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A Novel Population of Cardiovascular Progenitors Persist in Neonates as Mesendodermal Cells

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A Novel Population of Cardiovascular Progenitors Persist in Neonates as Mesendodermal Cells

by

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

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ABBREVIATIONS

ALPL	Alkaline phosphatase
c-KIT	KIT proto-oncogene receptor tyrosine kinase
CM	Cardiomyocyte
COL1A1	Collagen type 1
CPC	Cardiovascular progenitor cell
EC	Endothelial cell
EGFP	Enhanced green fluorescent protein
EOMES	Eomesdermin
FHF	First heart field
Flk-1	Fetal liver kinase-1
FS	Fractional shortening
GATA4	GATA binding protein 4
GSC	Goosecoid
HCN4	Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4
hCO	Human cardiac organoid
hESC	Human embryonic stem cell
ISL1	Islet-1
KDR	Kinase insert domain receptor
LVED	Left ventricular end-diastole
LVEF	Left ventricular ejection fraction
MESP1	Mesoderm posterior homolog 1
MIXL1	Mix paired-like homeobox

MLC2V	Myosin light chain 2
MPC	Multipotential progenitor cell
NANOG	Nanog homeobox
NKX2-5	NK2 homeobox 5
OCN	Osteocalcin
OCT-4	Octamer-binding protein 4
OPN	Osteopontin
PDGFR	Platelet derived growth factor receptor
RUNX2	Runt related transcription factor 2
SHF	Second heart field
SM	Smooth muscle actin
SMA	Smooth muscle cell
SOX2	SRY-box 2
T	Brachyury
TBX5	T-box 5
TGF-β	Transforming growth factor, beta
TNNT2	Troponin T2
vWF	Von Willebrand factor

ABSTRACT OF THE THESIS

The Mesendodermal Stage of Development is Sustained within a Novel Population of Human Neonatal Cardiovascular Progenitor Cells

by

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Master of Science, Graduate Program in Biochemistry
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Dr. Mary Kearns-Jonker, Chairperson

The rise in mortality due to cardiovascular disease has increased the need to develop an efficient regenerative therapeutic for heart failure. Numerous cell-based therapies have been investigated for myocardial regeneration; however, an optimal progenitor has yet to be discovered. Identifying a resident cell population with enhanced ability to differentiate into multiple lineages would greatly contribute to the field of stem cell-based regenerative therapy. Evidence suggests that endogenous cardiovascular progenitor cells (CPCs) that have been isolated from the heart itself express *ISL1, KDR,* and *MESP1,* and are capable of differentiating into all major cardiac lineages. The earlier developmental stage at which endogenous CPCs reside may be associated with enhanced multipotency and differentiation capacity. A clonal population of human neonatal CPCs expressing markers of early cardiovascular development was therefore selected for analysis. Based upon recent reports of differentiation into osteoblasts by cells expressing *ISL1*, we performed differentiation of the selected neonatal CPC into cardiovascular lineages as well as the mesodermal derivative, osteoblasts. Gene expression analysis and flow cytometry demonstrated differentiation into both cardiovascular and osteoblast lineages. To further address the relationship between an earlier developmental stage and

differentiation capability, we measured the expression of mesendodermal regulatory markers, and discovered that this population of progenitor cells may reside earlier in development than previously recognized. These results suggest that neonatal cardiovascular progenitors expressing transcripts of the mesendoderm persist within the newborn human heart, exhibit enhanced differentiation capacity into various mesodermal lineages, and can be expanded for further assessment of functional and regenerative ability

CHAPTER ONE

INTRODUCTION

For the last fifteen years, cardiovascular disease has been the leading cause of death worldwide[1]. Heart failure is predominantly associated with the loss of cardiomyocyte number and function following myocardial infarction, leading to cardiomyocyte hypertrophy, pathological remodeling of the heart, and ultimately to cardiac dysfunction (Lam et al., 2009). The heart's limited regenerative capacity has prompted the investigation of stem cell-based therapeutics for cardiac repair (Cyganek et al., 2013; Garbern et al., 2013; Karra et al., 2008). Among the myriad of stem cell populations that have been investigated, endogenous cardiac stem cells show great promise for the treatment of heart failure (Fuentes et al., 2013; Mayfield et al., 2014; Nadal-Ginard et al., 2014). However, the origins of these progenitor cells and the mechanisms governing lineage specification and differentiation have yet to be fully elucidated (Chong et al., 2014). A comprehensive understanding of the identity and differentiation capacity of endogenous CPCs would greatly advance the field of stem cell-based regenerative therapy.

The heart has long been thought to originate from two bilateral heart fields during early gastrulation (Brand, 2003); whereby the first heart field (FHF) contributes to the formation of the left ventricle and atria, and the second heart field (SHF) contributes predominantly to the right ventricle, outflow tract, and parts of the atria (Bruneau, 2013). Recently, however, an alternative school of thought regarding the origin of the heart has

[1] Available at: http://www.who.int/mediacentre/factsheets/fs310/en/ Accessed March 4, 2017.

developed. Several studies have provided key insight into population(s) of multipotent cardiovascular progenitors that exist during the early stages of embryonic development, suggesting that the heart may actually be derived from a single primordial precursor with the ability to differentiate into all cardiac lineages rather than two separate progenitor populations (Abu-Issa et al., 2004; Meilhac et al., 2004; Moorman et al., 2007). Emerging evidence of an inverse relationship between the developmental stage and regenerative capacity of cardiac progenitors supports the need to investigate endogenous cardiovascular cell populations that reside at early stages of development (Porrello et al., 2011; Fuentes et al., 2013; Jaszczak et al., 2016).

Although little is known regarding the molecular identity of a single primordial precursor, advances in cell fate-mapping techniques revealed that CPC populations may be found prior to formation of the primitive streak in the developing mouse embryo (Audo-Boucher et al., 2000). Various signaling molecules and transcription factors play critical roles in promoting differentiation of CPCs that are present during this "pre-streak" stage. Expression of the molecular markers *Mesp1, Brachyury (T),* and *Flk-1* (also known as *KDR* in humans) have been suggested to mark a population of cardiogenic primordial cells during murine embryonic development that are capable of differentiating into functional cardiomyocytes, endothelial, and smooth muscle cells (Kattman et al., 2006; Meilhac et al., 2014; Chiapparo et al., 2016; Liu et al., 2016). The temporal appearance of *Brachyury* and *Mesp1* have been shown to regulate the specification of the primitive streak (Bondue et al., 2008). Furthermore, knock out studies in mice support evidence suggesting that *Mesp1* and *Flk-1* are required for proper differentiation into the cardiovascular lineage (Saga et al., 1999; Kitajima et al., 2000; Abunnaja et al., 2014).

