stem cells during differentiation, can increase the number of cardiac cells produced 3-fold (Fonoudi et al., 2013). This highlights the utility of ISL1 expression in cardiogenesis. *In vitro,* ISL1+ progenitor cells are capable of forming all three cardiovascular lineages (Bu et al., 2009). Despite their clear role in cardiovascular development, the ability to isolate ISL1+ cardiac progenitors from the human heart decreases with age. In the young heart there are abundant levels of ISL1+ progenitor populations, yet with increasing age, ISL1+ cardiac progenitors are reduced to reportedly nonexistent levels (Simpson et al., 2012). Comparison of neonatal ISL1+ cardiac progenitors with similarly isolated adult progenitors that are negative for ISL1 revealed that, after transplantation into the infarcted myocardium, neonatal ISL1+ cardiac progenitors had a significantly higher ability to preserve myocardial function, prevent

adverse remodeling, and enhance blood vessel formation (Simpson et al., 2012). This suggests that ISL1+ cardiac progenitors derived from the young heart may be more ideal for cardiovascular regeneration.

In this dissertation we utilize a novel stem cell population characterized by the expression of both ISL1 and ckit. In humans, this population of cells was thought to only exist in the fetal heart (Serradifalco et al., 2011), however, we have isolated cardiovascular stem cells that co-express both ISL1 and ckit from the adult heart (57-75 year olds). Because of the essential role of both ISL1 and ckit in cardiogenesis, the co-expression of these markers could potentially identify a progenitor population with high regenerative capacity. The fact that this vital population persists throughout life, yet, the aged heart still has limited regenerative capacity suggests that these cells are either too few in the adult heart, not activated sufficiently after injury and/or there are underlying biological differences between adult ISL1+ ckit+ cardiac progenitors and phenotypically-matched cardiovascular progenitors from the young heart.

In this dissertation we created single cell clones from our newly isolated adult ISL1+ckit+ cardiovascular progenitors and compared them to similarly isolated and cloned ISL1+ ckit+ cardiac progenitors from the neonatal (<1month old) heart. Comparison of single cell clones allows us to define how age impacts epigenetic, phenotypic, and functional parameters within ISL1+ ckit+ cardiovascular stem cells. This will provide a better mechanistic understanding of the superior regenerative ability of neonatal ISL1+ ckit+ cardiac progenitors and will provide novel therapeutic targets to improve myocardial regeneration.

In chapter 2, we will address the following hypotheses:

Hypothesis 1: Phenotypically-matched neonatal and adult cardiovascular progenitors differ in their ability to invade in response to growth factor stimulation and in their ability to differentiate into endothelial cells and cardiomyocytes.

Hypothesis 2: Superior neonatal functional ability is aided by the expression of microRNAs which are altered with stem cell age specifically in pathways influencing cell cycle progression and cellular senescence.

As previously stated, full regeneration of the injured heart may not only require carefully selected cell types, but will most likely require optimization of cell function by pretreatment methods. Therefore, in chapter 3, we will address the following hypothesis:

Hypothesis 3: Treatment of neonatal and adult cardiovascular progenitors with short-term hypoxia and/or prostaglandin E_2 (PGE_2) will alter MAPK/ERK pathway activation and increase cardiac progenitor cell cycle progression, migration and paracrine factor expression.

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CHAPTER TWO

HUMAN NEONATAL CARDIOVASCULAR PROGENITORS: UNLOCKING THE SECRET TO REGENERATIVE ABILITY

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Running Title: Human Neonatal Cardiovascular Progenitors

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Abstract

Although clinical benefit can be achieved after cardiac transplantation of adult c-kit+ or cardiosphere-derived cells for myocardial repair, these stem cells lack the regenerative capacity unique to neonatal cardiovascular stem cells. Unraveling the molecular basis for this age-related discrepancy in function could potentially transform cardiovascular stem cell transplantation. In this report, clonal populations of human neonatal and adult cardiovascular progenitor cells were isolated and characterized, revealing the existence of a novel subpopulation of endogenous cardiovascular stem cells that persist throughout life and co-express both c-kit and isl1. Epigenetic profiling identified 41 microRNAs whose expression was significantly altered with age in phenotypically-matched clones. These differences were correlated with reduced proliferation and a limited capacity to invade in response to growth factor stimulation, despite high levels of growth factor receptor on progenitors isolated from adults. Further understanding of these differences may provide novel therapeutic targets to enhance cardiovascular regenerative capacity.

Introduction

Endogenous cardiac progenitor cells (CPCs) are being carefully investigated to determine whether they have the ability to repair the heart when expanded *in vitro* and re-administered as a cell-based treatment after myocardial infarction in human clinical trials (Bolli et al., 2011; Makkar et al., 2012). As CPCs age, however, they lose the ability to efficiently regenerate damaged heart tissue. Telomerase activity is reduced with chronological age and an associated decline in the number of functionally-competent cardiac progenitor cells results in a dramatic loss of growth reserve within the adult heart (Cesselli et al., 2011; Torella et al., 2004). Functional studies in mice have shown that neonatal, not adult, c-kit+ cardiac progenitors support post-infarct myogenesis (Jesty et al., 2012). The molecular basis underlying the enhanced capacity for regeneration that distinguishes human neonatal cardiovascular progenitor cells from adults has not been defined.

As a fetus matures into a neonate, several developmental changes impact the CPC. Lineage tracing studies using embryonic stem cells show that early cardiovascular progenitors expressing MESP1 differentiate into two separate classes of Nkx2.5+ progenitor populations, one characterized by the expression of Isl1 and another characterized by the absence of Isl1 (Bondue et al., 2011). The Isl1+ cardiac progenitors can be differentiated into all three cardiac lineages including endothelial cells, smooth muscle cells, and cardiomyocytes (Laugwitz et al., 2005). The differentiation capacity of Isl1- CPCs is limited to smooth muscle cells and cardiomyocytes (Bondue et al., 2011). Histological analysis suggests that cells positive for Isl1, and SSEA-4 (an early stem cell marker) are abundant in the fetus and are only sporadically found in the neonate. Cells

expressing c-kit and Nkx2.5 decline in number significantly as a neonate transitions into an infant (Amir et al., 2008; Mishra et al., 2011). A gradual reduction of proliferation occurs in the heart at this time; during the neonatal period there are 3 times as many proliferating cells as those identified in children >2 years of age (Mishra et al., 2011). After the first month of life, the dynamics of the CPC population changes dramatically, highlighting the neonatal window as an optimal time during which progenitor cells can be isolated for therapy. The biological features that distinguish neonatal cardiovascular progenitor cells in humans will provide new insight that can be used to improve the outcome of stem cell-based treatment.

In this report, the epigenetic, phenotypic and functional changes that distinguish neonatal from adult cardiovascular progenitor cells are detailed within a newly-defined population of Isl1, c-kit co-expressing cardiovascular progenitor cells. By comparing matched, clonal cardiovascular progenitor cell populations that differ only by age, we identify significant differences in microRNA regulation and gene expression that correlate with functional limitations in the adult cardiovascular progenitor cell population.

Results

Phenotypic Profiling and Identification of Cardiovascular Progenitor Cell Clones Isolated from Human Neonates and Adults

The surface marker profile of cardiovascular progenitor cell clones residing within the heart of human neonates $\leq$1 month old and 57-75 year old adults was directly compared by flow cytometry (Fig. 1, Appendix; Supplementary Table 1). Over 240

Figure 1

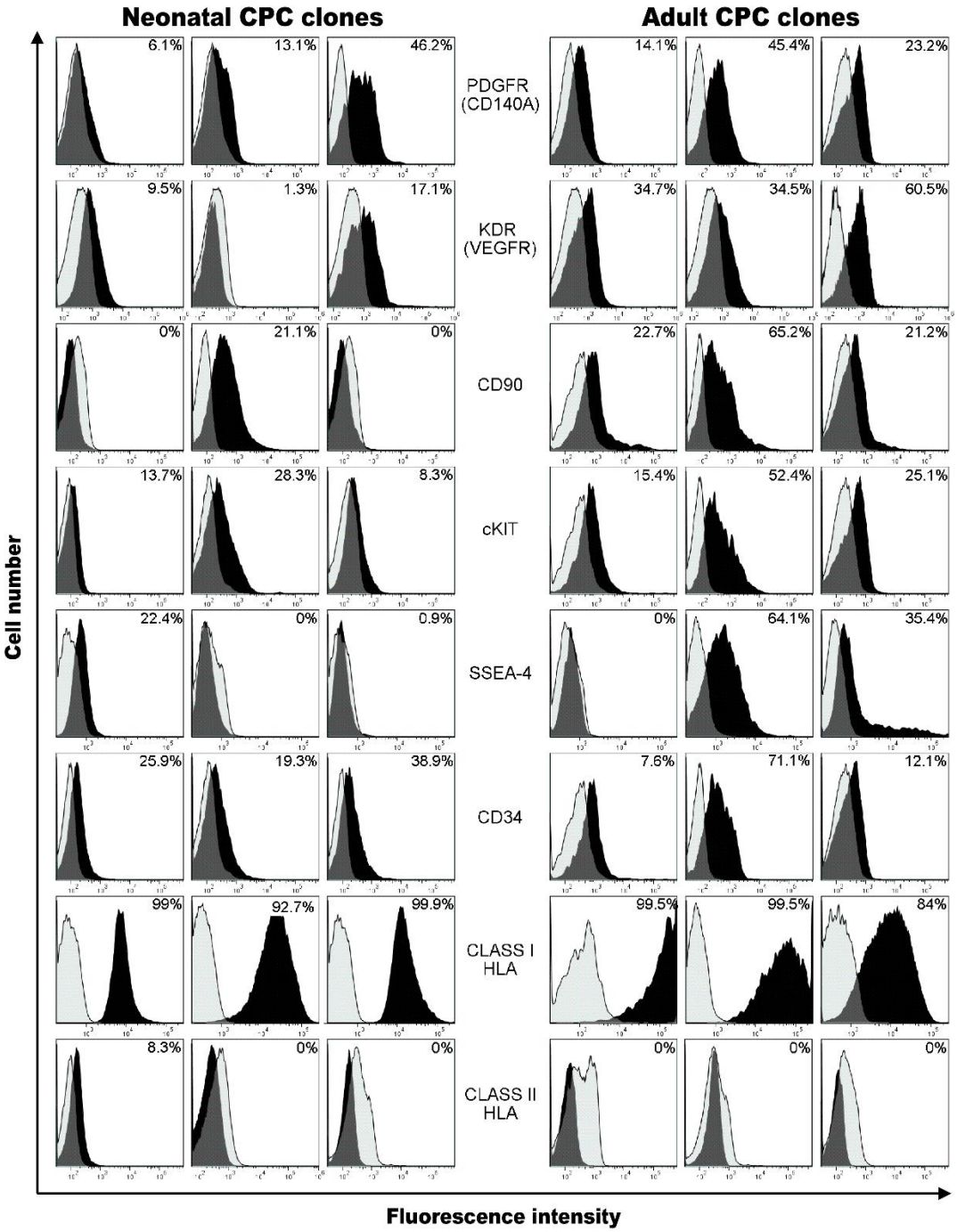
Neonatal CPC clones
Adult CPC clones
PDGFR (CD140A)
KDR (VEGFR)
CD90
cKIT
SSEA-4
CD34
CLASS I HLA
CLASS II HLA
6.1%
13.1%
46.2%
14.1%
45.4%
23.2%
9.5%
1.3%
17.1%
34.7%
34.5%
60.5%
0%
21.1%
0%
22.7%
65.2%
21.2%
13.7%
28.3%
8.3%
15.4%
52.4%
25.1%
22.4%
0%
0.9%
0%
64.1%
35.4%
25.9%
19.3%
38.9%
7.6%
71.1%
12.1%
99%
92.7%
99.9%
99.5%
99.5%
84%
8.3%
0%
0%
0%
0%
0%
Cell number
Fluorescence intensity

Figure 1. Phenotypic characterization of surface markers on neonatal and adult CPC clones. CPC clones from human neonates and adults were compared using flow cytometry to identify the surface phenotype of these cells. Labeling for select markers on representative CPC are shown in the figure where positive staining above the isotype control is shown in black. Each column represents a separate and representative clonal population. The surface phenotype of 24 clones was examined in total.

cardiovascular cell clones were isolated by single cell expansion. Phenotypic profiling using seventeen different antibodies (Appendix; supplementary table 2), specific for surface antigens reported to be present on functionally competent cardiovascular progenitors, provided a basis for identifying comparable cardiovascular progenitor cells residing in the heart of both newborns and adults. All clones expressed moderate to high levels of CD105 (60.6 – 99.8%), CD73 (41.0 – 98.3%), CD44 (60.6 – 99.8%), CD13 (73.7 - 99.9%), IGF1R (58.0 – 99.1%), and CD146 (35.7 – 99.9%). c-kit was expressed at lower levels (2.5 – 52.4%), and expression of KDR (0 – 75.1%), PDGFR (2.4 – 57.9%), CD34 (4.9 – 78.8%) and SSEA4 (0 –95.7%) was variable, thereby distinguishing specific populations. Interestingly, the majority of surface antigens were not expressed at significantly different levels in adult and neonatal cardiac progenitors. Of the 17 surface antigens profiled, only CD31 was expressed at significantly lower levels in adult cardiovascular progenitors ($p=0.04$). The progenitors were positive for the expression of HLA class I antigens (82.6 – 99.9%) and HLA class II antigens were either not expressed (0 - 3.9% in 21 CPC clones) or expressed at low to moderate levels (3 CPC clones 8.3%, 28.6%, 34.1%).

Coexpression of Isl1 and C-Kit on Human Neonatal and Adult Cardiac Progenitors

Expression of Isl1 and c-kit identifies cells with cardiomyogenic potential (Ferreira-Martins et al., 2012; Moretti et al., 2006). Characterization by flow cytometry and PCR revealed that Isl1 and c-kit were co-expressed on CPC clones isolated from both neonates and adults (Fig. 2). The majority of CPC clones expressed moderate levels of c-kit (23 ± 3% in neonates, 27 ± 3% in adults). In neonates, Isl1 was present on most, but

Figure 2

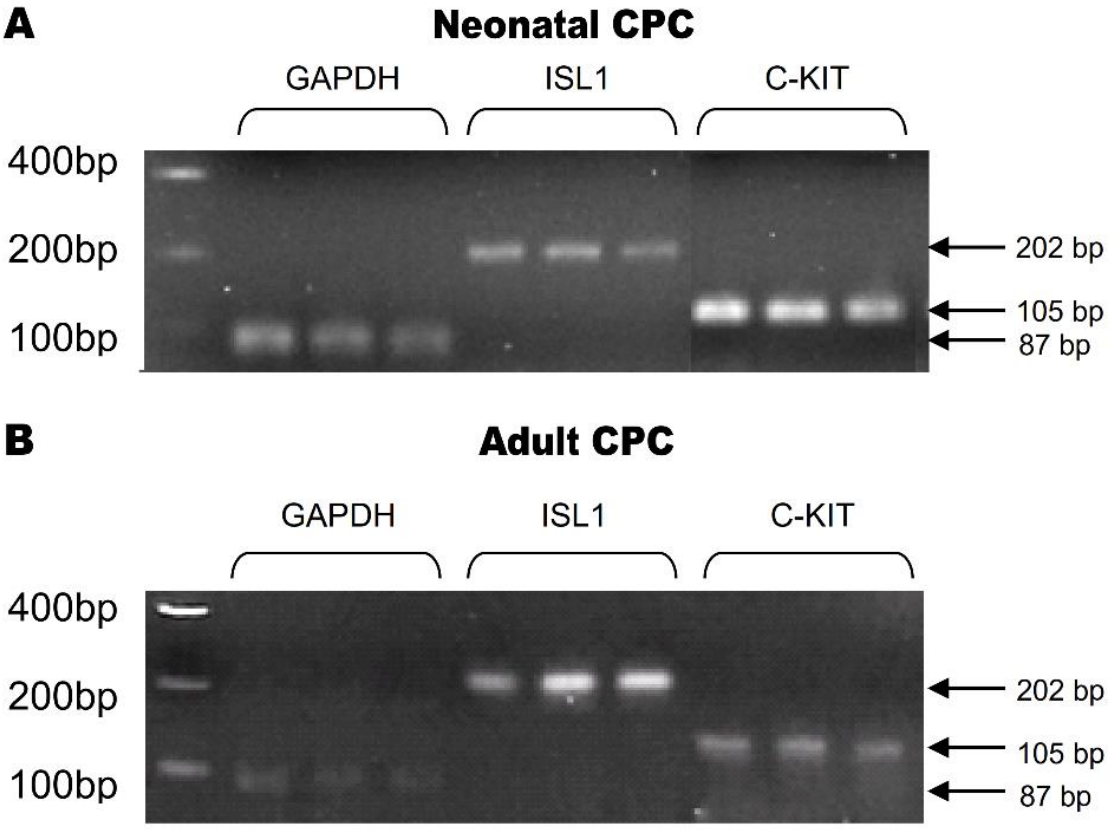
A
Neonatal CPC
GAPDH
ISL1
C-KIT
400bp
200bp
100bp
202 bp
105 bp
87 bp
B
Adult CPC
GAPDH
ISL1
C-KIT
400bp
200bp
100bp
202 bp
105 bp
87 bp

Figure 2. Co-expression of Isl1 and c-kit on neonatal and adult cardiovascular progenitors. Adult and neonatal cardiovascular progenitor cell clones were examined by PCR and flow cytometry to determine whether Isl1 was expressed in these cells. c-kit expression, initially identified by flow cytometry, was confirmed by PCR. PCR products were run on a gel, transcripts for Isl1 (size=202bp) and c-kit (size=105bp) were co-expressed on both neonatal (A) and adult (B) cardiovascular progenitor cell clones. Flow cytometry confirmed the expression of Isl1 protein (C & D) and the coexpression of Isl1 and c-kit protein (E) on neonatal and adult cardiovascular progenitors.

not all kit+ clones (78%, N=13). In adults, cardiac progenitor clones were all c-kit+ and Isl1+ (N=16).