Analogous studies on human embryonic stem cells (hESCs) have revealed that *MESP1* and *KDR* are expressed in human CPCs and are essential for heart formation (Yang et al., 2008; Ardehali et al., 2013). Cardiovascular progenitors expressing *MESP1* contribute to both first and second heart field derivatives (Bondue et al., 2010; Lescroart et al., 2014), and *MESP1*-null hearts lead to abnormalities during heart morphogenesis, resulting in cardia bifida. (Saga et al., 1996; 1999). Piccini and colleagues published data demonstrating that *MESP1* expression peaks at day 2 of hESC differentiation, thereby indicating early and temporal expression of *MESP1* in cardiac development (Piccini et al., 2016). Den Hartogh and Passier investigated the differentiation events of cardiovascular development in a dual MESP1$^{mCherry/w}$-NKX2-5$^{eGFP/w}$ reporter line generated from hESCs and reported the gene expression profile of *MESP1*-positive cells compared to that of *MESP1*-negative cells. Results from this study revealed that early cardiac genes were enriched during peak expression of *MESP1* (Den Hartogh et al., 2014). These results are consistent with earlier findings that *MESP1* expression occurs at early stages of development and serves as a key regulator of cardiovascular differentiation. Identifying a population of CPCs with multipotential capability would provide access to an optimal cell source for regenerating vital organs such as the heart.

There has been an increasing emphasis on understanding the molecular role of *MESP1* and *KDR* in the context of cardiovascular development; and more specifically, their interactions with cardiac transcription factors, such as *ISL1* (Solloway et al., 2003; Kattman et al., 2007). Interestingly, *ISL1* has been reported to be co-expressed with *MESP1* in a subset of early *MESP1*-expressing cells (Bondue et al., 2008 & 2011). The LIM-homeodomain transcription factor, *ISLET1*, plays a critical role in cardiovascular

development (Laugwitz et al., 2005; Moretti et al., 2006, Wu et al., 2006). *ISL1* marks a population of CPCs capable of giving rise to all cardiac lineages (Moretti et al., 2006; Ma et al., 2008; Fonoudi et al., 2013), and *Isl1*-null hearts in mice fail to undergo looping morphogenesis and are completely devoid of major components of the heart, including the outflow tract, right ventricle, and parts of the atria (Cai et al., 2003). *ISL1* expression is required for the survival, proliferation and migration of progenitor cells into the cardiac tube during heart development (Laugwitz et al., 2008). A recent report on the engraftment of human *ISL1*-CPC spheroids after myocardial infarction in murine hearts showed that mice treated with *ISL1*-CPC spheroids exhibited reduced infarct area, increased blood vessel formation, and increased left ventricular contractile function compared to control animals (Bartulos et al., 2016). These results indicate that an $MESP1^{+}/ISL1^{+}$ CPC population is capable of demonstrating multipotential capability and could serve as a promising candidate for stem cell-based regenerative therapeutics.

The efficacy of regeneration by CPCs expressing markers that are prevalent at varying stages of development have been investigated in both murine and human hearts. Studies in rodent hearts revealed a developmental stage-dependent discrepancy, whereby cell sources expressing early developmental markers demonstrated greater increases in regenerative capability than cells expressing late-stage markers (Table 1). Furthermore, cardiovascular progenitors expressing early developmental markers such as *KDR*, *PDGFR*α, and *ISL1* demonstrated better functional outcomes as measured by fractional shortening and/or ejection fraction (Table 1). Comparable results were shown in both mouse and human hearts following myocardial infarction. Such findings support the concept that early cardiovascular stem cells are better candidates for stem cell-based

therapies than late-stage cardiovascular stem cells. Moreover, CPCs provide a promising cell source for regenerative therapies due to minimal risk for teratoma formation following engraftment of cardiomyocytes relative to hESCs (Ardehali et al., 2013; Shiba et al., 2012; Chong et al., 2014; Harman et al., 2016).

Table 1. Animal and human studies of next-generation cell-based regenerative therapies.

Study	Dev Stage	Cell source	Clinical effects
Rodent (Fernandes et al., 2015)	Early	*KDR*$^+$/*PDGFR*α^+ hESC-derived CPCs	+28% FS (at 28 days)
Rodent (Li et al., 2017)	Early	*Isl1*$^+$ CPCs	+20% LVEF (at 28 days)
Rodent (Tang et al.., 2010)	Late	*c-Kit*$^+$/*EGFP*$^+$ CPCs	+4% FS +8% LVEF (at 35 days)
Human (Menasché et al., 2015)	Early	*Isl1*$^+$/SSEA-1$^+$ hESC-derived CPCs	+10% LVEF (at 3 months)
Human: CADUCEUS (Makkar et al., 2012)	Late	*c-Kit*$^+$ cardiospheres	+2% LVEF (at 6 months)
Human: SCIPIO (Bolli et al., 2011)	Late	*c-Kit*$^+$ Adult CPCs	+12.3 ± 2.1% LVEF (at 4 months)

Abbreviations: FS, fractional shortening; LVEF, left ventriclular ejection fraction; LVED, left ventricle at end-diastole volume.

Among the wide array of cell populations studied within the field of stem cell-based therapy for heart repair, endogenous CPCs from neonatal sources are considered to be the most promising. Cardiomyocyte exchange has been shown to occur more often in early childhood with a gradual decline throughout life (Bergmann et al., 2015), and

compelling results on the functional recovery of the human neonatal heart have been reported by numerous case studies (Saker et al., 1997; Farooqi et al., 2012; Cesna et al., 2013; Haubner et al., 2016). Recent analysis of the injury response of human cardiac organoids (hCOs) revealed that, following cyroinjury *in vitro*, hCOs resembled neonatal-like human cardiac tissue and underwent functional recovery in the absence of fibrosis or hypertrophy (Voges et al., 2017). Furthermore, neonatal CPCs demonstrate enhanced capacity for regulatory processes critical for regeneration, including proliferation, migration, and invasion (Fuentes et al., 2013; Mollova et al., 2013). MicroRNA analysis of neonatal CPCs co-expressing *ISL1* and *c-KIT*, a population that is the focus of work done in our laboratory, revealed significant upregulation of transcripts that promote cell invasion compared to adult CPCs, and distinct clonal populations of neonatal CPCs expressed moderate to high levels of the pre-cardiac mesoderm markers *KDR* and *PDGFR* (Fuentes et al., 2013). Thus, this neonatal *ISL1*$^{+}$/*c-KIT*$^{+}$ population expressing markers of early cardiac development could serve as a cell source similar to that of a single primordial precursor with enhanced efficacy for heart repair.

Stem cells expressing *ISL1* have been shown to be capable of differentiation into an osteoblastic phenotype (Eberhardt et al., 2006; Itzhaki-Alfia et al., 2009; Akiyama et al., 2014). *MESP1* expressing cells can differentiate into mesodermal derivatives including skeletal muscle and bone (Chan et al., 2013; Bondue et al., 2011). Emerging evidence suggests that *MESP1* plays an important regulatory role in development (Liu, 2017). Moreover, studies show that cells at early stages of development adopt their fate by location (Meilhac et al., 2014). Understanding the molecular properties contributing to mutipotency, commitment, and differentiation in cells that express all of these early

transcription factors is therefore necessary to identify the specific population of neonatal CPCs that may serve as optimal candidates for cardiac repair.

The hunt for an ultimate progenitor has begun. With the rise in mortality due to heart disease, there is a growing need to develop an efficient regenerative therapeutic. The field of stem cell-based therapy has performed extensive analyses on numerous cell populations with limited efficacy. Studies reveal that endogenous human neonatal CPCs possess greater potential as a source of cells to regenerate the heart compared to adult CPCs (Simpson et al., 2012; Fuentes et al., 2013). *ISL1, MESP1,* and *KDR* are expressed on CPCs that can differentiate efficiently into all cardiovascular lineages (Moretti et al., 2006; Lescroart et al., 2014; Bulatovic et al., 2016). Furthermore, neonatal CPCs co-expressing *ISL1, MESP1, KDR,* as well as *c-KIT* have been isolated in our laboratory and may represent a candidate with multipotential differentiation capability as well as enhanced regenerative function. Thus, the work proposed in my specific aims has been directed towards identifying whether or not this *ISL1, MESP1, KDR,* and *c-KIT* expressing clonal population of human neonatal CPCs isolated by our laboratory has multipotential differentiation capability. Results from these studies will determine the capability of these stem cells to differentiate into cardiac as well as osteoblast lineages. Additionally, findings from this study will provide innovative and essential information that will contribute to better functional outcomes in future stem-cell based therapeutic studies for patients with heart disease.

CHAPTER TWO

MATERIALS AND METHODS

Ethics Statement/ Maintenance of CPCs

The Institutional Review Board of Loma Linda University approved the protocol for the use of discarded tissue during cardiovascular surgery, without identifiable information, for this study with a waiver of informed consent. Cell isolation and expansion protocols were performed in previous studies for further use in the laboratory (Fuentes et al., 2013). In brief, discarded atrial cardiac tissue obtained during cardiovascular surgery of human neonates was cut into ~1mm^3 segments and collagenase digested (Roche Applied Science, Indianapolis, IN) for 2 hours at 37°C at a proportion of 1:2.5 tissue volume vs. collagenase. This solution was passed through a 40μm cell strainer (Smits et al., 2009) and resulting cells were cloned by limiting dilution at a concentration of 0.8 cells per well to create clonal populations.

Neonatal CPC Culture and *in vitro* Differentiation

Neonatal CPCs were cultured in 6-well plates coated with 0.1% gelatin. Control cells were cultured in CPC growth medium and passaged using 0.25% trypsin solution upon reaching 80% confluency. Cardiomyocyte differentiation of cardiac progenitor cells was performed by treatment with 5-azacytidine, ascorbic acid, and TGF-β in differentiation media for 14 days (Smits et al., 2009). In brief, CPC growth medium was exchanged for differentiation medium once cells reached >80% confluency. After 6-8 hours of changing media, 5μM 5-azacytidine was added directly to the cells for three consecutive days with a media change on day 4. Six days after the start of the

cardiomyocyte differentiation assay, 1000x stock 10^{-4} M ascorbic acid and 1ng/mL TGF-β were added to the medium. Following the first treatment, ascorbic acid was added every 2 days, and TGF-β was added twice weekly either during media changes or directly to the cells. Cells were taken at day 14 for qRT-PCR analysis. Endothelial tube formation was performed by thawing concentrated Matrigel (Corning) overnight in a 4°C refrigerator on ice. The next day, pipette tips were cooled in -20°C for 30 minutes and used to plate 50µL of thawed matrigel in each well of a 96-well plate. Plated matrigel was allowed to harden in a CO_2 incubator for 30-60 minutes and 20,000 cells were plated per well in 150µL EGM-2 + 10% FBS and incubated for an additional 5 hours. Cells were washed twice with PBS and 3.75µL Calcein AM was added to 1mL of EGM-2. The staining solution was added to each well and incubated for a final 30 minutes. Staining medium was replaced with fresh EGM-2 +10% serum prior to imaging endothelial tubes at the 4x objective using an EVOS imaging system (Arnaoutova et al., 2009). To induce osteogenic differentiation, cells were cultured in DMEM +10% FBS supplemented with 50µM ascorbic acid, 10mM β-glycerophosphate and 100nM dexamethasone. Medium was changed twice weekly for 16 days, and cells were analyzed for qRT-PCR and flow cytometry analysis for osteoblast markers, RUNX2, OPN, COL1A1, ALPL, and OCN, at days 7 and 14-16 (Jaiswal et al., 1997, Sottile et al., 2003) (Figure 1).

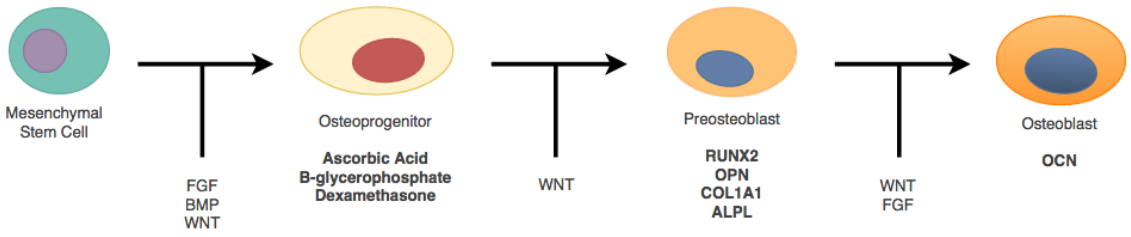

Figure 1. Osteogenic differentiation by ascorbic acid, β-glycerophosphate, and dexamethasone treatment.

RNA Extraction

RNAprotect (Qiagen, Valencia, CA) and TRIzol reagent (Life Technologies, Grand Island, NY) were used to store cells. RNA was extracted from cells in RNAprotect using the Qiagen RNeasy Kit. Cells were homogenized in TRIzol reagent and RNA was isolated as per the manufacturer's instructions. First strand cDNA synthesis was performed from 2μg total RNA using Superscript® III Reverse Transcriptase (Invitrogen, Grand Island, NY).

qRT-PCR Analysis

Quantitative RT-PCR was performed on a BioRad iQ™5 Cycler as previously described (Fuentes et al., 2013). PCR conditions were as follows: 94°C for 10 minutes, followed by cycles of 15 seconds at 94°C for denaturation, annealing for 60 seconds at 56°C, and extension for 30 seconds at 72°C for a total of 40 cycles for most primer pairs. Human primer pairs were designed using NCBI Primer Blast as well as taken from previously published literature (Sottile et al., 2003). Fold changes were calculated using the ΔΔCt method (Schmittgen et al., 2008). A list of primers that were used for qRT-PCR analysis may be found in supplemental table 1 (Table S1).