The Isl1+ c-kit+ neonatal and adult CPC clones were differentiated into cardiomyocytes using previously reported protocols (Smits et al., 2009). Successful differentiation was supported by the expression of mRNA transcripts for NK2 homeobox 5, gata binding protein 4, cardiac myosin light chain 2, cardiac myosin heavy chain alpha and troponin T, which were induced during the differentiation protocol (N=12, Appendix; Supplementary Fig. 1). There were no significant differences in transcription of these proteins between neonatal and adult Isl1+ c-kit+ cardiovascular progenitors. Cardiac progenitor cells were shown by immunocytochemistry to express cardiac Troponin I (Appendix; Supplementary Fig. 2). When treated with 10nM dexamethasone for 6 days (D'Amario, Fiorini, et al., 2011), CPCs were successfully differentiated into all three cardiovascular lineages as demonstrated by a shift in mean fluorescence intensity when the cells were treated with antibodies to identify binding to smooth muscle actin, von Willebrand Factor, cardiac Troponin T and cardiac Troponin I using flow cytometry.

MicroRNA Profiling Predicts Functional Differences in Neonatal and Adult Cardiovascular Progenitors.

Unique differences in epigenetic regulation emerged when comparing cardiovascular progenitor cell clones by microRNA profiling. MicroRNAs (miRNAs) function to negatively regulate mRNA expression by either translational inhibition or degradation. When comparing neonatal and adult CPC clones, 41 out of 88 microRNAs analyzed were

Figure 3

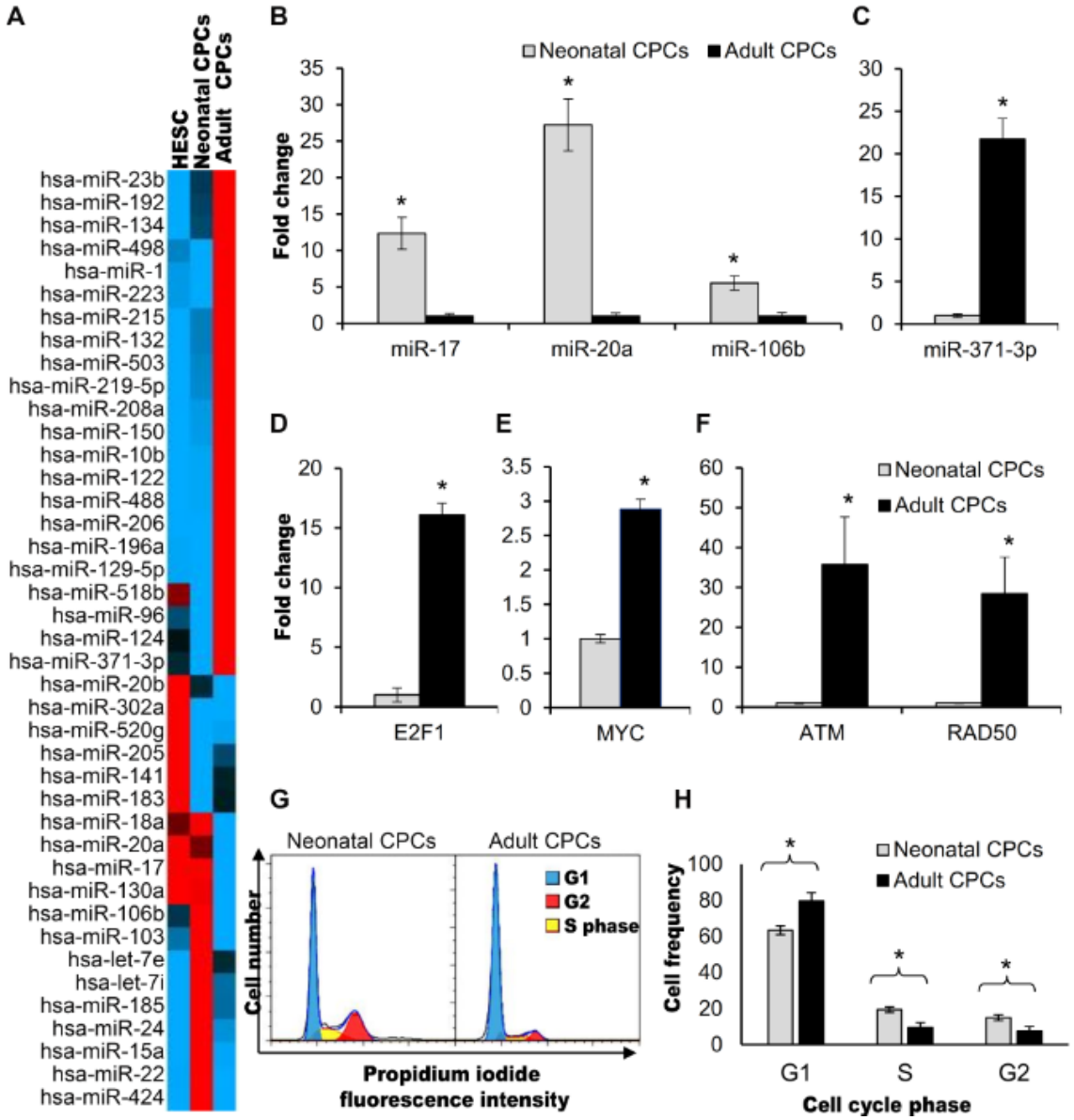
A
HESC
Neonatal CPCs
Adult CPCs
hsa-miR-23b
hsa-miR-192
hsa-miR-134
hsa-miR-498
hsa-miR-1
hsa-miR-223
hsa-miR-215
hsa-miR-132
hsa-miR-503
hsa-miR-219-5p
hsa-miR-208a
hsa-miR-150
hsa-miR-10b
hsa-miR-122
hsa-miR-488
hsa-miR-206
hsa-miR-196a
hsa-miR-129-5p
hsa-miR-518b
hsa-miR-96
hsa-miR-124
hsa-miR-371-3p
hsa-miR-20b
hsa-miR-302a
hsa-miR-520g
hsa-miR-205
hsa-miR-141
hsa-miR-183
hsa-miR-18a
hsa-miR-20a
hsa-miR-17
hsa-miR-130a
hsa-miR-106b
hsa-miR-103
hsa-let-7e
hsa-let-7i
hsa-miR-185
hsa-miR-24
hsa-miR-15a
hsa-miR-22
hsa-miR-424
B
Neonatal CPCs
Adult CPCs
Fold change
miR-17
miR-20a
miR-106b
C
miR-371-3p
D
Fold change
E2F1
E
MYC
F
Neonatal CPCs
Adult CPCs
ATM
RAD50
G
Neonatal CPCs
Adult CPCs
G1
G2
S phase
Cell number
Propidium iodide
fluorescence intensity
H
Neonatal CPCs
Adult CPCs
Cell frequency
G1
S
G2
Cell cycle phase

Figure 3. MicroRNA profiling and gene expression predicts functional differences when comparing neonatal and adult cardiovascular progenitors. A) Heat map of 41 microRNAs differentially regulated in neonatal (N=8) and adult (N=3) cardiac progenitors. hESC were also profiled as a means for comparison. Red color identifies maximum expression, black color represents average expression, blue color identifies microRNAs with minimum expression. Sets of co-regulated microRNAs were grouped together by RT2 Profiler PCR Array Data Analysis Version 3.5 software (SABiosciences). B) MicroRNAs expressed at significantly different levels in adult vs. neonatal CPCs suggest that proliferation is higher in neonatal CPCs. MicroRNA 17, miR-20a, and miR-106b levels, positively correlated with proliferation, were significantly higher in neonatal CPCs ($p=0.0094$, $p=0.0030$, $p=0.0085$, respectively). C) MicroRNA-371-3p, associated with senescence, was expressed at significantly higher levels in adult CPCs ($p=0.0238$). D) Messenger RNA transcripts for E2F1 (N=6) and E) Myc (N=5) were significantly higher in adult cardiovascular progenitors ($p=0.0075$, $p=0.0086$ respectively). E2F1 regulates the G1 to S transition of the cell cycle. F) Expression of DNA repair proteins ATM (N=5) and RAD50 (N=5) were significantly higher in adult CPCs ($P=0.0099$, $P<0.0001$, respectively). G) Representative cell cycle analysis of neonatal and adult CPCs. H) Quantification of cell cycle analysis. The frequency of adult CPCs (N=5) in G1 was significantly higher than neonatal CPCs ($P=0.0038$). The frequency of neonatal CPCs (N=5) in the S and G2 phases of the cell cycle was significantly higher than adult CPCs ($p=0.0066$, $p=0.0051$ respectively).

expressed at significantly ($P<0.05$) different levels (Fig. 3a, Appendix; Supplementary Table 3). MicroRNA expression levels in hES-3 embryonic stem cells were also identified and the results are shown in Figure 3 for comparison. The microRNA expression pattern of neonatal cardiac progenitors was more similar to that of human embryonic stem cells, highlighting a number of shared characteristics. DIANA mirPath computational software identified forty-six pathways that were significantly ($P<0.05$) impacted by differentially-expressed microRNAs (Appendix; Supplementary Table 4). Fourteen of these pathways were relevant to proliferation, including Wnt signaling, MAPK signaling, p53 signaling, TGF-β signaling, VEGF signaling, and base excision repair. Functional differences in proliferation would be anticipated based on the expression of microRNAs 17, miR-20a, and miR-106b which were expressed at significantly higher levels in neonatal cardiovascular progenitors (Fig. 3b) (B. Li et al., 2011; Petrocca et al., 2008; Pickering, Stadler, & Kowalik, 2009; Trompeter et al., 2011). High levels of expression of these microRNAs promotes cell cycle progression by suppressing the inappropriate accumulation of E2F1 transcription factors that lead to G1 arrest (Petrocca et al., 2008; Pickering et al., 2009). E2F1 regulates the G1 to S transition of the cell cycle to induce proliferation (Wu et al., 2001). The 16-fold reduction in mRNA transcripts for E2F1 in neonatal cardiac progenitors was confirmed by RT-PCR ($p=0.0075$, Fig. 3c).

Replicative Senescence in Adult Cardiac Progenitors Predicted by MicroRNA and Gene Expression Differences

Replicative senescence is defined by a progressive loss in proliferative ability despite normal viability and metabolic activity (Morgado-Palacin, Llanos, & Serrano,

2012) and is associated with an increased DNA damage response and increased cell size (Rodier & Campisi, 2011). Several microRNAs that were highly expressed in neonatal CPCs are linked to proliferative ability and play a role in preventing cellular senescence. MicroRNAs 20a and 17, which were upregulated in neonatal CPCs (27.3 fold, P=0.0030 and 12.5 fold, P=0.0094 Fig. 3b), function to rescue cells from Ras-induced cellular senescence (L. Li et al., 2012) and reduce DNA double-stranded breaks (Pickering et al., 2009). In contrast, microRNA-371-3p (Wagner et al., 2008), upregulated 27.9 fold (p=0.0238) in adult cardiovascular progenitor cells, is induced during replicative senescence. Myc expression has been associated with the induction of cellular senescence (Campaner et al., 2010) due to DNA stress (Robinson, Asawachaicharn, Galloway, & Grandori, 2009). Transcripts for Myc and DNA repair proteins, RAD50 and ATM were significantly elevated in adult cardiac progenitors (2.9 fold, P=0.0086, 28.5 fold, P<0.0001, 35.7 fold, P=0.0099 respectively, Fig. 3c&d). Comparison of cell size by flow cytometry in three separate experiments using forward scatter gating demonstrated that adult cardiac progenitors had a greater percentage of large cells when compared with neonatal CPCs (56.3% vs. 40.2%, N=5, p=0.0073).

Rate of Progression through the Cell Cycle Differs in Neonatal and Adult Cardiovascular Progenitors

To further investigate the predicted proliferative differences demonstrated by microRNA profiling, propidium iodide (PI), a DNA intercalating agent, was used to identify the percentage of cells in each phase of the cell cycle. Using flow cytometry to detect PI fluorescence intensity, a higher frequency of adult cardiac clones were

Figure 4

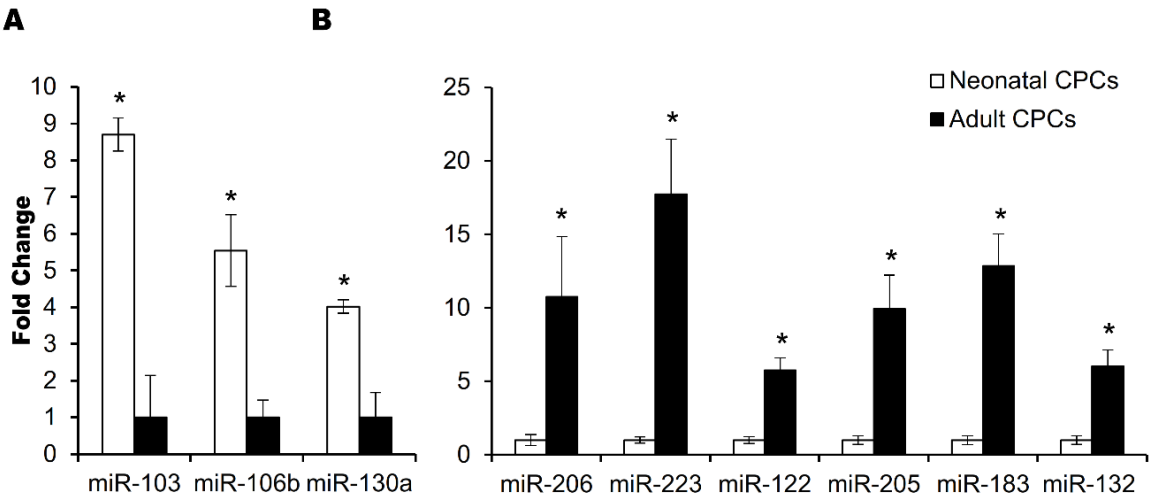
A
B
Fold Change
10
9
8
7
6
5
4
3
2
1
0
*
*
*
miR-103
miR-106b
miR-130a
25
20
15
10
5
0
Neonatal CPCs
Adult CPCs
miR-206
miR-223
miR-122
miR-205
miR-183
miR-132

Figure 4 –MicroRNAs expressed at significantly different levels ($p<0.05$) impact invasion. MicroRNAs that both promote (A) and inhibit (B) invasion were expressed at significantly different levels when comparing neonatal and adult CPC by RT-PCR. MicroRNAs that promote the ability to invade are transcribed at significantly higher levels in neonatal CPCs.

identified in G1 (82.5% vs. 63.3%, P=0.0046) and a higher frequency of neonatal clones were identified in S phase (19.4% vs. 7.0%, P=0.0026) and in G2 (15.0% vs. 7.5%, P=0.0051) (Fig. 3e). Neonatal CPCs proliferate more actively when compared to adult CPCs.

Cardiovascular Progenitors from Neonates and Adults Differ in Their Ability to Respond to Growth Factor Stimulation

Cardiac regeneration requires CPC migration away from its stem cell niche, followed by invasion into the area of injury in response to external stimuli. Nine microRNAs reported to regulate invasion (Aigner, 2011; Xuwei Hou et al., 2012; Jain et al., 2012; Ucar et al., 2010) were expressed at significantly ($P<0.05$) different levels in neonatal and adult CPCs (Fig. 4). To investigate the possibility that neonatal and adult progenitors differ in their ability to invade the site of injury within the heart, transwell invasion assays were performed to test the response of cells to SDF-1α (stromal cell-derived factor-1). SDF-1α is secreted in the infarcted heart and recruits endogenous cardiac stem cells to the site of injury (J. M. Tang et al., 2011; Y. L. Tang et al., 2009). Fewer adult cardiac progenitors (6.7×10^3) were able to invade through the basement membrane extract when compared to neonatal CPCs (14.6×10^3, p=0.0463, Fig. 5a). The inability of adult CPCs to invade in response to SDF-1α was not due to the lack of SDF-1α receptor expression on the surface of these CPCs, as demonstrated by flow cytometry. The surface expression of CXCR4 and CXCR7, both of which are receptors for SDF-1α (Tarnowski et al., 2010), was comparable on progenitors isolated from neonates and adults (Fig. 5b).