Flow Cytometry

Following induction, cells were trypsinized, fixed with 4% paraformaldehyde, and labeled with antibodies at concentrations recommended by the manufacturers. Cells for osteoblast detection were labeled with mouse anti-human Osteocalcin Alexa Fluor 488 conjugated antibody (R&D Systems, MN). FACS analysis was performed on a

MACSquant analyzer (Miltenyi Biotec, Auburn, CA). Forward- and side-scatter gating was used to gate out any small particles and/or dead cells. FlowJo software (Ashland, OR) was used for quantification.

Statistical Analysis

Data were processed using Microsoft Excel and Graphpad Prism 7.02 and expressed as the mean ± S.E.M. Data were tested for normality using the Sharpiro-Wilk normality test. Statistical significance was determined using a Student's *t* test for qRT-PCR analysis. *P*-values less than 0.05 were assumed to be significant.

CHAPTER THREE

RESULTS

Human Neonatal CPCs Express a Distinct Set of Early Developmental Markers

Clonal populations of human neonatal cardiovascular progenitor cells co-expressing *ISL1* and *c-Kit* were previously isolated and characterized for further use in the laboratory (Fuentes et al., 2013). Phenotypic profiling revealed distinct populations of neonatal CPCs expressing pre-cardiac mesoderm markers *ISL1, KDR,* and *PDGFRa* (Fuentes et al., 2013). In order to investigate the developmental stage of the neonatal CPC co-expressing *ISL1, MESP1, KDR,* and *c-KIT,* we performed gene expression analysis on neonatal CPCs for molecular markers associated with the mesoderm.

All neonatal CPC clones that were examined for this study expressed the pluripotency marker *OCT4*, as well as markers of the mesoderm and mesendoderm including brachyury (*T*) and goosecoid (*GSC*), respectively. One of the clonal populations of neonatal CPCs uniquely expressed demonstrable levels of *MESP1*, along with its mesendodermal precursor, *EOMES* (Figure 2). This clone presented a gene expression profile similar to that of a primitive cardiogenic cell population. Based upon expression of early molecular markers associated with the mesoderm and pre-cardiac mesoderm, we sought to assess the differentiation capacity of this *MESP1*$^+$ neonatal CPC (Figure 3). Previous studies have shown *ISL1*$^+$/*c-KIT*$^+$ co-expressing neonatal CPCs can differentiate into cardiomyocyte and endothelial cells. Differentiation into the cardiac and osteoblast lineage was performed on this *ISL1*$^+$, *c-KIT*$^+$, *KDR*$^+$, and *MESP1*$^+$ clone to investigate the differentiation and multipotential capacity.

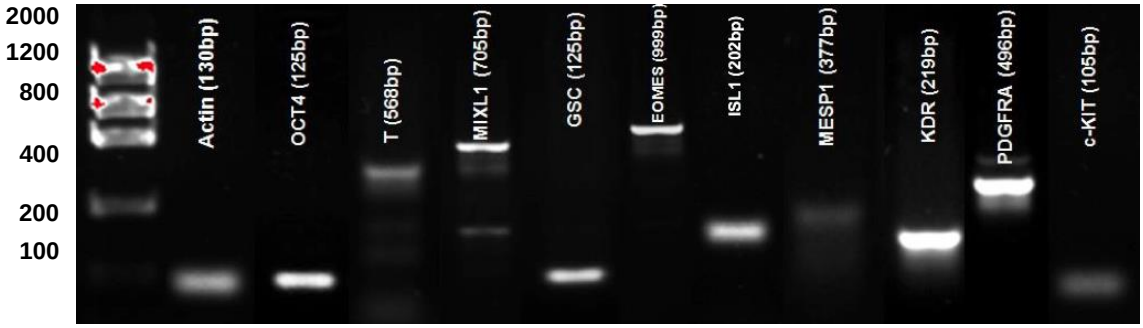

Figure 2. mRNA analysis of molecular markers associated with mesoderm, precardiac mesoderm, and cardiac progenitors. Relative mRNA expression of pluripotent marker, OCT4, mesoderm (T, MIXL1, GSC, EOMES) and pre-cardiac mesoderm (MESP1, KDR, PDGFRa) in the select $ISL1^{+}$/c-KIT^{+}/KDR^{+} and $MESP1^{+}$ clone by RT-PCR and resolved using an agarose gel.

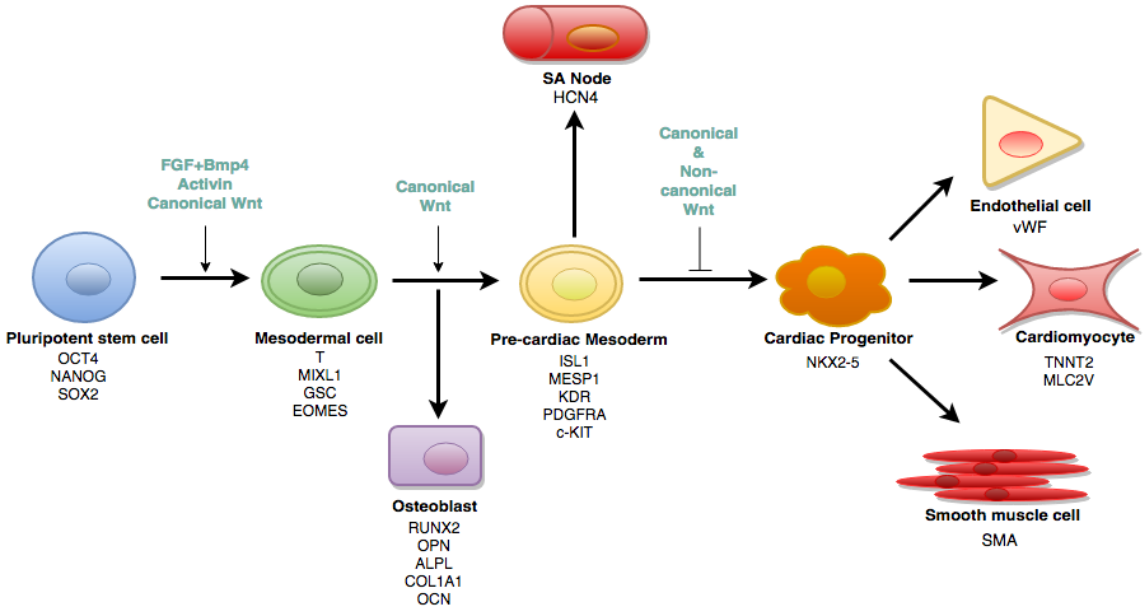

Figure 3. Summary of the stage-specific expression of molecular markers associated with cardiovascular development. Blue text signifies activation. Sharp arrows represent stimulatory effects; blunt arrows depict inhibitory effects.

Differentiation of an *ISL1*$^+$/*c-KIT*$^+$ and *MESP1*$^+$ Neonatal CPC into the Cardiac Lineage

Cardiomyocyte differentiation was performed in order to confirm the differentiation capacity of the *ISL1*$^+$/*c-KIT*$^+$/*MESP1*$^+$/KDR$^+$ neonatal CPC along the cardiovascular lineage. The cardiomyocyte differentiation protocol was adapted from a previous report involving treatment of human cardiomyocyte progenitor cells with 5-azacytidine, TGF-β, and ascorbic acid (Smits et al., 2009). In addition, an endothelial tube formation assay was conducted to assess endothelial cell differentiation. Formation of tubes has been shown to determine the capability of cells to differentiate along the endothelial cell lineage (Arnaoutova et al., 2009). Endothelial cells respond to proangiogenic signals and migrate rapidly to form capillary-like tubes when plated over Matrigel (DeCicco-Skinner et al., 2014).

The *ISL1*$^+$/*c-KIT*$^+$/*KDR*$^+$ and *MESP1*$^+$ clone was capable of differentiation into cardiomyocytes and endothelial cells. Cardiomyocyte differentiation was demonstrated by a significant induction of the cardiomyogenic-specific transcript, Troponin T (*TNNT2*) (Figure 4). Furthermore, the neonatal clone effectively formed tubes (Figure 5), indicating that these cells have endothelial differentiation capacity. Following tube network formation, cells were imaged and documented using an EVOS FL imaging system set at the 4x objective.

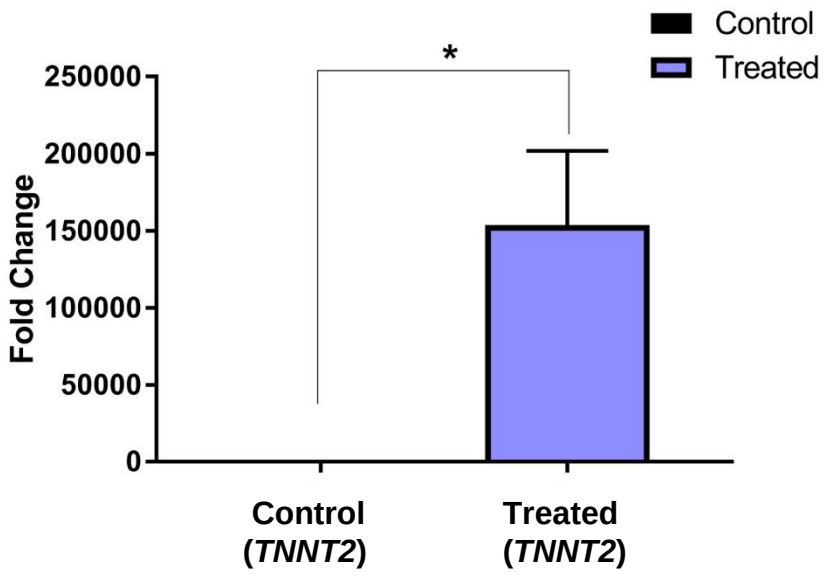

Figure 4. Differentiation of ISL1^{+}/c-KIT^{+}/MESP1^{+}/KDR^{+} human neonatal CPC into cardiomyocytes. After 14 days of culture in differentiation medium, gene expression levels for Troponin T (*TNNT2*) were elevated in the neonatal CPC expressing markers of early cardiovascular development (N=3, run in triplicate $P < 0.05$).

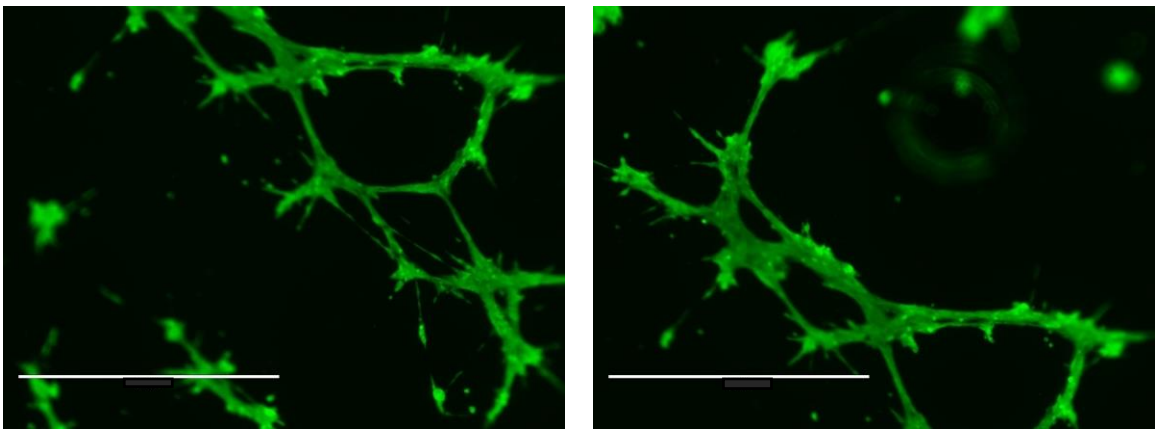

Figure 5. Tube formation by the $ISL1^{+}$/$c\text{-}KIT^{+}$/ KDR^{+} and $MESP1^{+}$ neonatal CPC clone verifies differentiation along the endothelial cell lineage. Representative images of endothelial cell tube formation over Matrigel in a select neonatal CPC exhibiting early developmental characteristics. Scale bar = 1000 μm (N = 3).

A Neonatal CPC Co-Expressing *ISL1, c-KIT, KDR* and *MESP1* Differentiates into a Mature Osteoblast Phenotype

In order to assess the differentiation capacity of the early *ISL1*$^{+}$/*c-KIT*$^{+}$/*MESP1*$^{+}$/*KDR*$^{+}$ neonatal CPC clone into mesodermal derivatives, differentiation into the osteoblast lineage was performed. Neonatal CPCs were cultured in 6-well plates at 5 x 10^3 cells per well in DMEM/10% FBS with osteogenic supplements, 50uM ascorbic acid, 10mM β-glycerophosphate and 100nM dexamethasone (Figure 6) (Bielby et al., 2004; Jaiswal et al., 1997; Sottile et al., 2003). Dexamethasone acts to induce the expression of the transcription factor, *RUNX2*, through a beta-catenin-mediated pathway, while ascorbic acid and β-glycerophosphate have been shown to play roles predominantly in establishing the extracellular matrix by increasing collagen type 1 secretion and serving as a phosphate source, respectively (Langenbach et al., 2013).

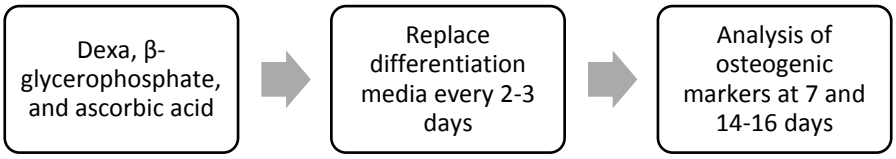

Figure 6. Timeline of neonatal CPC differentiation into osteoblasts. Cells were expanded in 6-well plates for 7 to 14/16 days.