Figure 5

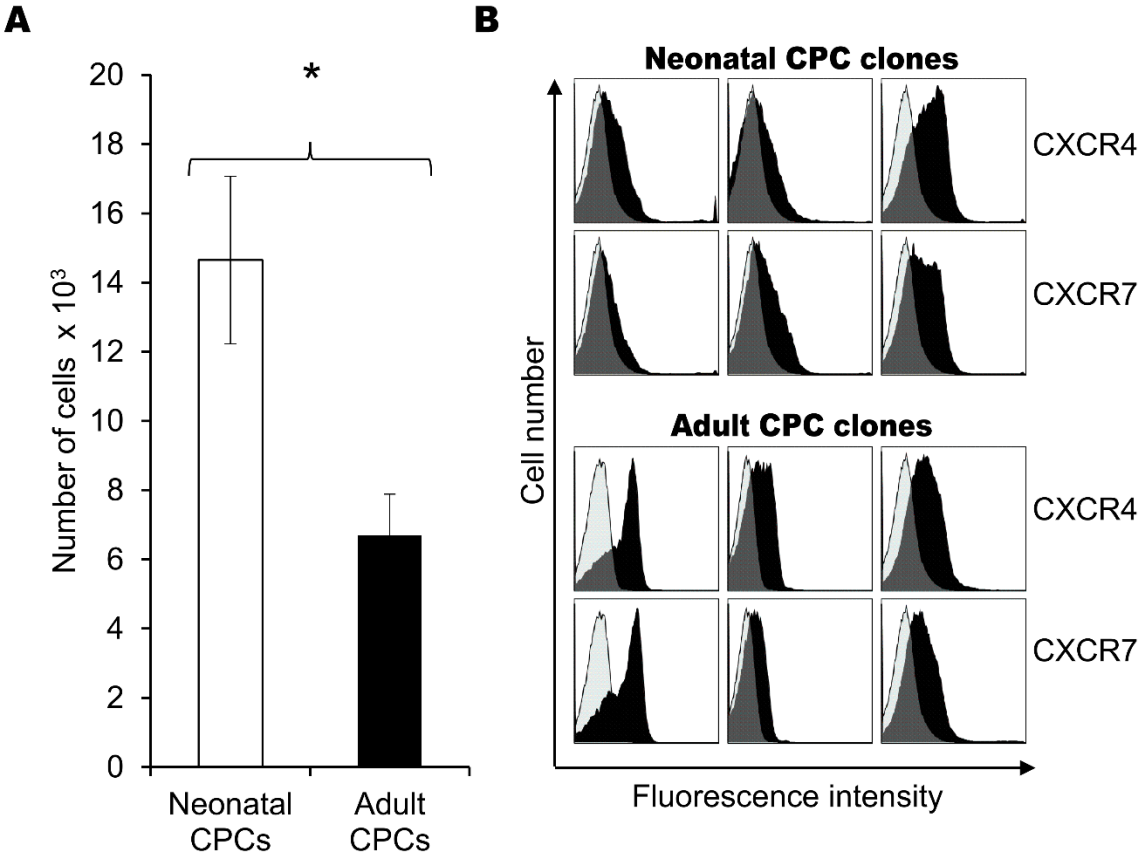
A
B
*
20
18
16
14
12
10
8
6
4
2
0
Number of cells x 10³
Neonatal CPCs
Adult CPCs
Neonatal CPC clones
CXCR4
CXCR7
Adult CPC clones
CXCR4
CXCR7
Cell number
Fluorescence intensity

Figure 5. Cell invasion in response to SDF-1. A) Phenotypically similar neonatal and adult clones were run on a transwell invasion assay. The adult CPC clones were less invasive than their neonatal counterparts (n=5, run in triplicate) in response to SDF-1α, results were significant to P = 0.0463. B) SDF-1α receptors, CXCR4 and CXCR7, were expressed on both neonatal and adult cardiovascular progenitor cell clones. Each column represents a separate and representative clonal population. Isotype control labeling is shown in white, positive labeling is shown in black.

SSEA-4+ Cardiovascular Progenitors Invade More Readily

SSEA-4 was not expressed on all CPC clones. Inherent differences were noted in neonatal and adult cardiac progenitors that were found, by flow cytometry, to express SSEA-4. MicroRNA profiling revealed 26 microRNAs expressed at significantly different levels between SSEA4+ adult and neonatal progenitors ($P<0.05$, Fig. 6A, Appendix; Supplementary Table 5). The top ten pathways, regulated by these microRNAs that impact cardiovascular stem cell function are shown in figure 6b. Six of these pathways impact invasion (marked with an arrow). In functional studies, the SSEA-4+ progenitors were more highly invasive than SSEA4- clones within the neonatal cardiovascular progenitor cell population (2.0×10^4 vs 9.1×10^3, $p=0.0186$, Fig. 6C). Adult CPC clones expressing SSEA4+ were similarly more responsive to SDF-1 (8.1×10^3 vs 3.9×10^3, $p=0.0297$). SSEA4+ cardiovascular progenitors display an enhanced capacity to invade infarcted tissue in response to injury.

Discussion

In the present study, Isl1+ c-kit+ cardiovascular progenitor cells, identified in both human neonates and adults, were expanded as clonal populations and utilized as a resource to unravel the phenotypic and epigenetic features that distinguish neonatal and adult cardiac progenitors.

This population of cells was not previously described within the endogenous cardiovascular progenitor cell population in adult humans and challenges current dogma suggesting that Isl1+ CPCs are abundant only in the fetal or neonatal heart (X. Hou et al., 2012; Simpson et al., 2012). Previous reports comparing neonatal and adult

Figure 6

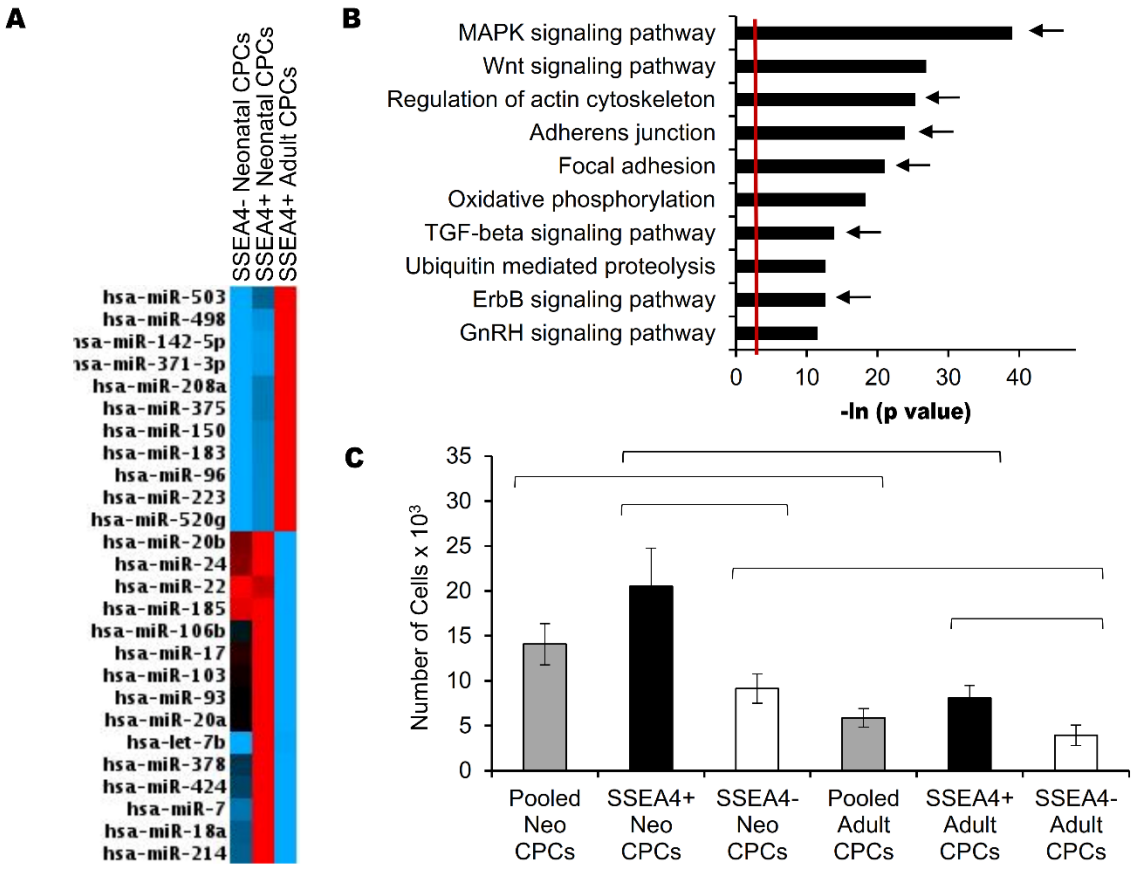
A
SSEA4- Neonatal CPCs
SSEA4+ Neonatal CPCs
SSEA4+ Adult CPCs
hsa-miR-503
hsa-miR-498
hsa-miR-142-5p
hsa-miR-371-3p
hsa-miR-208a
hsa-miR-375
hsa-miR-150
hsa-miR-183
hsa-miR-96
hsa-miR-223
hsa-miR-520g
hsa-miR-20b
hsa-miR-24
hsa-miR-22
hsa-miR-185
hsa-miR-106b
hsa-miR-17
hsa-miR-103
hsa-miR-93
hsa-miR-20a
hsa-let-7b
hsa-miR-378
hsa-miR-424
hsa-miR-7
hsa-miR-18a
hsa-miR-214
B
MAPK signaling pathway
Wnt signaling pathway
Regulation of actin cytoskeleton
Adherens junction
Focal adhesion
Oxidative phosphorylation
TGF-beta signaling pathway
Ubiquitin mediated proteolysis
ErbB signaling pathway
GnRH signaling pathway
0
10
20
30
40
-ln (p value)
C
Number of Cells x 10^3
0
5
10
15
20
25
30
35
Pooled Neo CPCs
SSEA4+ Neo CPCs
SSEA4- Neo CPCs
Pooled Adult CPCs
SSEA4+ Adult CPCs
SSEA4- Adult CPCs

Figure 6 – SSEA-4 separates cardiovascular progenitors by the ability to invade. A) Heat map of 26 microRNAs whose expression was differentially regulated in SSEA4+ neonatal and adult cardiovascular progenitors ($p<0.05$). Red color identifies microRNAs with maximum expression, black color indicates average expression, blue color shows microRNAs with minimum expression. Sets of similarly expressed microRNAs are grouped together by RT2 Profiler PCR Array Data Analysis Version 3.5 software (SABiosciences). B) MicroRNAs expressed at significantly different levels when comparing SSEA4+ adult and neonatal CPCs were analyzed using DIANA mirPATH software. The top ten pathways that impact cardiovascular stem cell function, and are regulated by these microRNAs, are shown. Six of the top ten pathways impact invasion and are marked with an arrow. The red line denotes significance of $P=0.05$. C) The invasive response of SSEA4+ neonatal and adult cardiovascular progenitor cell clones, run on a transwell migration assay, was compared. Both SSEA4+ neonatal and adult CPCs were significantly more responsive to SDF-1α when compared to SSEA4- neonatal and adult CPC, respectively ($P=0.0186$, $P=0.0297$). SSEA4+ neonatal cardiovascular progenitor cell clones were most responsive to SDF-1. These cells were significantly more invasive when compared to both SSEA4+ adult and SSEA4- neonatal CPC clones ($p=0.0183$, $p=0.0186$).

cardiosphere-derived cells (CDCs) for Isl1 expression indicated that Isl1 is abundant in neonatal CDCs but not in CPCs isolated from adults (36.2% of neonatal CPCs versus 3.2% adult CPCs) (Simpson et al., 2012). Cardiac progenitors co-expressing c-kit and Isl1, identified in the fetal heart (Serradifalco et al., 2011), have not been isolated from the adult myocardium. Stem cells isolated from the adult heart were reported to have no overlap in expression of c-kit and Isl1 (0.01% coexpression by flow cytometry) (Itzhaki-Alfia et al., 2009). The very existence of an Isl1+ c-kit+ progenitor in human adults has been questioned (Sussman, 2012). Our results demonstrate, for the first time, that the Isl1+ c-kit+ cardiac progenitor phenotype exists in both the neonatal and adult human heart. As in the embryonic heart (Serradifalco et al., 2011), all progenitors that expressed Isl1 expressed c-kit, however not all c-kit+ cells expressed Isl1, suggesting that the Isl1+ c-kit+ progenitor may be a subpopulation of c-kit+ progenitors. PDGFR and IGF1R, which are present on subpopulations of c-kit+ progenitors with superior regenerative capacity (D'Amario, Cabral-Da-Silva, et al., 2011; Hidaka et al., 2010), were expressed at moderate to high levels on these cells.

HLA Class II antigens were not expressed on the majority of neonatal and adult CPC clones. MHC class II expression activates acute T-cell mediated graft rejection (Pietra, Wiseman, Bolwerk, Rizeq, & Gill, 2000). Allogeneic transplantation of class I positive, class II negative cardiac progenitors would be expected to elicit a minor, but transient local immune response (Malliaras et al., 2012).

Based on findings reported here, the capacity for cardiac regeneration after transplantation of CPCs in neonates and adults is impacted by underlying differences in epigenetic regulation. Forty-one microRNAs were expressed at significantly different

levels as a consequence of age. Pathways significantly impacted by these microRNAs fell into broad categories such as proliferation (ex. p53 signaling, base excision repair, MAPK signaling) and migration/invasion (ex. Regulation of Actin Cytoskeleton, TGF-β signaling, VEGF signaling) suggesting that regulatory mechanisms governing these processes differed significantly in neonatal and adult progenitors. In rodents, microRNA profiling similarly identified mechanisms by which a proliferative difference occurs when comparing neonatal and adult CPCs (Sirish et al., 2012). Human embryonic CPCs directly isolated from the heart without culture have a proliferative advantage over adult CPCs (Sirish et al., 2012). Our studies take this work a step further by using matched clonal populations to document distinct differences in microRNA expression when comparing neonatal and adult human cardiovascular progenitor cells.

A possible mechanism for decreased proliferation in adult cardiac progenitors is complex regulation of the Myc-E2F1 axis by microRNAs leading to an increase in cellular senescence. E2F1 is a transcriptional activator that is important in the G1/S transition; inappropriate accumulation of E2F1 increases DNA damage response and significantly impacts the ability of cells to enter the S phase of the cell cycle (Pickering et al., 2009). High levels of Myc expression may result in senescence by initiating cellular stress (Campaner et al., 2010). This stress leads to upregulation of DNA repair proteins and cell cycle arrest (Robinson et al., 2009), both of which were observed in adult cardiac progenitors. MicroRNA-371-3p, which was highly expressed in adult CPCs, is correlated with induction of senescence (Wagner et al., 2008). Pro-proliferative microRNAs such as miR-106b, mir-20a, and mir-17 also play a role; these microRNAs help reduce G1 arrest through regulation of E2F1 transcription factors (Pickering et al., 2009; Trompeter

et al., 2011). Transcripts for all three microRNAs were significantly elevated in neonatal CPCs and mir-17 and mir-20a are directly regulated by Myc expression (Coller, Forman, & Legesse-Miller, 2007; Pickering et al., 2009).

MicroRNA profiling and pathway analysis also predicted differences in the capacity to invade, results that were confirmed *in vitro*. Fewer adult CPCs responded to SDF-1α, despite having adequate CXCR4 and CXCR7 receptor levels on their surface. SDF-1α is secreted in the damaged heart and recruits both exogenous and endogenous cardiovascular stem cells to the site of injury (Y. L. Tang et al., 2009). Interestingly, not all neonatal and adult cardiac progenitors responded equally to SDF-1α; we identified a subpopulation of neonatal and adult progenitors that expressed SSEA-4. SSEA-4 is a stem cell marker that identifies cells in early stages of progenitor development (Henderson et al., 2002). In our study, SSEA-4 expression was correlated with differential expression of microRNAs involved in invasion-related pathways. SSEA-4 is expressed on cancer cells that are more highly invasive (Van Slambrouck & Steelant, 2007), however little is known about the impact of SSEA-4 on SDF-1α-induced invasion in cardiovascular cells. Our study shows that SSEA-4+ progenitors invaded more readily in response to SDF-1α when compared to their SSEA-4- counterparts. Comparison of SSEA4+ neonatal and adult CPCs demonstrated that age negatively impacts invasion.