Osteogenic-specific molecular markers were analyzed by qRT-PCR following 7 days of induction which revealed a significant increase in the expression of early markers of osteoblast differentiation (Figure 7). *RUNX2*, the most upstream transcription factor essential for osteoblast differentiation (Komori, 2017), was found to be significantly elevated (519-fold, P=0.01) following the initial induction period. Osteopontin (*OPN*), one of the non-collagenous extracellular structural proteins secreted in the bone, has been shown to behave bimodally, by which peak expression occurs during active proliferation, decreases post-proliferatively, and peaks once more at the onset of mineralization (Lian et al., 1995; Twine et al., 2014). Similar to these findings, *OPN* expression was significantly induced in the neonatal CPC at the initial proliferative stages of osteoblast differentiation (Figure 7A). Alkaline phosphatase (*ALPL*) and Collagen type 1 (*COL1A1*) are important components for establishing the mineralized matrix present in bone (Stein et al., 1993). Expression of both mineralization and extracellular matrix-related transcripts were induced following 7 days of differentiation in osteogenic supplemented media. *OCN* expression has been shown to occur at later stages of osteoblast development and is reflective of a mature osteoblast phenotype (Lian et al., 1995; Ducy et al., 1995; Huang et al., 2007). In order to confirm osteoblast lineage commitment, gene and protein expression analyses of *OCN* were performed following 14-16 days of osteoblast induction. The neonatal CPC demonstrated an increase in the expression of the late-stage osteoblast marker by both qRT-PCR (Figure 8) and flow cytometry (Figure 9), and therefore verified differentiation of the selected *ISL1*$^{+}$*/c-KIT*$^{+}$*/KDR*$^{+}$ and *MESP1*$^{+}$ neonatal CPC into a mature osteoblast phenotype. Differentiation into the osteoblast lineage thereby confirms mutlipotential differentiation capability of this early progenitor.

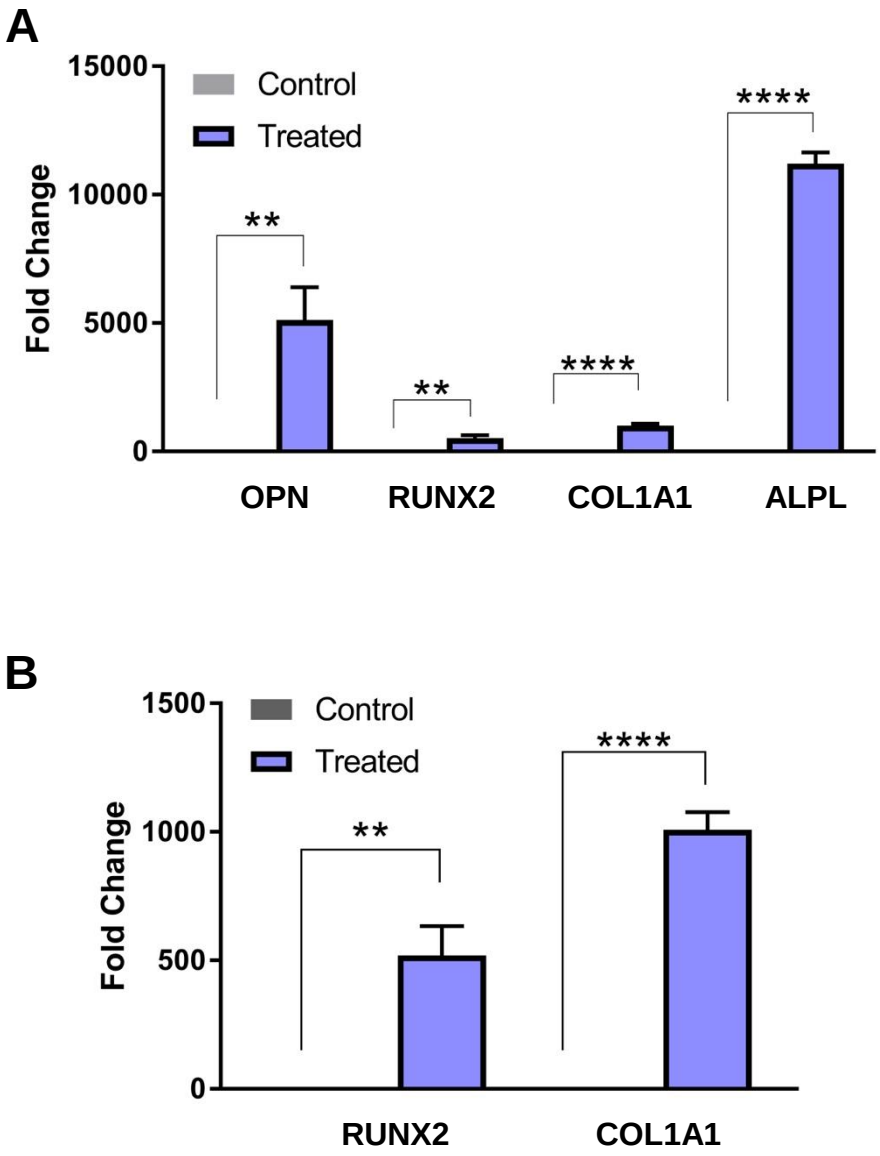

Figure 7. Neonatal CPC co-expressing ISL1, c-KIT, KDR, and MESP1 treated with osteogenic supplements for 7 days showed enhanced expression of osteogenic markers. **(A)** Representative gene expression analysis of early developmental markers *OPN* ($P \leq 0.01$), *RUNX2* ($P \leq 0.01$), *COL1A1* ($P \leq 0.0001$), and *ALPL* ($P \leq 0.0001$) following 7 days of differentiation. **(B)** *RUNX2* and *COL1A1* expression was induced ~500 and ~900 fold, respectively. Abbreviations: OPN, Osteopontin; OCN, Osteocalcin; Runx2, Runt related transcription factor 2; Col1A1, Collagen Type 1; ALPL, Alkaline Phosphatase (N=3).

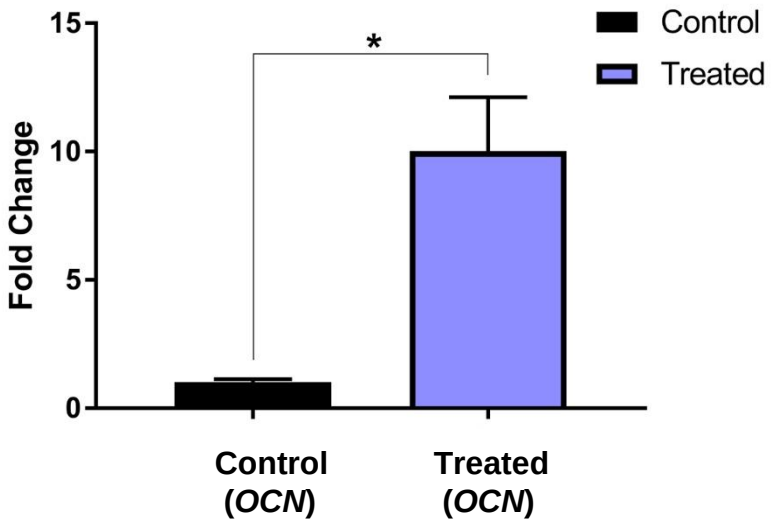

Figure 8. The ISL1^{+}/c-KIT^{+}/KDR^{+} and MESP1^{+} Neonatal CPC treated with osteogenic supplements for 14-16 days showed enhanced expression of late-stage osteoblast differentiation marker, osteocalcin. Expression of the transcript for osteocalcin (*OCN*) was induced at 14-16 days of osteoblast differentiation (N=3, P <0.05).

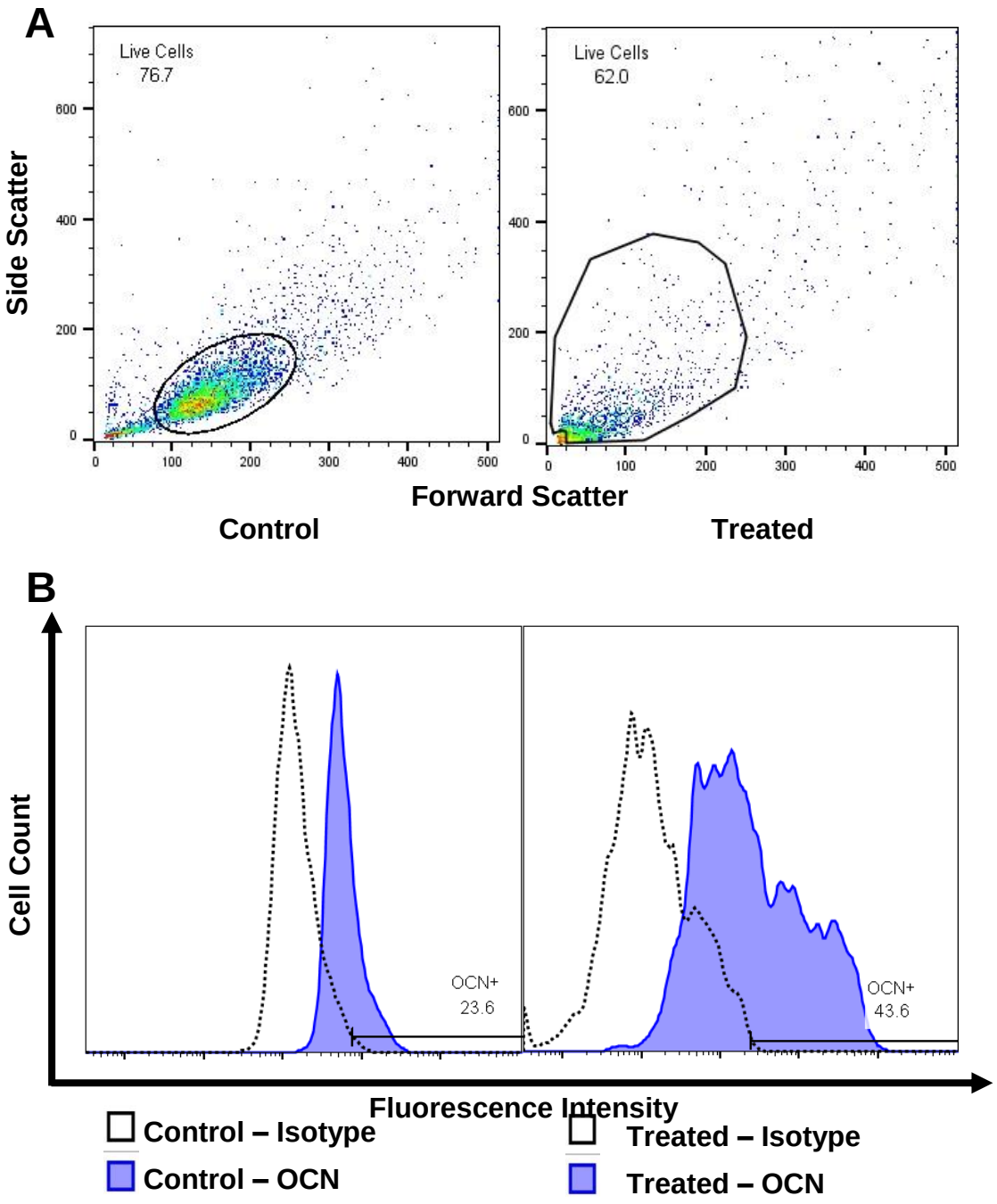

Figure 9. The ISL1^{+}/c-KIT^{+}/KDR^{+} and MESP1^{+} Neonatal CPC treated with osteogenic supplements for 14-16 days showed enhanced expression osteocalcin by flow cytometry **(A)** Forward and side scatter dot plot of untreated and treated CPCs. **(B)** Flow cytometry histogram of increased OCN expression. Positive staining is represented by the colored histogram, dotted histogram is representative of the isotype control (N=3).

CHAPTER FOUR

DISCUSSION

In the present study, we demonstrated that neonatal CPCs isolated from the human heart co-expressing *MESP1, c-KIT, KDR,* and *ISL1* can be induced to differentiate into various mesodermal lineages, including cardiovascular and bone. Differentiation into the osteoblast lineage by neonatal cardiac progenitors expressing this array of early developmental markers demonstrated enhanced multipotential capability. Osteoblasts, the specialized mesenchymal cells that synthesize bone are, in addition to the cardiovascular lineage, one of the mesodermal derivatives regulated by the Wnt-signaling pathway (Bakre et al., 2007; Yavropoulou et al., 2007). A lineage tracing study in mice demonstrated that development of the heart and hindlimb may be regulated by a common pathway in *Isl1*-expressing progenitors (Yang et al., 2006). This early study supports the concept that *Isl1* marks a subset of heart and hindlimb progenitors, and *Isl1* may be upstream in common heart/hindlimb pathways (Yang et al., 2006). Similarly, human cardiac progenitor cells expressing *ISL1* have been reported to differentiate into the osteoblast lineage, further demonstrating the multipotential capability of *ISL1*-expressing progenitor populations. However, the findings from this group did not demonstrate clonogenicity of progenitor cells, nor were the cell populations examined for co-expression of early developmental markers such as *MESP1* and/or *KDR* that have been reported in the current study. Furthermore, the cardiac progenitor cells investigated did not exhibit an overlap between *c-KIT* and *ISL1* expression (Itzhaki-Alfia et al., 2009). Taken together, these findings support the novel concept that isolation of cells on the basis of *ISL1* and *c-KIT* coexpression selects for a subpopulation of cardiac progenitors

with enhanced multipotential differentiation capacity, and that these cells may be sustained within the mesendodermal stage of development.