There may be several reasons for this age-related functional discrepancy. SDF-1α signals primarily through Akt signaling or ERK1/2 signaling. Depending on the cell type, these pathways can signal independently, one regulating the survival and proliferation functions of SDF-1 signaling, the other regulating invasion and migration (Peng et al., 2005). After activation by SDF-1α, CXCR4 dimerizes and is phosphorylated

by JAK2 and JAK3 which create docking sites for transcription factors to propagate signaling (Vila-Coro et al., 1999). Adult CPCs may demonstrate decreased CXCR4 dimerization, reduced numbers of cosignaling molecules, or lower levels of phosphorylation and activation of the receptor. Additionally, the cells themselves may secrete different levels of growth factors which could contribute to lower levels of receptor activation.

MicroRNAs play a role in SDF-1α signaling and invasion. Significant differences in microRNA expression in aged cardiac progenitors influences functional parameters relevant for cardiovascular repair. For example, activation of the SDF-1α receptor, CXCR4, induces the expression of proteases such as matrix metallopeptidase 9 (MMP9) which help to degrade the extracellular matrix, allowing cells to invade (Yu et al., 2011). The expression of MMP9 is inhibited by miR-132 (Ucar et al., 2010) and miR-206 (H. Liu, Cao, Ye, & Sun, 2010). The expression of these microRNAs were significantly upregulated in adult CPCs when compared with their expression level in neonatal CPCs (6.0 fold, p=0.0005, 9.6 fold, p=0.0166 respectively). Inhibitors of invasion, such as metallopeptidase inhibitor (TIMP3) (Jain et al., 2012) and homeo box A5 (HOXA5) (X. H. Liu et al., 2012) are targeted by microRNAs upregulated in neonatal cardiac progenitors. MicroRNA-103 represses the expression of TIMP-3 (Jain et al., 2012) (elevated 8.7 fold in neonatal CPCs, p=0.0027) and microRNA-130a represses the expression of HOXA5 (Chen & Gorski, 2008) (elevated 2.4 fold in neonatal CPCs, p=0.0288). If adult progenitors cannot effectively invade the site of injury, a dramatic difference in regeneration will occur with age.

MicroRNA-mediated regulation of gene expression is a novel, rapidly expanding area of research which has opened up new therapeutic options for the reversal of heart disease. Pretreatment of cardiac progenitors prior to transplantation, or direct administration into the heart may activate stem cell recruitment from endogenous sources. In neonatal rodents, microRNA mimics had a positive effect on cytokinesis, DNA synthesis, and cell cycle re-entry (Eulalio et al., 2012). MicroRNAs whose expression levels are altered in aged CPCs may be manipulated *in vivo* to promote recovery from myocardial damage. For example mir-24 (upregulated 8.4 fold in neonatal CPCs) when introduced after myocardial infarction reduces infarct size (Boregowda et al., 2012). Conversely, inhibition of mir-208a (upregulated 12.4 fold in adult CPCs) reduced cardiac remodeling, improved cardiac function, and survival after hypertension-induced heart failure (Abdelalim & Tooyama, 2012). The potential for microRNA-based therapeutics to promote stem cell mobilization, combined with an understanding of the role of microRNAs in cardiac regeneration, promises to open up new treatment options that may improve the outcome of stem cell-based therapies.

Cardiovascular progenitor cells co-expressing c-kit and Isl1 can be identified and expanded *in vitro* from neonatal and adult heart tissue. Epigenetic differences highlight the mechanism by which neonatal cardiovascular progenitor cells can proliferate and invade in response to cytokine stimulation, whereas the adult cells have a diminished capacity for mobilization. Neonatal cardiac progenitor cell clones expressing SSEA-4 are more responsive to stimulation and may be optimal for cardiac regeneration.

Methods

Ethics Statement/ Cell Isolation and Expansion

The Institutional Review Board of Loma Linda University approved the protocol for use of tissue that was discarded during cardiovascular surgery, without identifiable private information, for this study with a waiver of informed consent. Discarded atrial cardiac tissue from human neonates (<1 month old) and adults (57-75 years old), was cut into small clumps (approximately $1mm^3$) and collagenase digested (Roche Applied Science, Indianapolis, IN) for approximately 2 hours at 37 degrees at a proportion of 1:2.5 tissue volume vs. collagenase. This solution was then passed through a 40μm cell strainer to isolate cardiac progenitors (Smits et al., 2009). Resulting cells were cloned by limiting dilution at a concentration of 0.8 cells per well to create clonal populations which were expanded for further study. Over two hundred and forty clones were isolated from human patients by this procedure; seventeen neonatal and sixteen adult cardiovascular cell clones were compared in detail for this study. The human embryonic stem cell line hES-3 was cultured as previously published (Kearns-Jonker et al., 2012). Cardiac progenitor cell clones were differentiated both by treatment with 5-azacytidine followed by ascorbic acid and TGF- β to induce cardiomyogenic differentiation (Smits et al., 2009) and by treatment with 10nM dexamethasone in DMEM/F12 media supplemented with 10% fetal bovine serum to induce differentiation into all three cardiovascular lineages (D'Amario, Fiorini, et al., 2011). Cardiomyogenic differentiation was confirmed by measuring the induction of mature cardiac-specific transcripts by RT-PCR. Expression of endothelial, smooth muscle, and cardiomyocyte markers induced by dexamethasone treatment were quantified by flow cytometry.

Flow Cytometry Experiments

Cells were labeled using antibody concentrations that were recommended by the manufacturer(s). Fluorescently labeled cells were analyzed using a MACSquant analyzer (Miltenyi Biotec, Auburn, CA). FlowJo software (Ashland, OR) was used for quantification. Dead cells and small particles were gated out using forward-scatter, side-scatter gating. Isotype controls (MS IgG1) were used to define negative and positive populations. Antibodies used included CD105-PE, IGF1R-PE, CXCR4-PE, CXCR7-PE, CD140a-PE, CD146-PE, SSEA-4-FITC, Pan HLA-FITC, CD309-PerCP/Cy5.5 (Biolegend, San Diego, CA), CD44-FITC, CD13-PE, CD31-PE, HLA-Dr-PE, CD73-PE, CD34-PE (BD Biosciences San Jose, CA), CD90-PE (Immunotech, Brea, CA), CD117-PE (Millipore, Billerica, MA). Additional antibody data can be found in appendix; supplementary table 2. Relative percentage of large neonatal and adult cardiovascular progenitors was assessed by flow cytometry. Large cells were defined by forward scatter gating that was uniformly applied to all samples and the percentage of cells within this gate was compared. Each test was run in duplicate.

RT-PCR

Total RNA was extracted from neonatal and adult cardiovascular progenitor cell clones and reverse transcribed into cDNA using superscript III (Invitrogen, Grand Island, NY). Real-time PCR was performed using Go Taq® Green Master Mix (Promega, Madison, WI). The PCR conditions were: 94°C for 10 minutes, 94°C for 15 seconds, 56°C for 60 seconds, 72°C for 30 seconds for a total of 40 cycles. Human primers were created using NCBI primer blast and included tyrosine-protein kinase (c-kit),

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ISL LIM homeobox 1 (Isl1). Primer sequences are listed in appendix; supplementary table 6.

For microRNA profiling experiments, total RNA was extracted from representative neonatal (N=8) and adult (N=3) CPC clones and converted to cDNA using the RT^2 miRNA First Strand Kit (SABiosciences, Valencia, CA). The Cell Development & Differentiation miRNA PCR array plates (SABiosciences, Valencia CA) and RT^2 SYBR Green qPCR Mastermix (SABiosciences, Valencia, CA) was used. Plates were run on an iCycler iQ5 PCR Thermal Cycler (Bio-Rad, Hercules, CA) for 94°C for 10 minutes, 94°C for 15 seconds, 60°C for 60 seconds, 72°C for 30 seconds for a total of 40 cycles. Fold change was calculated using the ΔΔCt method(Rodier & Campisi, 2011). Data from representative cardiovascular cell clones was analyzed individually, then pooled within age groups. The microRNA expression data has been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev, & Lash, 2002) and are accessible through GEO series accession number GSE49235. miRNAs that were expressed at significantly different levels when comparing neonatal CPC with adult CPC were analyzed using DIANA mirPath computational software (Athens, Greece) which performs an enrichment analysis of multiple microRNA target genes, comparing each set of microRNA targets to all known KEGG pathways (T. M. Liu et al., 2013).

Cell Cycle Analysis

Cells were trypsinized at 60-80% confluency, counted, and concentrated to 10^5 cells/0.3ml PBS. Ethanol (0.7ml) was added dropwise to fix the cells. Cells were stored for at least 1 hour at -20 degrees, washed, incubated at 37 degrees with RNase A

(0.5mg/ml Invitrogen, Grand Island, NY) and propidium iodide was then added at a final concentration of 10µg/ml. Samples were run on MACSquant analyzer (Miltenyi BIotec, Auburn, CA). Cell cycle analysis was done using FlowJo software (Ashland, OR).

Transwell Invasion Assay

Cells were plated in the top well of Costar Transwell plates (8µm pores), coated with Cultrex™ basement membrane extract (Trevigen, Gaithersburg, MD) mimicking extracellular matrix. Cells were plated at a density of 100,000 cells per 100µl in starved medium. Stromal cell-derived factor-1α (SDF-1α, Invitrogen, Grand Island, NY) was used as a chemoattractant at a concentration of 100ng per ml of M199 plus EGM-2 in the bottom chamber of a transwell plate. After 24 hours, cells in the bottom wells were trypsinized and counted using a flow cytometer. The response of neonatal and adult CPC clones was analyzed individually then pooled by age and subdivided according to the presence or absence of SSEA-4 expression.

Immunocytochemistry

Neonatal and adult CPC were plated on gelatin coated Lab-Tek II cc^2 chamber slides (Nunc, Rochester, NY) and grown at 37º for 4 days, fixed in 4% paraformaldehyde and stained using a primary anti-troponin I antibody at 10µg/ml (Millipore #MAB1691, Billerica, MA) and FITC-conjugated, goat anti-mouse IgG secondary antibody at 2µg/ml (Southern Biotech, Birmingham, Alabama). Coverslips were mounted using Prolong Gold antifade with DAPI (Invitrogen, Grand Island, NY). Slides were imaged using a Zeiss confocal LSM 710 NLO laser-scanning, confocal microscope (GmbH, Germany).

Statistics

RT^2 Profiler PCR Array Data Analysis software (SABiosciences, version 3.5) was used to calculate statistical significance for microRNA profiling. Data was tested for normal distribution using the Anderson-Darling normality test calculator Version 6.0 with an alpha of 0.05 (faculty.missouri.edu/~glaserr/3700s11/AD-Test_Calculator.xls). For normally distributed data, an unpaired, two-tailed student's t test was performed. For data that was not normally distributed, a Mann-Whitney Rank Sum Test was performed. Statistically significant differences were identified as $P < 0.05$. Data was reported as the mean +/- standard error.

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CHAPTER THREE

INVESTIGATION OF A NOVEL THERAPEUTIC STRATEGY TO IMPROVE ENDOGENOUS CARDIAC STEM CELL FUNCTION

Introduction

The ERK signaling pathway is the classical mitogen-activated protein kinase signaling pathway that is stimulated by a variety of growth factors including SDF-1α (Hu et al., 2007; Yin et al., 2011). Changes in the phosphorylation of ERK can have functional consequences on cell proliferation, invasion, and secretory phenotype (Howe, Aplin, & Juliano, 2002; H. J. Lee, Cho, Kwon, Park, & Kim, 2013; Xie et al., 2014). ERK signaling is required for cardiosphere formation and subsequent VEGF production, and inhibition of ERK results in increased levels of stemness associated genes such as OCT4 (Cho et al., 2012). Tight regulation of the ERK signaling pathway in CPCs is essential, as small increases in phosphorylation can result in functional benefits whereas large increases in ERK phosphorylation are associated with replicative senescence (Choi et al., 2013). Duration and intensity of ERK phosphorylation can also impact downstream functional consequences (Murphy & Blenis, 2006).

In this chapter we investigated the potential for a novel combination of short-term hypoxia and prostaglandin E_2 treatment to improve endogenous CPC function through regulation of the MAPK/ERK signaling pathway. There is evidence that treatment of with a combination of hypoxia and PGE_2 can result in additive functional improvements (Jung, Choi, Yoo, Baek, & Kwon, 2013) through overlapping signaling. In human cancer cells, 24 hours of hypoxia (1% oxygen) activated the cyclooxygenase-2-PGE_2 synthase axis (J. J. Lee et al., 2010). Conversely, treatment with PGE_2 induced hypoxia-inducible

factor-1α (HIF-1α) stabilization and nuclear localization (X. H. Liu et al., 2002). In endothelial cells, exogenous administration of PGE_2 augmented hypoxia-induced proliferation and VEGF secretion (Zhao et al., 2012) and in hematopoietic stem cells, increased HIF-1α was required for increased migration through PGE_2 mediated upregulation of CXCR4 (Speth, Hoggatt, Singh, & Pelus, 2014). Both migration and invasion are important CPC functions required for cardiac regeneration. In response to external stimuli, cardiac progenitors must first migrate through the blood and extracellular fluid towards the site of injury. Once at the site of injury, CPC must then invade, through the use of proteases and other proteins and infiltrate the injured area. In this chapter, we report the response of neonatal and adult cardiovascular progenitors treated with short-term hypoxia and prostaglandin E_2. We measured the relative phosphorylation of ERK and the functional consequences of altered signaling after cell treatment, specifically, cell cycle progression, expression of paracrine factors, and migration.

The portion of this work that was prepared for publication is included in this chapter. The data summarizing the treatment of cells with hypoxia alone and treatment of cells with a combination of hypoxia and prostaglandin E_2 is included in the appendix. In brief, exposure to hypoxia (1% oxygen) increased the phosphorylation of ERK (Appendix, supplementary figure 3) and combination of prostaglandin E_2 with hypoxia further increased the phosphorylation of ERK (Appendix, supplementary figure 4). Migration, which was not altered by hypoxia exposure, was increased with a combination of prostaglandin E_2 and hypoxia when compared with untreated cells (9803 vs 1987 cells, $p=0.07$, appendix, supplementary figure 5). Cell cycle progression, on the other hand was

not significantly altered by either hypoxia treatment alone or a combination of hypoxia and prostaglandin E_2 (Appendix, supplementary figure 6). Therefore, the combination of prostaglandin E_2 with hypoxia can increase CPC ERK phosphorylation and migration when compared with hypoxia treatment alone.

Prostaglandin E_2 Treatment Reduces Paracrine Factor Expression and ERK Phosphorylation in Human Endogenous Cardiac Stem Cells

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Running Title: Prostaglandin E_2 and cardiac stem cells

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Abstract

Prostaglandin E_2 improves functional outcome when injected into the heart or co-transplanted with stem cells after a myocardial infarction. This effect may be mediated through activation of select populations of existing stem cells. The signaling and functional events associated with treatment of human endogenous cardiac stem cells with prostaglandin E_2 is unknown. In this report we used clonal populations of Islet-1+ c-kit+ cardiac stem cells that were derived from the human heart to study the effect of prostaglandin E_2 on migration, cell cycle progression, gene expression and signaling. Islet-1+ c-kit+ cardiac progenitors expressed transcripts for prostaglandin receptors EP1, EP2, and EP4 but not EP3 by PCR. A significant increase in migration of cardiac progenitors was identified after 24 hours of prostaglandin E_2 treatment, using a transwell migration assay. On the other hand, cell cycle progression (as measured by flow cytometry) was unchanged and differentiation along the cardiac specific lineage as identified by transcripts for troponin T and myosin light chain 2v was not significantly induced by a short term exposure to prostaglandin E_2. Transcripts for genes encoding paracrine factors that play a key role in cardiac stem cell regenerative ability, including insulin-like growth factor 1, stromal cell-derived factor 1α, and vascular endothelial growth factor A, were expressed at significantly lower levels in endogenous cardiac progenitors following prostaglandin E_2 treatment. Phosphorylation of ERK and the prosurvival protein, AKT, was also reduced. Our studies show that prostaglandin E_2 treatment for 24 hours improves Islet-1, c-kit+ progenitor cell migration but reduces cytokine expression and the activation of key pathways involved in cardiac stem cell regenerative ability.

Introduction

The inability of the adult human heart to completely repair itself after injury, and the growing number of patients with heart disease, has fueled the investigation of stem cell-based therapeutics to enhance cardiac regeneration. Recent clinical trials using endogenous cardiac stem cells administered after myocardial infarction have demonstrated some benefit following transplantation, however outcomes are varied and do not completely ameliorate dysfunction (Bolli et al., 2012; Malliaras et al., 2014). Pre-treating stem cells prior to transplantation can lead to increased cardiac regeneration after myocardial infarction (Hahn et al., 2008; Li et al., 2010) and several approaches have been taken to optimize stem cell function *in vitro*. One concept is to harness the innate response to injury as a means of developing new methods that may further enhance regeneration. For example, in response to cellular injury such as myocardial infarction, prostaglandin E_2 is secreted within the infarct zone. Prostaglandin E_2 (PGE_2) is a lipid compound derived from arachidonic acid through a reaction catalyzed by COX-2. Although prostaglandin E_2 is immunomodulatory (Dhingra et al., 2013; Kalinski, 2012) and has been shown to promote tolerance when co-transplanted with stem cells into the heart (Dhingra et al., 2013), there is evidence that it may also facilitate cardiac repair (Xiao et al., 2004). After myocardial infarction, injection of prostaglandin E_2 increases the number of cardiomyocytes in the border zone (Hsueh, Wu, Yu, Wu, & Hsieh, 2014), whereas knockout of prostaglandin receptor EP4 increases infarct size (Xiao et al., 2004) and microsomal deletion of prostaglandin E_2 synthase-1 impairs restoration of left ventricular systolic and diastolic function (Degousee et al., 2008). Prostaglandin E_2, therefore, is crucial to repair and may play a role in activating endogenous stem cells.

We hypothesized that pretreatment of cardiac stem cells with PGE_2 prior to transplantation may enhance their regenerative capacity.

We have previously identified an early cardiovascular progenitor cell population that can be isolated from the human heart and expresses both the early stem cell marker Islet-1 (Isl-1) as well as tyrosine protein kinase, c-kit (Fuentes et al., 2013). In this report, we characterized the effect of prostaglandin E_2 on gene expression, signaling, cell cycle progression and migration in clonal populations of human Isl-1+c-kit+ cardiac progenitor cells (CPCs).

Materials and Methods

Ethics Statement/Cell Isolation and Expansion

The Institutional Review Board of Loma Linda University approved the protocol for use of tissue that was discarded during cardiovascular surgery, without identifiable private information, for this study with a waiver of informed consent. All experiments were performed according to the principles expressed in the Declaration of Helsinki. Discarded cardiac tissue from the right atrium was cut into ~$1mm^3$ clumps then incubated at 37^oC for 2 hours with collagenase (Roche Applied Science, Indianapolis, IN). This solution was then passed through a 40μM cell strainer to isolate cardiac progenitors (Smits et al., 2009). To create clonal populations for further study, cells were grown at a concentration of 0.8 cells/well and expanded for further study.

Prostaglandin E_2 Dilution

Lyophilized prostaglandin E_2 (Cayman Chemical, Ann Arbor, MI) was dissolved

in DMSO at a concentration of 10mM and stored at -20°C. Prior to adding PGE_2 to cell culture medium, the pH of the media was adjusted to approximately 6.8-7.0.

Cell Cycle Progression

To investigate relative cell cycle progression, cells were treated for 24 hours with normal growth medium supplemented with 10µM PGE_2. After 24 hours, cells were trypsinized and counted. One hundred thousand cells were fixed in 70% ethanol, incubated at 37°C with RNAse A (0.2mg/ml, Fisher Scientific Pittsburg, PA), then labeled with propidium iodide. Stained samples were run on a MACSquant analyzer (Miltenyi Biotec, Auburn, CA) and analyzed using Flowjo software.

Flow Cytometry

We used flow cytometry to measure phosphorylated proteins. Quantitation of phosphorylated proteins by flow cytometry is consistent with data obtained by the more conventional western blot technique and has the added advantage of being able to assess phosphorylation levels on single cells (Abrahamsen & Lorens, 2013; Jun, Lee, Song, Mansfield, & Chou, 2011; Krutzik & Nolan, 2003). This technique can also determine whether or not the level of response is homogenous within the population (Krutzik, Irish, Nolan, & Perez, 2004). Cardiovascular progenitor cell clones were grown for 24 hours in starving medium then treated with 10µM PGE_2 for 15 minutes. Following treatment, cells were trypsinized, fixed with 4% paraformaldehyde, permeabilized with 90% methanol, and labeled with antibodies against phosphorylated ERK 1/2 (Thr202/Tyr204, Cell Signaling Technology, Danvers, MA) at a 1/200 dilution or pAKT (Ser473, Cell

Signaling Technology, Danvers, MA) at a 1/100 dilution. FITC-labeled goat anti-rabbit IgG (BD Biosciences San Jose, CA) at a dilution of 1/150 was used as a secondary antibody. After antibody incubation, cells were analyzed using MACSquant analyzer (Miltenyi Biotec, Auburn, CA). Small particles and dead cells were gated out using forward-scatter, side-scatter gating. FlowJo software (Ashland, OR) was used for quantification.

Migration

Migration experiments were performed after CPCs were grown in normal growth medium supplemented with 10μM PGE_2 for 24 hours. Cardiac progenitors were then trypsinized, counted and plated at a density of 50,000 cells per well in a 96 well transwell migration assay with 8 micron pores (Corning, Union City, CA). Migration experiments were performed according to manufacturer's instructions. In brief, CPCs were resuspended in starving medium and plated in the top chamber. Normal growth medium supplemented with 100ng/mL of SDF-1α (Life Technologies, Grand Island, NY) was used as a chemoattractant in the bottom chamber. After 6 hours, migrated cells in the bottom chamber were stained using Calcein AM (Fisher Scientific Pittsburg, PA) and fluorescence was measured using a FLX800 fluorescent plate reader (Bio-Tek, Winooski, VT). Fluorescence was converted to cell number using a standard curve as per manufacturer's instructions.

Quantitative RT-PCR

Trizol reagent (Life Technologies, Grand Island, NY) was used to store cells after

PGE_2 treatment (10µM for 24 hours). RNA was isolated using the RNeasy Mini-spin Columns (Qiagen, Valencia, CA) and 500ng of RNA was used to make cDNA using the Superscript III protocol (Life Technologies, Grand Island, NY). Beta actin was used as a housekeeping control. RT-PCR was run on an IQ5 machine (Bio-rad, Hercules, CA). The PCR conditions were: 94°C for 10 minutes, 94°C for 15 seconds, 56°C for 60 seconds, 72°C for 30 seconds for a total of 45 cycles or 94°C for 10 minutes, 94°C for 15 seconds, 55°C for 60 seconds, 72°C for 30 seconds for a total of 45 cycles. Primer sequences can be found in appendix, supplementary table 7. Fold change was derived using the ΔΔCt Method (Schmittgen & Livak, 2008).

Statistics

A paired student's t test was used to calculate significance in cell cycle, migration, and flow cytometry experiments. A student's t test was used for RT-PCR experiments. Significance was set at $p<0.05$. Data is represented as the mean +/- the standard error.

Results and Discussion

The vital role of prostaglandin E_2 in heart regeneration (Degousee et al., 2008; Hsueh et al., 2014; Xiao et al., 2004) lends credence to the idea that PGE_2 can activate the human cardiac progenitor population. However, this has not been thoroughly investigated. It is unknown whether the majority of benefits after prostaglandin E_2 administration are mediated through direct activation of existing stem cells within the heart, or are mediated through other cell types. In this study, we used clonal populations

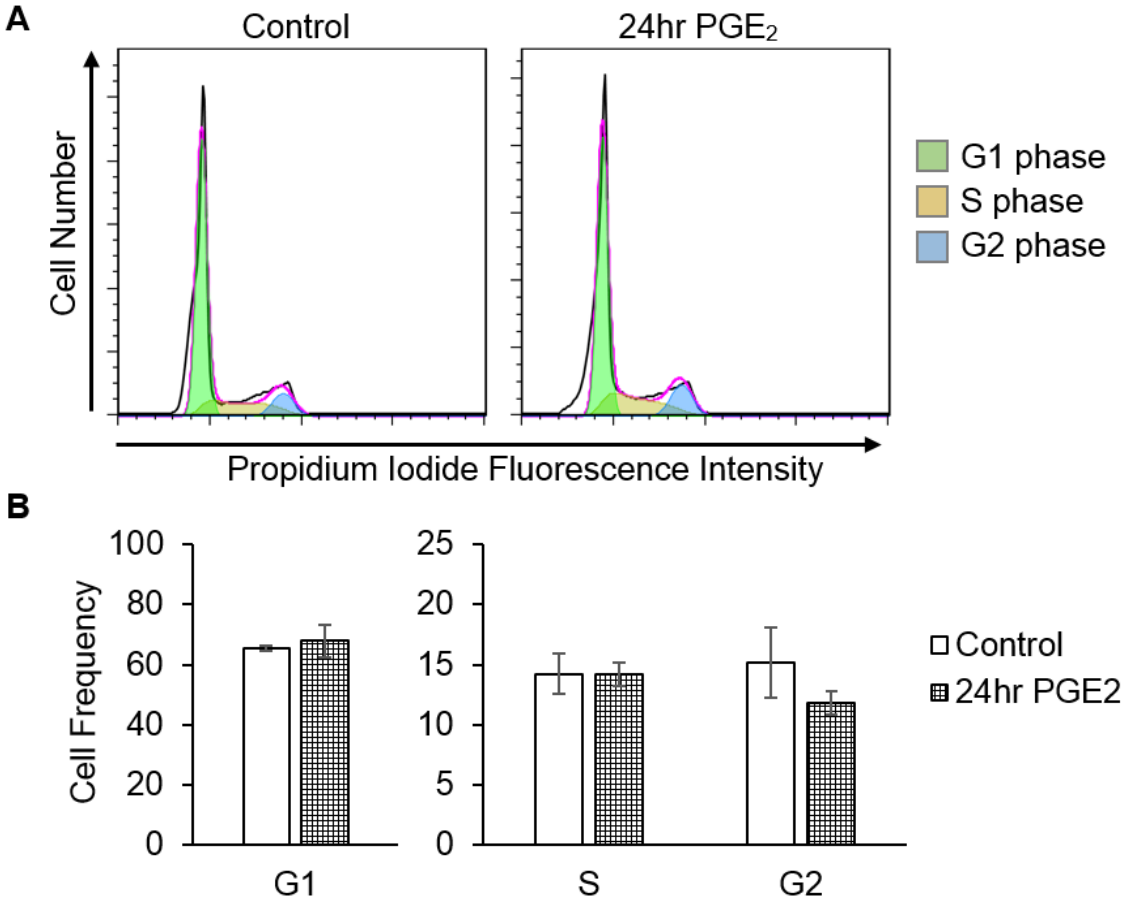

Fig. 7. Cell cycle progression after 24 hour treatment of cardiac progenitors with 10µM PGE_2. A) Representative histogram of cell cycle progression in a cardiac progenitor cell clone. B) Quantification of cell cycle analysis. There was no significant difference in the cell cycle progression in cardiac progenitor cell clones (n=4) after 24 hours of 10µM PGE_2 treatment.

of human endogenous cardiac progenitors that co-express both c-kit and Isl-1 to elucidate whether PGE_2 could be used as a pretreatment to improve CPC function. The use of single cell clones with a clearly defined phenotype (Fuentes et al., 2013) allows us to correlate functional response after PGE_2 exposure to a single cell type that normally resides within the heart.

Methods to promote efficient cardiac regeneration will ideally optimize functions such as cell proliferation and migration. The ability of stem cells to effectively divide and repopulate damaged tissue is key to their capacity for cardiac repair. We measured the effect of PGE_2 treatment on proliferation of Isl-1+c-kit+ cardiac progenitors using a flow cytometry-based approach. After 24 hours, 10μM PGE_2 treatment did not alter the percentage of dividing cells. There was no significant change in the frequency of cells in G1, S, or G2 phase of the cell cycle (n=4, Fig. 7). This outcome is consistent with results from other studies using other cell types, such as neuroectodermal stem cells that do not demonstrate changes in cell proliferation after PGE_2 treatment (Wong, Ahmad, Li, & Crawford, 2014). By and large, the cellular response to PGE_2 appears to be dependent on variables that include cell source, cell type and dose of PGE_2. In certain populations of mesenchymal stem cells, treatment with 10μM of PGE_2 for 24 hours increases proliferation (Jang et al., 2012). In other cell types, proliferation is inversely related to dose, high doses of prostaglandin E_2 decreases proliferation and low doses increases proliferation (Kleiveland, Kassem, & Lea, 2008; Zhang & Wang, 2014). It is important to note that cell cycle progression, although unchanged, was not adversely affected by prostaglandin E_2 treatment.

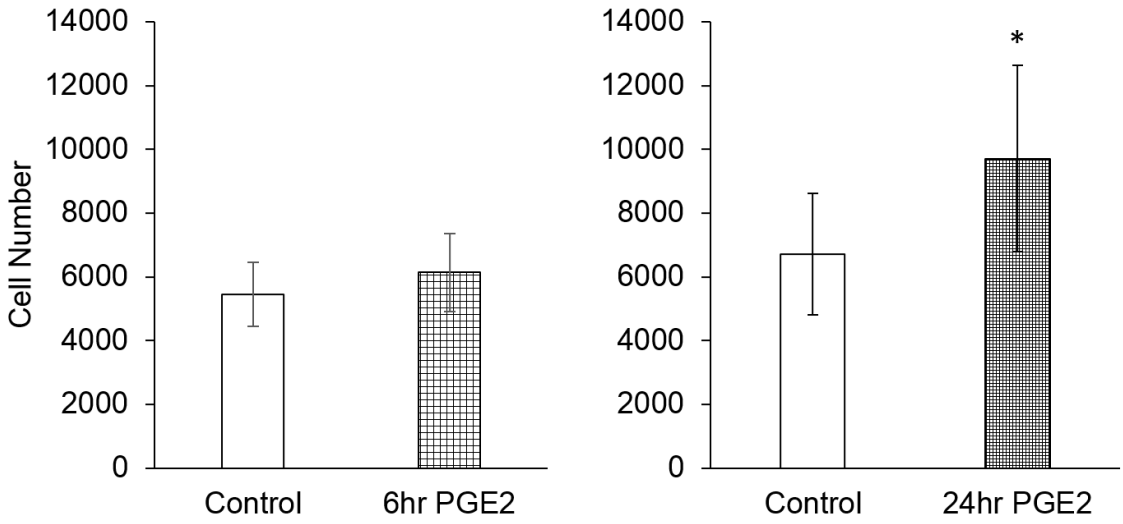

Fig. 8. Migration after PGE_2 treatment in cardiac progenitor cell clones. Treatment with 10μM PGE_2 significantly altered cardiac progenitor cell migration after 24hrs (n=4, p=0.049) but not after 6 hours (n=4, p=0.32).

Migration is also key to regeneration. After injury, cardiac progenitors must migrate away from the stem cell niche towards the site of injury. One of the factors that can induce CPC migration after myocardial infarction is SDF-1α (J. M. Tang et al., 2011). Previously, we have demonstrated that Isl-1+c-kit+ cardiac progenitor cell clones have ample expression of the SDF-1α receptor responsible for cell migration, CXCR4 (Fuentes et al., 2013). We therefore tested the effect of prostaglandin E_2 on migration of human Isl-1+c-kit+ cardiac stem cells in response to normal growth medium supplemented with SDF-1α. We compared the effect of both a 6 hour, and a 24 hour treatment with 10μM PGE_2. As with other cell types (Hoggatt, Singh, Sampath, & Pelus, 2009; X. H. Liu et al., 2002; Speth et al., 2014; Weller et al., 2007), prostaglandin E_2 significantly increased SDF-1α-induced migration of Isl-1+c-kit+ cardiac stem cells after 24 hours ($9.7x10^3$ cells, PGE_2-treated CPCs vs $6.7x10^3$ cells, untreated CPCs, n=4, p=0.049, Fig. 8). A six hour treatment, however, did not alter cell migration ($6.1x10^3$ cells, PGE_2-treated CPCs vs $5.5x10^3$ cells, untreated CPCs, n=4, p=0.32, Fig. 8), suggesting that time may be a factor in stem cell activation. The increased chemotaxis of CPCs after PGE_2 treatment may explain the elevated expression levels of stem cell markers (noted by others) within the heart after prostaglandin E_2 injection in mice (Hsueh et al., 2014). This suggests that prostaglandin E_2 may play a role in homing resident cardiac stem cells to the site of injury and could hypothetically be used as a pretreatment to increase cell migration.