The *ISL1*$^{+}$/*c-KIT*$^{+}$/*MESP1*$^{+}$/*KDR*$^{+}$ neonatal CPC expressed transcripts for modulators of the mesendoderm, such as *GSC, MIXL1,* and *EOMES*, and therefore may reside in a stage of development that is earlier than the pre-cardiac mesoderm. *GSC* is strongly expressed in cell populations identified as mesendodermal regulators during the formation of the anterior primitive streak (Blum et al., 1992; Tada et al., 2005), and furthermore has been shown to be expressed in a majority of *ISL1*$^{+}$/*c-KIT*$^{+}$ neonatal CPCs that were examined in the present study. The mesendoderm has been characterized as a diverging point of the mesoderm and definitive endoderm during hESC differentiation (Tada et al., 2005). Interestingly, *MESP1* has been shown to be involved in the regulation of the mesendodermal stage of embryogenesis (Soibam et al., 2015). Soibam and colleagues identified early cell lineages regulated by *MESP1*, and found that *MESP1* targets mesendoderm modulating markers, giving rise to progenitors of the mesoderm and endoderm during gastrulation (Soibam et al., 2015). Several mesendoderm regulatory factors that are activated by *MESP1* include *EOMES, GATA4, GSC, T,* and *MIXL1,* suggesting that this *ISL1*$^{+}$/*c-KIT*$^{+}$/*MESP1*$^{+}$/*KDR*$^{+}$ neonatal CPC resides within the mesendodermal stage of development.

The bipotent mesendoderm has been established as a very early developmental stage in many species, including *C. elegans,* drosophila, xenopus, and zebrafish (Rodaway and Patient, 2001). Fate mapping experiments have demonstrated that definitive endoderm progenitors are specified in close proximity to cardiovascular progenitors (Costello et al., 2015). The close association between mesoderm and

endoderm formation within the primitive streak in addition to the co-expression of markers specific for mesoderm and endoderm precursors in early progenitor cells, supports evidence of a common bipotential progenitor population originating at the onset of gastrulation (Rodaway et al., 1999; Kimelman and Griffin, 2000). Moreover, these reports suggest that a population of progenitor cells within this region may be capable of differentiating into both mesodermal and endodermal lineages. Further research to define the differentiation and regenerative properties of neonatal CPCs is ongoing.

The present study sought to identify a population of human neonatal CPCs with enhanced differentiation and multipotential capability for applications in stem cell-based regenerative therapy. Our findings demonstrate that the *KDR*$^{+}$, *MESP1*$^{+}$, *Isl1*$^{+}$, and *c-KIT*$^{+}$ endogenous human neonatal CPC are associated with the early stages of embryonic development and preserves characteristics of the mesendoderm, with the potential for enhanced efficacy. Analysis of several *ISL1*$^{+}$/*c-KIT*$^{+}$ adult CPCs revealed expression of transcripts for *GSC*, suggesting that the clonal populations from both neonatal as well as adult sources co-expressing *Isl1* and *c-KIT* sustain transcripts of the mesendoderm. These exciting results provide valuable information regarding the identity of a cardiovascular stem cell population that may be implemented as an optimal source for numerous stem cell-based regenerative therapies. Future studies will focus on determining the differentiation propensity of endogenous neonatal CPCs into the endodermal lineage to verify the functionality of the expressed mesendodermal transcripts.

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SUPPLEMENTAL TABLES/FIGURES

Table S1. List of primers used to detect gene expression by qRT-PCR

Amplicon	Product Size	Tm-Fwd	Tm-Rev	Forward and Reverse Sequence (5'-3')
ACTIN	130bp	63	62	**Fwd:** TTTGAATGATGAGCCTTCGTCCCC **Rev:** GGTCTCAAGTCAGTGTACAGGTAAGC
ALPL	543bp	62	59	**Fwd:** GGGGGTGGCCGGAAATACAT **Rev:** GGGGGCCAGACCAAAGATAG
BRACHYURY	568bp	67	66	**Fwd:** ACTGGATGAAGGCTCCCGTCTCCTT **Rev:** CCAAGGCTGGACCAATTGTCATGGG
C-KIT	105bp	63	62	**Fwd:** ATTCCCAAGCCCATGAGTCCTTGA **Rev:** ACACGTGGAACACCAACATCCT
COL1	461bp	57	59	**Fwd:** GGACACAATGGATTGCAAGG **Rev:** TAACCACTGCTCCACTCTGG
EOMES	381bp	59	59	**Fwd:** TTGAAAAAGGGCAGAAAGGCG **Rev:** TTTGCAAAGCGCAGACGG
GATA4	189bp	58	59	**Fwd:** GACAATCTGGTTAGGGGAAGC **Rev:** ACACAATGCAAAACCCACGG
GSC	158bp	60	59	**Fwd:** AACGCGGAGAAGTGGAACAA **Rev:** AGCATCGTCTGTCTGTGCAA
ISL1	202bp	59	56	**Fwd:** CACAAGCGTCTCGGGATTGTGTTT **Rev:** AGTGGCAAGTCTTCCGACAA
KDR	219bp	62	61	**Fwd:** CCTCTACTCCAGTAAACCTGATTGGG **Rev:** TGTTCCCAGCATTTCACACTATGG
MESP1	377bp	60	60	**Fwd:** CGCTATATCGGCCACCTGTC **Rev:** GGCATCCAGGTCTCCAACAG
MIXL1	501bp	57	60	**Fwd:** TTTTCTCCCCTCTTCCAGGTA **Rev:** CGTCATTGACAAAGTGGCGG
NKX2-5	111bp	57	61	**Fwd:** CGCCGCTCCAGTTCATAG **Rev:** GGTGGAGCTGGAGAAGACAGA
OCN/ BGLAP	294bp	60	65	**Fwd:** ATGAGAGCCCTCACACTCCTC **Rev:** GCCGTAGAAGCGCCGATAGGC
OCT4/ POU5F1	123bp	65	63	**Fwd:** AACCTGGAGTTTGTGCCAGGGTTT **Rev:** TGAACTTCACCTTCCCTCCAACCA
OPN/ SPP1	355bp	55	56	**Fwd:** CATCTCAGAAGCAGAATCTCC **Rev:** CCATAAACCACACTATCACCTC
PDGFRA	496bp	62	61	**Fwd:** GCGCAATCTGGACACTGGGA **Rev:** ATGGGGTACTGCCAGCTCAC
RUNX2	460bp	60	59	**Fwd:** CCTCAGTGATTTAGGGCGCA **Rev:** GGTGTGGTAGTGAGTGGTGG
TGFB1	384bp	60	60	**Fwd:** GGGCTACCATGCCAACTTCT **Rev:** GACACAGAGATCCGCAGTCC
TNNT2	131bp	59	57	**Fwd:** GTGGGAAGAGGCAGACTGAG **Rev:** ATAGATGCTCTGCCACAGC