There are four receptors for prostaglandin E_2 including EP1, EP2, EP3, and EP4. In mice, EP4 is the most abundantly expressed prostaglandin receptor within the heart (Xiao et al., 2004). Previous reports have demonstrated expression of all four

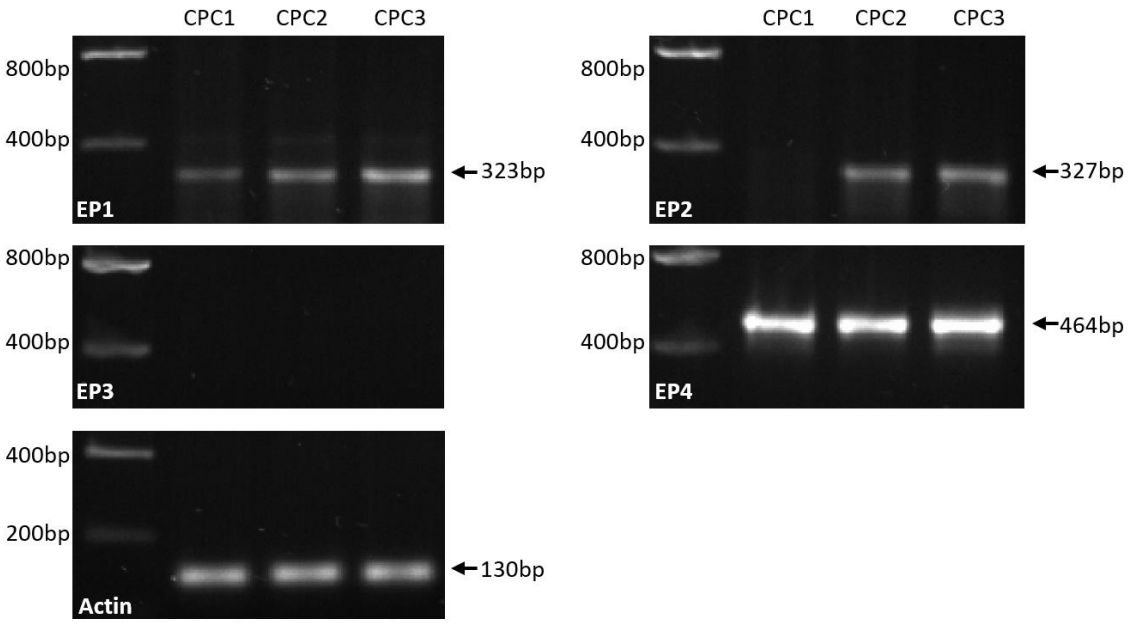

Fig. 9. Expression of prostaglandin E_2 receptors by PCR. Cardiac progenitor cell clones were examined by PCR to determine whether they expressed prostaglandin E_2 receptors. PCR products were run on a gel. Transcripts for EP1 (size = 323bp) EP2 (size = 327bp) and EP4 (size = 464bp) were expressed on cardiac progenitor cell clones. EP3 (size = 384 bp) transcript expression was not found.

prostaglandin E_2 receptors on other human stem cell types such as mesenchymal (Jang et al., 2012) and hematopoietic stem cells (Hoggatt et al., 2009), however whether resident human cardiac progenitors express receptors for prostaglandin E_2 is unknown. We therefore measured transcripts for the four prostaglandin receptors by PCR. Isl-1+ c-kit+ cardiac progenitors expressed transcripts for EP1 (product size 323bp), EP2 (product size 327bp), and EP4 (product size 464bp). EP3 (product size 384bp), however, was not expressed in any of the CPC clones tested (Fig. 9). This suggests that the response of human CPCs to prostaglandin E_2 is possibly mediated through signaling in one or more of the prostaglandin receptors including EP1, EP2, and EP4, but not likely EP3.

Enhanced migration in cardiac progenitors after prostaglandin E_2 treatment could involve signaling in the PI3K-AKT pathway (Guo et al., 2014). Prostaglandin E_2 treatment (in cancer cells, tendon stem cells, and dendritic cells) can increase PI3K-AKT signaling as evidenced by increased levels of phosphorylated AKT (George, Sturmoski, Anant, & Houchen, 2007; J. Liu, Chen, Tao, & Tang, 2013; Vassiliou, Sharma, Jing, Sheibanie, & Ganea, 2004). In this study, we quantified the activation of the PI3K-AKT signaling pathway after prostaglandin E_2 treatment by measuring the level of phosphorylated AKT by flow cytometry. Surprisingly, treatment with 15 minutes of 10μM PGE_2, which can increase AKT phosphorylation in other cell types (Buchanan et al., 2006; Wang & Klein, 2007), decreased AKT phosphorylation levels in CPCs (3968 MFU decrease, n=6, p=0.056, Fig. 10A&C). This suggests that increased migration after PGE_2 treatment in endogenous CPCs, is likely independent of PI3k-AKT signaling. This decrease in phosphorylated AKT could hypothetically impact functions such as cell survival (Liou et al., 2007; Vassiliou et al., 2004).

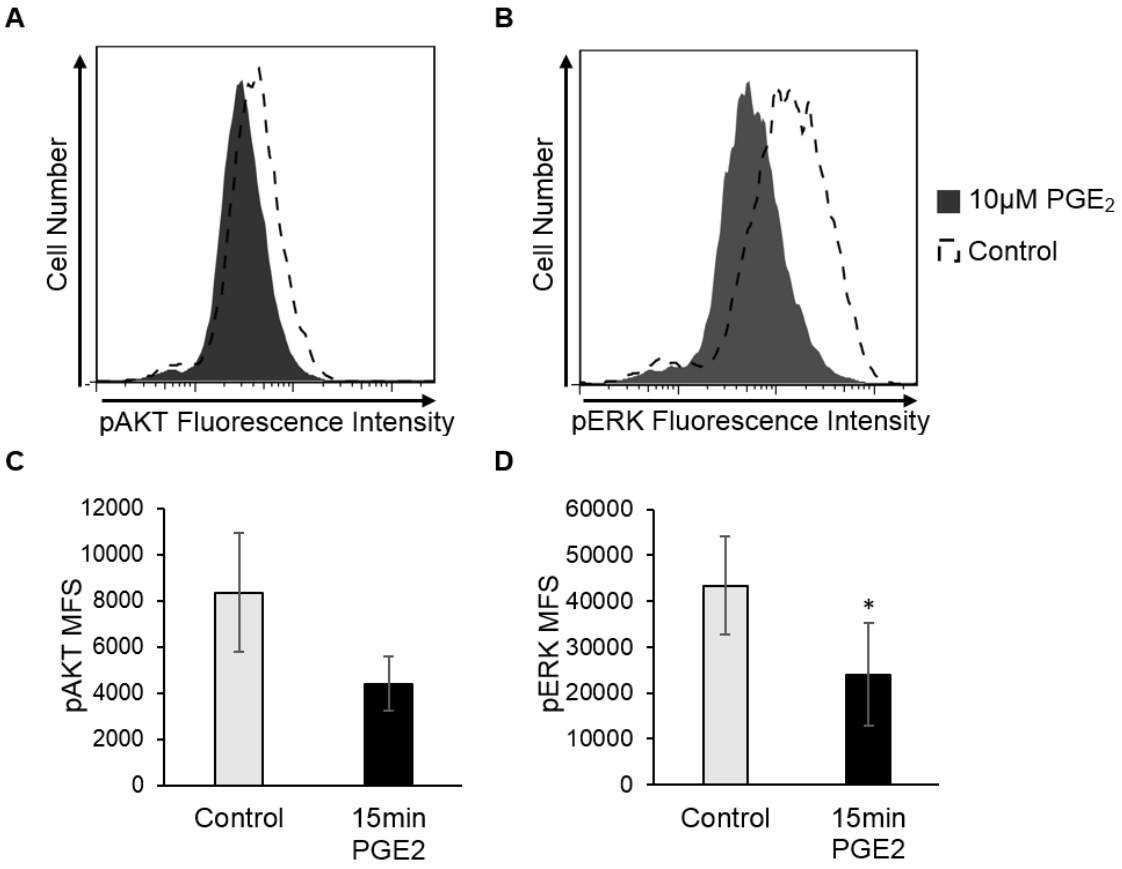

Fig. 10. Changes in ERK and AKT phosphorylation after PGE_2 treatment in starved medium. Representative histograms displaying A) phosphorylated AKT and B) phosphorylated ERK protein expression levels by flow cytometry after 15 minutes of PGE_2 treatment. C) Levels of phosphorylated AKT were decreased by PGE_2 treatment but not significantly (n=6, p=0.056). D) Levels of phosphorylated ERK were significantly decreased after prostaglandin E_2 treatment in cardiac progenitors (n=6, p=0.035).

At the present time, stem cell survival after transplantation has been reported to be relatively low to negligible on a long-term basis (K. U. Hong et al., 2014; Malliaras et al., 2013). Due to this fact, paracrine mechanisms (rather than direct differentiation), have been reported to account for the majority of the beneficial effects of cardiac stem cell transplantation (Chimenti et al., 2010; Kyung U Hong et al., 2014). In other types of resident cardiac stem cells, the MAPK/ERK pathway regulates the secretion of beneficial paracrine factors (H. J. Lee et al., 2013). When cardiac stem cells are in ERK-stimulating conditions, growth factors, such as VEGF, are secreted at elevated levels, whereas in ERK-inhibitory conditions, cells are more prone to cardiomyogenic differentiation (H. J. Lee et al., 2013). The MAPK/ERK signaling pathway, if altered, can also affect cell proliferation, migration, stemness and secretory phenotype (Cho et al., 2012; H. J. Lee et al., 2013; Ryu et al., 2010; Xie et al., 2014). We measured changes in the MAPK/ERK signaling pathway by quantifying the level of phosphorylated ERK (pERK) by flow cytometry. After 15 minutes of exposure to 10μM PGE_2, the level of pERK declined significantly (19,406 MFU decrease, n=6, p=0.035, Fig. 10B&D). This same time frame increases ERK phosphorylation in other cell types (Krysan et al., 2005; Rao et al., 2007).

Under "ERK-inhibitory" conditions (H. J. Lee et al., 2013) we would expect decreased growth factor expression. After prostaglandin E_2 exposure we used RT-PCR to quantify the expression of paracrine factors in Isl-1+c-kit+ cardiac progenitors. These growth factors included hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), stromal cell-derived factor 1α (SDF-1α), and vascular endothelial growth factor A (VEGFA) which are all known to improve cell function and enhance cardiac

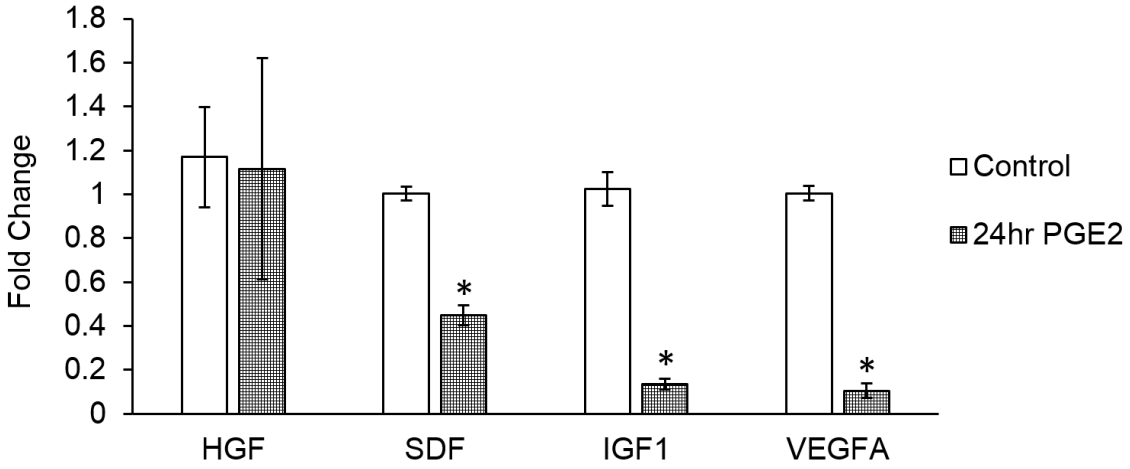

Fig. 11. Expression of growth factors in cardiac progenitor cell clones after PGE_2 treatment. After 24 hours of 10μM PGE_2, the expression of SDF-1, IGF1, and VEGFA were significantly downregulated in cardiac progenitor cell clones as shown by RT-PCR (n=3, run in triplicate, $p<0.05$).

regeneration after injury (Cheng et al., 2014; Hu et al., 2007; Linke et al., 2005; J. Tang et al., 2009). As expected (based on ERK phosphorylation levels), 24 hours of prostaglandin E_2 treatment significantly decreased the expression of transcripts for SDF-1α, IGF1, and VEGFA (2.3 fold, 7.4 fold, and 9.5 fold respectively, $p<0.0000001$, Fig. 11). This decline in growth factor expression levels is a novel finding that is inconsistent with reports in other cell types (Jang et al., 2012; Junpeng Liu, Chen, Liu, & Tang, 2014; Peng et al., 2013; Zhao et al., 2012) and raises the possibility that prostaglandin E_2 treatment, in Isl-1+c-kit+ cardiac stem cells, may hinder their therapeutic efficacy after transplantation and consequently their regenerative capacity.

In this study, prostaglandin E_2 treatment lowered both the phosphorylation level of ERK and the expression of transcripts for SDF-1α, IGF-1, and VEGFA. We therefore also measured the effect of PGE_2 on the expression of cardiac specific transcripts, Troponin T and MLC2v. After 24 hours of 10μM PGE_2, we identified a trend (less than 2-fold) towards increased expression levels of Troponin T and MLC2v that was not found to be significant when compared with untreated cells (Fig. 12, n=3, run in triplicate). This suggests that 24 hours is not sufficient to significantly upregulate cardiac-specific transcripts associated with cardiomyogenic differentiation.

Conclusions

In this report, we evaluated the effect of prostaglandin E_2 on Isl-1+c-kit+ cardiac progenitors derived from the human heart. Although prostaglandin E_2 is a crucial factor involved in cardiac regeneration (Degousee et al., 2008; Hsueh et al., 2014; Xiao et al., 2004), at the current doses it is not a preferable pretreatment option for CPCs. Resident

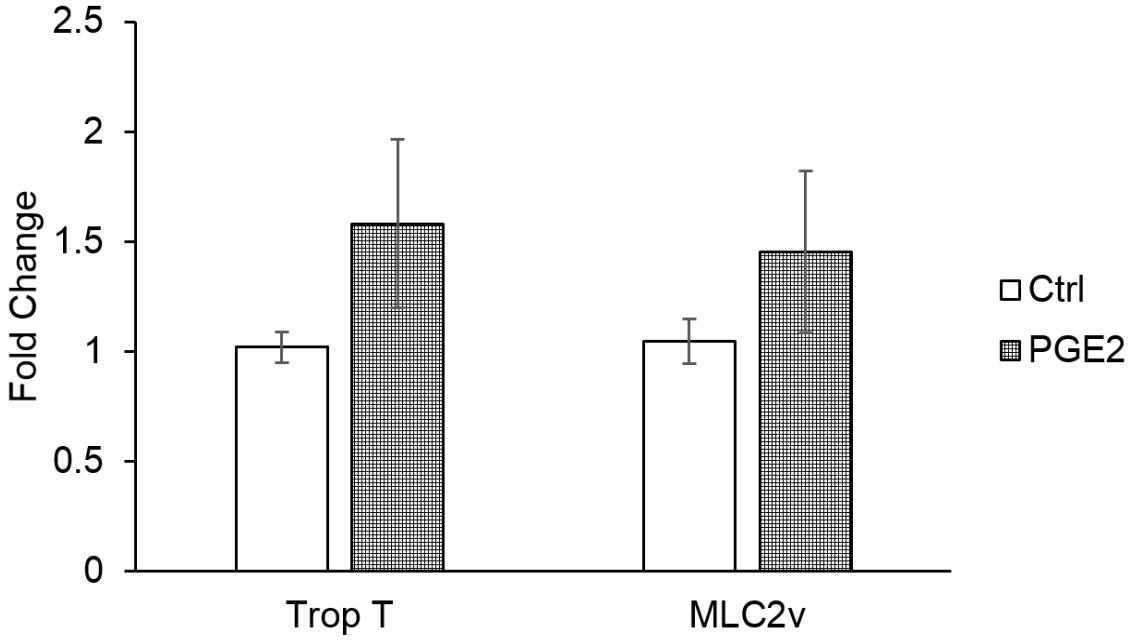

Fig. 12. Cardiac differentiation in CPCs after PGE_2 treatment. RT-PCR expression levels of cardiac-specific differentiation transcripts, cardiac troponin T (Trop T) and myosin light chain 2 (MLC2v), were not significantly altered after 24 hours of 10μM PGE_2 treatment in cardiac progenitor cell clones (n=3, run in triplicate).

cardiac stem cells show enhanced cell migration after exposure to 10μM prostaglandin E_2, however paracrine factor expression declines. Likewise, the phosphorylation of ERK and AKT, key proteins involved in cell function and survival were decreased in CPCs after short-term prostaglandin E_2 treatment.

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CHAPTER FOUR

CONCLUSIONS & FUTURE DIRECTIONS

Summary

The purpose of this dissertation was to define key epigenetic, phenotypic, and functional changes that distinguish neonatal and adult cardiovascular progenitors. In chapter 2, we determined that cardiac progenitors isolated from the adult human heart have a clear shift in microRNA expression profile with 41 significantly altered microRNAs. These epigenetic differences may contribute to functional disparities evident between neonatal and adult cardiac progenitors including significantly decreased cell cycle progression and reduced SDF-1α-induced cell invasion in adult cardiac progenitors. Interestingly, both neonatal and adult CPCs had similar cardiomyogenic differentiation ability as assessed by the expression of cardiac-specific transcripts during 5-azacytidine induced cardiac differentiation by RT-PCR. This change in cardiac stem cell functional ability is consistent with studies that have determined that neonatal CPCs have a higher regenerative ability *in vivo* when compared with adult CPCs (Simpson et al., 2012). We also distinguished a subpopulation of cardiac progenitors that express SSEA4, which have superior ability to invade in response to growth factor simulation.

In chapter 3, our goal was to investigate a novel strategy to improve cardiac progenitor cell function that could serve as a potential pre-treatment of cardiac progenitors prior to transplantation into the heart after injury. To this end, we assessed the effect of a combination of hypoxia and prostaglandin E_2 on CPC function. The treatment of CPC with hypoxia for 48 hours followed by an acclimatization period of normal culture conditions for 6 hours and then 6 hours of 10μM prostaglandin E_2 did

increase ERK phosphorylation and migration when compared with untreated CPCs and CPCs treated with hypoxia alone. However, treatment of CPCs with hypoxia (when administered alone) and the combination of hypoxia with prostaglandin E_2 did not significantly alter cell cycle progression.

In this chapter we were also able to elucidate novel changes in CPC function in response to prostaglandin E_2. Although PGE_2 treatment can benefit the heart *in vivo*, it may not uniformly improve cardiac stem cell function *in vitro*. We identified that CPCs expressed three of the four prostaglandin E_2 receptors including EP1, EP2, and EP4 by PCR. Twenty-four hours of 10μM PGE_2 treatment significantly increased cell migration, but decreased the expression of paracrine factors that play important roles in myocardial regeneration. Furthermore, short-term treatment with prostaglandin E_2 decreased the phosphorylation of AKT and ERK, key signaling molecules involved in stem cell regenerative ability.

Future Directions

At the end of this work, we are left with several important questions that may serve as building blocks for future studies. Our long-term goals are to define the mechanisms that contribute to cardiac stem cell-mediated myocardial regeneration and to optimize cardiac stem cell functional ability.

The significant change in microRNA expression profile with age lends credence to the idea that the packaging and secretion of expressed microRNAs is also affected in aged CPCs. MicroRNAs can act as paracrine factors by being packaged and secreted within small membrane vesicles of endocytic origin known as exosomes (Théry,

Zitvogel, & Amigorena, 2002). However, it is unknown whether age influences the level of exosomes secreted by cardiac stem cells, and secondly whether age can alter the type and levels microRNAs packaged within exosomes. To test this, we will first isolate exosomes and validate isolation by western blot marker CD63 as well as confirmation of relative size of isolated particles. We will then be able to compare relative secretion levels of neonatal and adult cardiac progenitors, isolate microRNAs from exosomes (Moldovan, Batte, Wang, Wisler, & Piper, 2013), and determine the relative profile of microRNAs within exosomes derived from the neonatal and adult CPC conditioned medium. Lastly, the relative ability of exosomes derived from neonatal and adult CPC to regulate gene expression and resistance to cell death in cardiomyocytes will be measured *in vitro*. This will provide novel information regarding the shift in microRNA expression within exosomes with age in CPCs, and will allow us to correlate this shift in microRNA expression with functional differences in cardiomyocytes after exosome treatment *in vitro*.

Exosomes can act as critical agents in cardiac regeneration (Chen et al., 2013); transplantation of CDC-derived exosomes into injured mouse hearts recapitulates the regenerative and functional effects produced by CDC transplantation, and this effect is partially mediated by the microRNAs packaged within them (Ibrahim, Cheng, & Marban, 2014). In our work, and work from other laboratories, we have demonstrated the superior functional capacity of cardiac stem cells derived from the neonatal heart when compared with cardiac stem cells from the adult heart (Fuentes et al., 2013; Simpson et al., 2012). It is unknown, however, whether exosomes secreted from neonatal CPCs have a superior ability to ameliorate cardiac injury when compared with exosomes from adult CPCs. To

evaluate this we could isolate exosomes from human neonatal and adult CPC conditioned medium and compare their therapeutic efficacy in a mouse model of myocardial infarction by intramyocardial delivery of conditioned medium-derived exosomes. Thirty days after exosome administration, relative infarct size and viable tissue as well as the level of apoptosis within the heart could be used to determine relative regenerative efficacy.

The response of cardiovascular progenitors to prostaglandin E_2 treatment outlined in chapter 3 in light of the overwhelming evidence of benefits of prostaglandin E_2 in cardiac injury (Hsueh, Wu, Yu, Wu, & Hsieh, 2014) leaves the question as to the mechanisms responsible for this disparity in effect. It is known, in other types of stem cells, that PGE_2 can have a biphasic effect with opposing responses at low and high doses (Kleiveland, Kassem, & Lea, 2008; Zhang & Wang, 2014). Although, in our study, we did not see uniformly beneficial effects of prostaglandin E_2 treatment, this could be due to dose, duration of treatment, and receptor levels. There are four known receptors for prostaglandin E_2 including EP1, EP2, EP3, and EP4. Interestingly, PGE_2 treatment can regulate the abundance of its own receptors (Jang et al., 2012). Therefore future work could contrast the effect of low and high doses of prostaglandin E_2 on cardiac progenitor cell function, and evaluate the receptors responsible for functional changes.

After low and high doses of PGE_2 we would assess the relative level of EP1-4 receptors and correlate receptor levels to changes in functional parameters such as cell stemness, cell differentiation, as well as cell cycle progression, migration, and growth factor expression. To further define the mechanism by which differing doses of PGE_2 mediates its effect on CPCs, we would use specific receptor blockers (Sugimoto &

Narumiya, 2007) for the EP receptors to assess which receptors are responsible for select cardiac progenitor cell responses to PGE_2. We would also assess downstream signaling transcripts associated with a given receptor to pinpoint the signaling responsible for the differing effects. This approach would further define the mechanism by which dose and PGE_2 receptor levels govern CPC response to PGE_2 treatment.

Conclusions

At the conclusion of this work, we have a better understanding of some of the various mechanisms underlying superior neonatal regenerative ability and have identified novel targets for improving adult CPC function. We have established that, with age, there is a shift in human CPC microRNA expression profile which correlates with functional differences between neonatal and adult CPCs *in vitro*. We have also evaluated a novel combination of prostaglandin E_2 and hypoxia and identified changes in CPC function in response to PGE_2 treatment including significantly reduced growth factor expression, and reduced signaling that may hinder CPC therapeutic efficacy.

The work outlined in this dissertation provides important advancements to the field of cardiac stem cell mediated regeneration, and provides insight into novel therapeutic targets and strategies to help repair the damaged heart.

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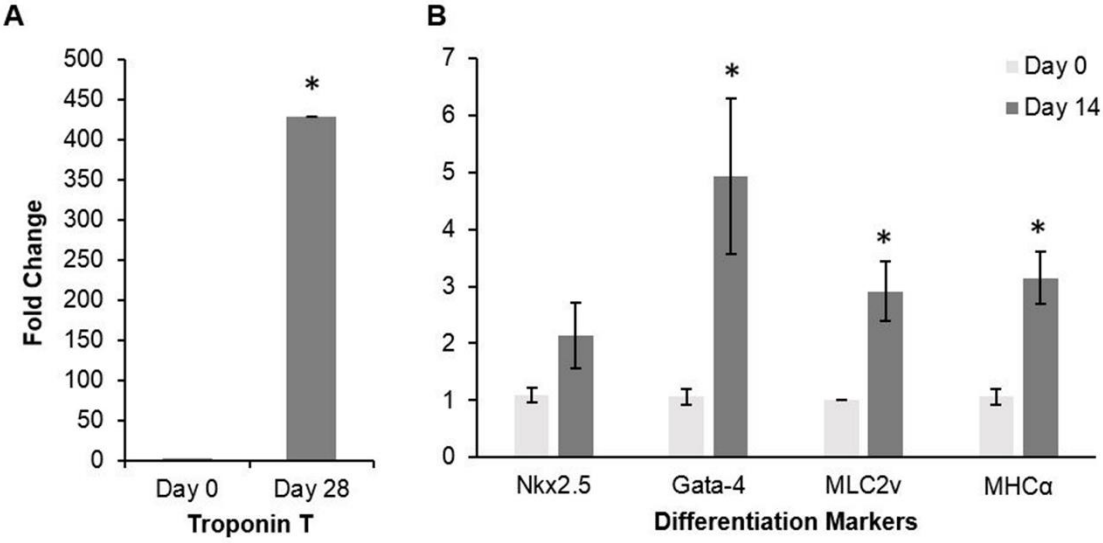
APPENDIX

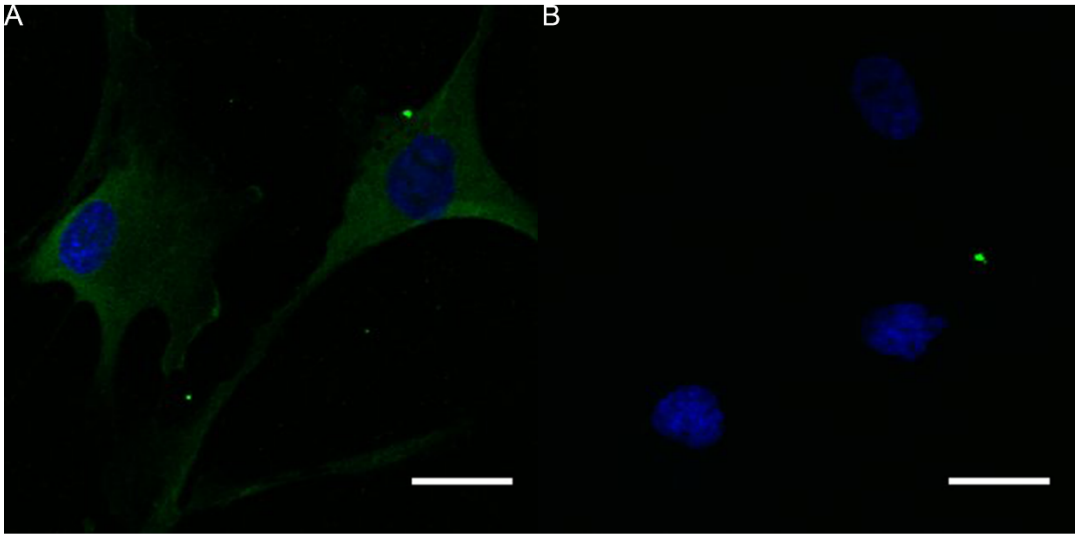
SUPPLEMENTARY FIGURES AND TABLES

Supplementary Figure 1 Expression of cardiac Troponin T, Nkx2.5, Gata-4, MLC2v and MHCα is induced after differentiation of Isl1+ c-kit+ cardiac progenitor clones.



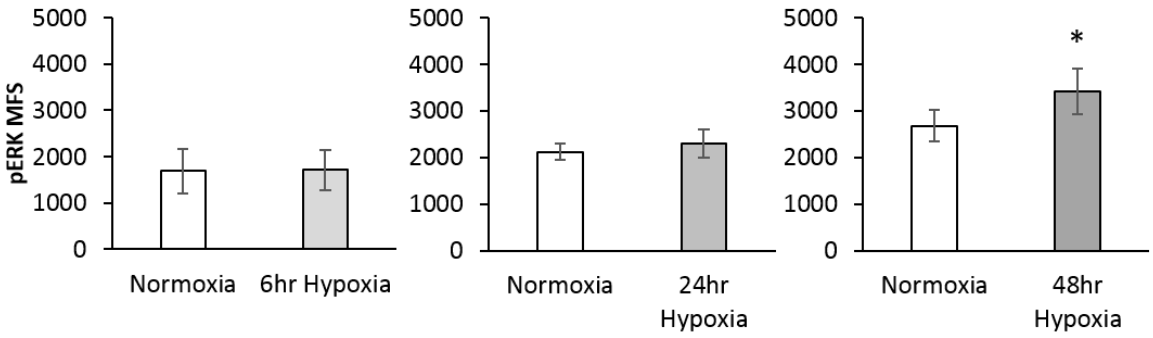
Differentiation of the cardiovascular progenitor cell clones is associated with elevated transcripts for Troponin T (A), Nkx2.5, Gata-4, MLC2v, and MHCα (B) as identified by real time PCR.

Supplementary Figure 2 Expression of cardiac Troponin I in Isl1+ c-kit+ cardiac progenitor clones.



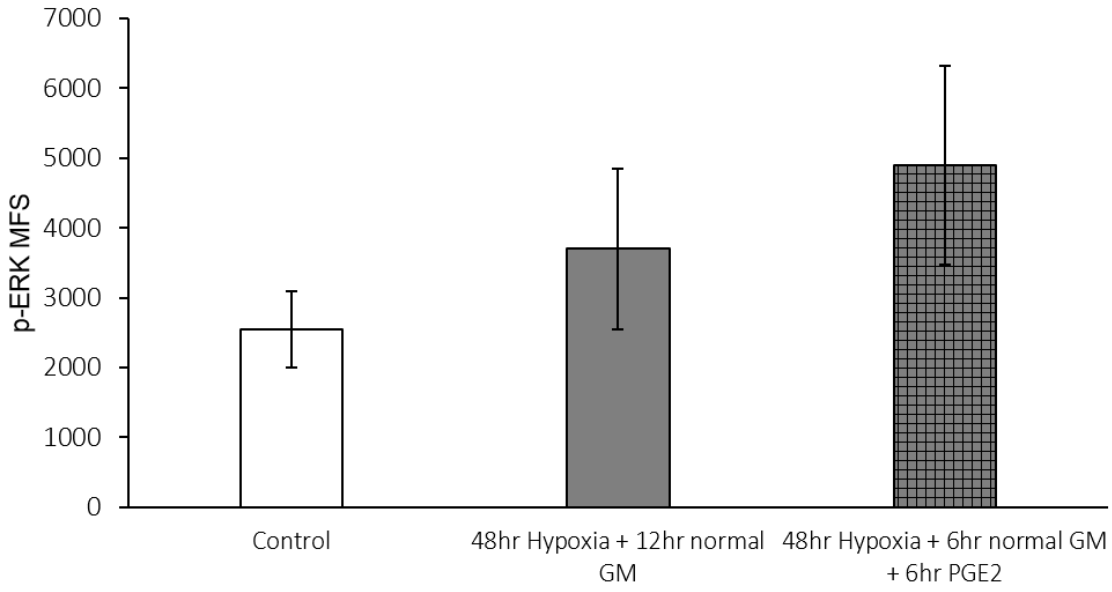
Troponin I is expressed in cardiovascular progenitor clones. Immunocytochemistry with FITC-conjugated anti-cardiac Troponin I -specific antibody (green) and DAPI (blue). Positive staining (A) and control staining using secondary antibody only (B) are shown above. Scale bar is 25μm.

Supplementary Figure 3 Changes in ERK phosphorylation after time course of cardiac progenitor cell exposure to 1% O_2.



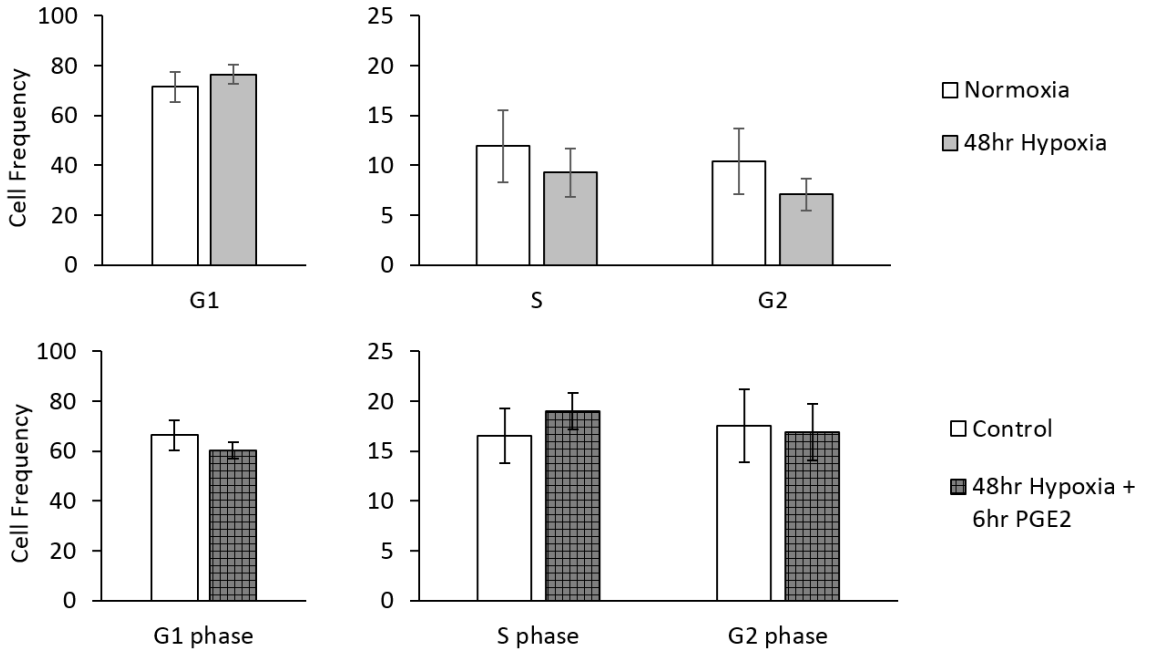
Cardiac progenitors were exposed to 6 hours (n=7), 24 hours (n=3), and 48 hours (n=8) of hypoxia (1% O_2) and ERK phosphorylation was measured by flow cytometry. The mean fluorescence shift above secondary antibody alone is shown above. Levels of ERK phosphorylation in response to hypoxia were only significantly elevated in cardiac progenitors after 48 hours of hypoxia (3423 vs 2683 MFU, p=0.03).

Supplementary Figure 4 ERK phosphorylation after combination of Prostaglandin E_2 and hypoxia treatment.



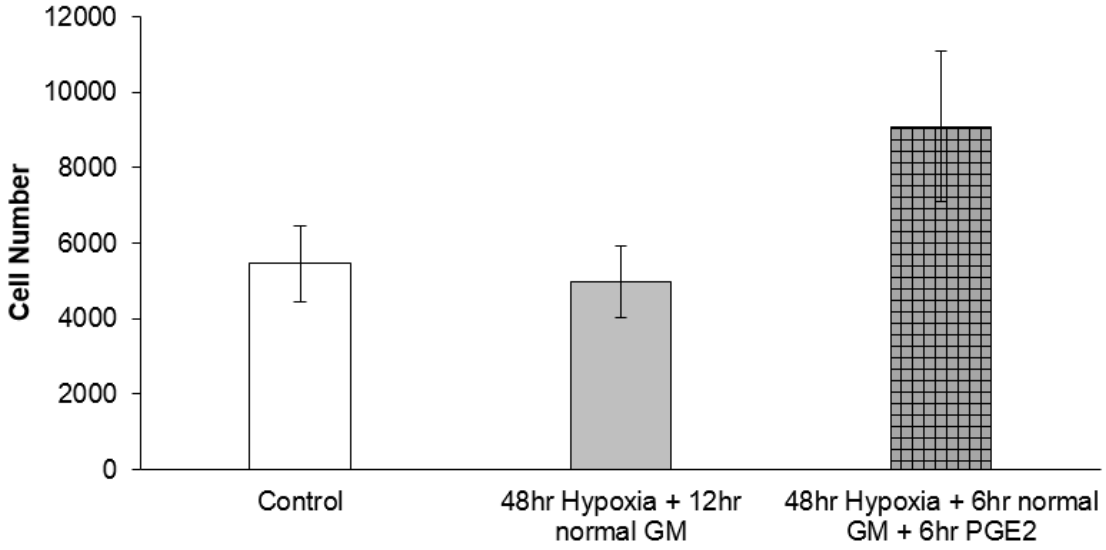
Cardiac progenitors were treated with 48 hours of 1% oxygen then 6 hours of normal growth medium (GM) followed by 6 hours of 10μM PGE_2. Flow cytometry was used to measure ERK phosphorylation, mean fluorescence shift above secondary alone is shown above. ERK phosphorylation was increased with a combination of hypoxia and prostaglandin E_2 treatment (n=4, p=0.15) when compared with untreated cells.

Supplementary Figure 5 Cell cycle progression after hypoxia or combination hypoxia and prostaglandin E_2 treatment.



Cell cycle progression after hypoxia or combination hypoxia and prostaglandin E_2 treatment. Cardiac progenitors were incubated for 48 hours with hypoxia (1% Oxygen) or a combination of hypoxia and prostaglandin E_2. For the combination treatment, cardiac progenitors were treated with 48 hours of hypoxia (followed by 6 hours of normal culture conditions for acclimatization) then 6 hours 10μM prostaglandin E_2 in normal growth medium. Cell cycle progression was not significantly altered by treatment with hypoxia alone (n=4), or a combination of hypoxia and PGE_2 (n=4) when compared with untreated cells.

Supplementary Figure 6 Migration in cardiovascular progenitors after consecutive treatment with hypoxia and PGE_2.



Cardiac progenitor cell clones were cultured in a hypoxia chamber for 48 hours, then 6 hours of normal culture conditions, followed by 6 hours of 10µM PGE_2. Migration was assessed using a transwell migration assay. Combination treatment increased migration but not significantly (9083 vs 5455 cells, p=0.07).

Supplementary Table 1 Cell surface phenotype of neonatal and adult CPCs as identified by flow cytometry.

Surface Antigen	**Neonatal CPC** Percent labeled	**Adult CPC** Percent labeled	**Range**
CD13	89 ± 2%	98 ± 1%	74 – 100%
CD31	81 ± 8%	43 ± 15%	21 – 95%
CD34	42 ± 5%	25 ± 7%	5 – 79%
CD44	79 ± 6%	89 ± 2%	61 – 100%
CD73	91 ± 3%	88 ± 3%	41 – 98%
CD90	19 ± 6%	32 ± 5%	0 – 95%
CD105	94 ± 2%	92 ± 2%	61 – 99%
CD146	85 ± 4%	96 ± 2%	36 – 100%
C-KIT	23 ± 3%	27 ± 3%	3 – 52.4%
CXCR4	37 ± 6%	50 ± 5%	13 –72%
CXCR7	29 ± 6%	44 ± 7%	3 – 69%
HLA I	87 ± 4%	92 ± 3%	83 – 100 %
HLA II	4 ± 2%	6 ± 4%	0 – 34%
IGF1R	87 ± 3%	89 ± 3%	58 – 99%
KDR	13 ± 7%	35 ± 14%	0 – 75%
PDGFR	20 ± 5%	35 ± 6%	2 – 58%
SSEA-4	12 ± 6%	27 ± 8%	0 – 96%

Supplementary Table 2 Antibodies used to characterize cell surface markers expressed on neonatal and adult CPC clones.

Antibody Against	Fluorophore	Company	Clone	Isotype	Cat #	Lot#
CD44	FITC	BD Biosciences	L178	MS IgC1	347943	502666
CD90	PE	Immunotech	F15-42-1-5	MS IgG1	1840	3
CD13	PE	BD Biosciences	L138	MS IgG1	347837	12616
CD31	PE	BD Biosciences	WM59	Ms IgG1	555446	42184
HLA-Dr	PE	BD Biosciences	G46-6	MS IgG2a	347367	60070
CD73	PE	BD Biosciences	AD2	MS IgG1	550257	77302
CD34	PE	BD Biosciences	8G12	MS IgG1	348057	60098
IGF1R	PE	Biolegend	1H7/CD221	MS IgG1	351805	8152252
CD105	PE	Biolegend	43A3	MS IgG1	323205	B152252
CXCR7	PE	Biolegend	8F11-M16	MS IgG2b	331103	B146742
CD140a	PE	Biolegend	16A1	MS IgG1	323505	B154801
CD146	PE	Biolegend	SHM-57	MS IgG2a	342003	B140547
CXCR4	PE	Biolegend	12G5	MS IgG2a	306505	B139046
SSEA4	FITC	Biolegend	MC-813-70	MS IgG3	330409	B153640
Pan HLA	FITC	Biolegend	W6/32	MS IgG2a	311403	B150905
CD117	PE	Millipore	104D2	IgG1	FCKMAB214P	1995601
CD309	PerCP/Cy5.5	Biolegend	HKDR-1	MS IgG1	338915	B155679
IgG1	PE	BD Biosciences	G18-145	MS IgG1	555787	0000028013
IgG1	FITC	Sigma-Aldrich	MOPC 21	MS IgG1	F6397	015K4830
IgG1	PerCP	Biolegend	MOPC 21	MS IgG1	40018	B137528
ISL1	Mouse Monoclonal	Abcam	1H9	MS IgG1	Ab86472	GR120150-1
vWF	Rabbit Polyclonal	Dako	Polyclonal	n/a	A0082	00088175
SMA	Mouse Monoclonal	Dako	1A4	MS IgG2a kappa	M0851	00087780
Goat anti mouse IgG	FITC	Southern Biotech	Polyclonal	IgG	1030-02	D240-U141C
Goat anti Rabbit Ig	FITC	BD Biosciences	Polyclonal	Ig	554020	2200705

Supplementary Table 3 Relative expression of significantly altered microRNAs in neonatal and adult cardiovascular progenitors.

	Fold Change		St Error		
MicroRNA	Adult CPCs	Neonatal CPCs	Adult CPCs	Neonatal CPCs	P value
miR-22	1	26.87	0.01	0.16	0.0052
miR-424	1	23.92	0.02	0.25	0.0357
miR-20a	1	20.03	0.02	0.18	0.0084
miR-18a	1	14.74	0.03	0.44	0.0238
miR-20b	1	13.01	0.02	0.24	0.0351
miR-17	1	12.45	0.03	0.18	0.0094
miR-15a	1	9.48	0.08	0.23	0.0368
miR-103	1	8.70	0.05	0.13	0.0027
miR-24	1	8.41	0.05	0.10	0.0005
let-7i	1	6.53	0.03	0.37	0.0238
miR-106b	1	4.98	0.09	0.20	0.0295
miR-185	1	3.52	0.06	0.10	0.0022
miR-130a	1	2.41	0.28	0.08	0.0288
let-7e	1	2.23	0.17	0.14	0.0456
miR-192	2.16	1	0.11	0.28	0.0273
miR-134	3.13	1	0.44	0.16	0.0007
miR-23b	3.33	1	1.29	0.25	0.0395
miR-503	4.97	1	0.89	0.45	0.0238
miR-122	4.98	1	0.85	0.24	0.0005
miR-10b	5.48	1	2.34	0.36	0.0279
miR-219-5p	5.98	1	1.75	0.27	0.0045
miR-132	6.03	1	1.10	0.29	0.0005
miR-215	6.80	1	2.70	0.22	0.0145
miR-1	6.85	1	2.22	0.50	0.0238
miR-205	7.65	1	2.30	0.29	0.0038
miR-183	8.11	1	2.16	0.31	0.0020
miR-498	8.21	1	1.40	0.62	0.0238
miR-206	9.59	1	4.10	0.38	0.0166
miR-141	9.70	1	4.02	0.27	0.0137
miR-302a	9.98	1	4.20	0.30	0.0145
miR-520g	10.30	1	1.76	0.33	0.0001
miR-96	12.30	1	0.48	0.27	<0.0001
miR-208a	12.43	1	2.60	0.37	0.0004
miR-223	15.90	1	3.75	0.22	0.0005
miR-124	16.49	1	6.32	0.26	0.0074
miR-150	16.54	1	1.97	0.25	<0.0001
miR-488	17.74	1	7.93	0.50	0.0238
miR-371-3p	21.76	1	2.38	0.21	0.0238
miR-129-5p	23.28	1	10.81	0.18	0.0163
miR-196a	24.02	1	8.76	0.39	0.0052
miR-518b	45.95	1	21.44	0.19	0.0150

Supplementary Table 4 Pathway analysis associated with microRNAs that were differentially regulated when comparing neonatal and adult CPCs

Pathway	-ln(pvalue)*
MAPK signaling pathway	26.14
Ribosome	25.29
Regulation of actin cytoskeleton	23.61
Wnt signaling pathway	21.31
Adherens junction	21.23
Focal adhesion	20.5
Oxidative phosphorylation	17.34
TGF-beta signaling pathway	16.81
ErbB signaling pathway	12.21
Tight junction	10.14
Long-term potentiation	10.13
Ubiquitin mediated proteolysis	10.04
Arachidonic acid metabolism	9.77
Insulin signaling pathway	9.47
mTOR signaling pathway	8.59
p53 signaling pathway	8.52
Glycolysis / Gluconeogenesis	7.46
Androgen and estrogen metabolism	7.36
Pyruvate metabolism	7.31
GnRH signaling pathway	7.23
SNARE interactions in vesicular transport	6.99
Circadian rhythm	6.37
Complement and coagulation cascades	6.04

Tryptophan metabolism	5.73
Polyunsaturated fatty acid biosynthesis	5.53
Gap junction	5.38
Neuroactive ligand-receptor interaction	5.35
ECM-receptor interaction	5.33
Glycine, serine and threonine metabolism	5.3
Linoleic acid metabolism	5.14
Proteasome	4.74
Calcium signaling pathway	4.5
VEGF signaling pathway	4.32
Glycan structures – degradation	4.24
Methionine metabolism	4.24
Starch and sucrose metabolism	4.2
Glutathione metabolism	4.08
Phosphatidylinositol signaling system	4.01
Notch signaling pathway	3.88
Base excision repair	3.65
Adipocytokine signaling pathway	3.65
Valine, leucine and isoleucine degradation	3.64
Histidine metabolism	3.39
D-Glutamine and D-glutamate metabolism	3.12
Butanoate metabolism	3.1
Propanoate metabolism	3.04

*Only pathways found to be significantly different ($p<0.05$) are displayed and are listed from most significant to least significant.

Supplementary Table 5 Relative expression of significantly altered microRNAs in SSEA-4+ neonatal and adult cardiovascular progenitors

	Fold Change		St Error		
MicroRNA	SSEA-4+ Adult CPCs	SSEA-4+ Neonatal CPCs	SSEA-4+ Adult CPCs	SSEA-4+ Neonatal CPCs	P value
miR-142-5p	18.71	1	4.12	0.18	0.0127
miR-371-3p	13.36	1	1.46	0.20	0.0011
miR-498	8.81	1	1.50	0.21	0.0067
miR-223	7.59	1	1.79	0.27	0.0219
miR-96	7.23	1	0.28	0.03	<0.0001
miR-183	6.81	1	1.81	0.06	0.0327
miR-375	6.21	1	1.04	0.20	0.0079
miR-520g	6.03	1	1.03	0.28	0.0091
miR-150	5.77	1	0.69	0.46	0.0045
miR-208a	5.51	1	1.15	0.28	0.0189
miR-503	3.12	1	0.56	0.42	0.0383
let-7b	1	2.81	0.15	0.01	0.0117
miR-185	1	3.80	0.06	0.19	0.0217
miR-93	1	4.02	0.10	0.20	0.0268
miR-214	1	4.37	0.09	0.14	0.0101
miR-378	1	6.00	0.12	0.21	0.0259
miR-106b	1	7.91	0.06	0.07	0.0006
miR-24	1	10.63	0.04	0.13	0.0027
miR-7	1	12.59	0.04	0.32	0.0476
miR-103	1	14.18	0.03	0.17	0.0062
miR-17	1	16.46	0.02	0.12	0.0013
miR-20b	1	17.50	0.01	0.28	0.0268
miR-22	1	27.81	0.01	0.32	0.0412
miR-20a	1	31.21	0.01	0.04	<0.0001
miR-18a	1	31.89	0.02	0.25	0.0174
miR-424	1	57.54	0.01	0.18	0.0058

Supplementary Table 6 Primers used to detect gene expression by PCR

Gene	Forward Primer	Reverse Primer
ATM	GGGCGAGCCGCAAACGCTAA	TTCGGCCCGTCGGAGCAAAC
C-KIT	ATTCCCAAGCCCATGAGTCCTTGA	ACACGTGGAACACCATCCT
E2F1	GACCATCAGTACCTGGCCGAGAG	GACGACACCGTCAGCCGAGTG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
GATA 4	GATCTTCGCGACAGTTCCTC	GTCCCCGGGAAGGAGAAG
ISL1	CACAAGCGTCTCGGGATTGTGTTT	AGTGGCAAGTCTTCCGACAA
MHCα	GTCATTGCTGAAACCGAGAATG	GCAAAGTACTGGATGACACGCT
MLC-2v	TATTGGAACATGGCCTCTGGAT	GGTGCTGAAGGCTGATTACGTT
MYC	AAGACAGCGGCAGCCCGAAC	TGGGCGAGCTGCTGTCGTTG
RAD50	CTACGGCTTTGCGTCCCCGG	ACACCAGCTGCTTTCCCCGC
TROP T	GTGGGAAGAGGCAGACTGAG	ATAGATGCTCTGCCACAGC

Supplementary Table 7 Primers used for Real-time PCR

Gene	Forward Primer	Reverse Primer
β-Actin	TTTGAATGATGAGCCTTCGTCCCC	GGTCTCAAGTCAGTGTACAGGTAAGC
EP1	CTTGTCGGTATCATGGTGGTGTC	GGTTGTGCTTAGAAGTGGCTGAGG
EP2	ATGGGCAATGCCTCCAATGAC	GCACGCGCGGCTCTCGGGCGCCAG
EP3	GCATAACTGGGGCAACCTTTTCTTCGCC	CTTAACAGCAGGTAAACCCAAGGATCC
EP4	CGCTGTCCTCCCGCAGACGA	CCACCCCGAAGATGAACATC
HGF	CACGAACACAGCTTTTTGCC	TGATCCCAGCGCTGACAAAT
IGF1	CAGAGCAGATAGAGCCTGCG	CAGGTAACTCGTGCAGAGCA
MLC2v	TATTGGAACATGGCCTCTGGAT	GGTGCTGAAGGCTGATTACGTT
SDF-1α	CTACAGATGCCCATGCCGAT	GTGGGTCTAGCGGAAAGTCC
Trop T	GTGGGAAGAGGCAGACTGAG	ATAGATGCTCTGCCACAGC
VEGF	CAGCGAAAGCGACAGGGGCA	GCTGGAGCACTGTCTGCGCA