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Identification of SALL4 Expressing Islet-1+ Cardiovascular Progenitor Cell Clones

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IDENTIFICATION OF SALL4 EXPRESSING ISLET-1+ CARDIOVASCULAR PROGENITOR CELL CLONES

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

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ABSTRACT

The utilization of cardiac progenitor cells (CPC) has been shown to induce favorable regenerative effects. While there are various populations of endogenous CPCs in the heart, there is no consensus regarding which population is the most ideal for cell-based regenerative therapy. Identifying an Islet-1+ (Isl-1+) early-stage progenitor population with enhanced stemness, multipotency and differentiation potential would be beneficial for regenerative therapy. Spalt-like transcription factor 4 (SALL4) plays a role in embryonic development as well as proliferation and expansion of hematopoietic progenitor cells. We hypothesize that SALL4 will be co-expressed in Isl-1+ cardiac progenitor cell clones isolated from human cardiac tissue and represent a pre-mesendodermal progenitor population. Ingenuity Pathway Analysis revealed Isl-1+ human neonates exhibit enhanced stemness properties compared to Isl-1+ adult CPCs. We compared RNA-seq based transcriptomic data from human neonatal Isl-1+ CPC clones with that of published time-course specific RNA-Seq based transcriptomic data collected from various stages of cardiac differentiation from human pluripotent stem cells (hPSCs). This approach elucidated genes that are highly expressed at different stages of cardiac development giving insight into the developmental state of SALL4 expressing Isl-1+ neonatal CPCs. In addition to SALL4, evidence suggests that SOX2, EpCAM and TBX5 are expressed early along the cardiovascular pathway. CPCs were previously derived from human cardiac tissue discarded at surgery, clonally expanded, and screened for expression of Isl-1. RNA was isolated from

individual human neonatal (n=10) Isl-1+ CPCs, cDNA was synthesized, and real time PCR was done. We utilized RT-PCR to identify the expression of SALL4, SOX2, EpCAM and TBX5 in individual neonatal Isl-1+ CPC clones. Gel electrophoresis of the PCR products was used to confirm that transcripts of the correct size were amplified. Results demonstrate that 9 out of 10 Isl-1+ neonatal CPC clones tested expressed SALL4. To further substage neonatal Isl-1+ CPC clones that expressed SALL4, SOX2, EpCAM and TBX5, clones were tested for expression of TFAP2C. These features will allow for the identification and isolation of an optimal early-stage CPC clone that may be of value in regenerative therapeutic applications.

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CHAPTER ONE

INTRODUCTION

Cardiovascular disease (CVD) refers to a number of disorders of the heart and blood vessels. According to the World Health Organization, cardiovascular diseases are the number one cause of death globally. It is estimated that by 2030, nearly 23.6 million people will die of CVD per year (Mozaffarian et al., 2015). Prevalence of CVD in adults over the age of 20 is 49.2% and increases with age in both males and females (Virani et al., 2021). Interestingly, 1 out of every 4 deaths in the United States is a result of CVD with a death occurring every 37 seconds (Virani et al., 2021). With more than 100,000 people on the national heart transplant list, there is a need to address alternative options for cardiac repair. Current options for repair range from simple lifestyle changes to coronary artery bypass grafting, heart valve repair or replacement, insertion of a pacemaker, and more. Surgical repair of the heart is often successful but comes with risks such as, bleeding, infection, stroke, even death (Asahq). There is an unmet need to drive regenerative solutions into clinical trials. Current therapies for CVD are unable to replace dead cardiomyocytes. Cardiomyocytes are the main cardiac cell type lost in cardiovascular disorders, including heart failure, myocardial infarction, and ischemia, and replacing these cells could potentially restore heart function (Le et al., 2016). According to clinicaltrials.gov, there are 90 studies that use autologous stem cells as an intervention for heart repair (clinicaltrial.gov). Most interventions lack functional efficacy for cardiovascular

repair, and many do not reach completion. Consequently, there is a need to identify a cardiovascular progenitor cell (CPC) that can differentiate into a variety of cardiac subtypes and will demonstrate the ability to improve function.

Early heart development involves several types of progenitor cells that are derived from the mesoderm, proepicardium, and neural crest (Tan et al., 2020). Ultimately, this leads to the formation of the four chambered heart by gestational week seven via heart looping and complex cellular interactions (Tan et al., 2020). Heart development involves two distinct cardiac progenitor cell pools termed the first heart field (FHF) and second heart field (SHF) progenitor cells that are derived from the lateral plate mesoderm and neural crest cells. Overlap in expression of the FHF and SHF markers have led to debate as to where these two pools originate. It has been proposed that the FHF and SHF arise from a common progenitor that segregates into the heart fields early in development (Meilhac et al., 2004). Understanding the origin of CPCs may elucidate the markers that are expressed at various stages of CPC development and allow the identification of an early-stage progenitor with multi-lineage potential.

Fate mapping and lineage tracing experiments can track cell lineages and their progeny, aiding in the identification of an early-stage progenitor population. These experiments cannot be done in humans; therefore, the mouse is the most common model. Fate mapping studies have postulated that an early CPC exists at the primitive streak stage of CPC development and may have the ability to give rise to multiple cells of the heart (Peterson et al., 2021). According to the Jia group cell fate mapping experiments demonstrated that cardiac progenitor cells

in mice form from MESP1-expressing cells that leave the primitive streak during gastrulation on mouse development day E6.5 (Jia et al., 2018). Furthermore, fate mapping studies in mice have shown that cardiac mesoderm progenitors are the first to ingress through the primitive streak during gastrulation, later contributing to the FHF and subsequently the SHF (Meilhac et al., 2004). Specific markers such as Brachyury (T), Eomes, and MESP1 are some of the earliest markers to date that are present before or during ingression through the primitive streak and formation of the heart fields as evidenced in mouse lineage studies. Following the primitive streak stage, at E7.5, cells express NKX2-5 and the transcription factor Islet-1 (Isl-1+) and demonstrate multi-lineage potential generating cardiomyocytes, vascular smooth muscle cells, and endothelial cells (Jia et al., 2018). According to Cai et al., Isl-1+ identifies a distinct population of undifferentiated progenitors evidenced through fate mapping and in situ hybridization (Cai et al., 2003). In our lab, we isolate CPCs from discarded human cardiac tissue on the basis of Isl-1+ expression.

Isl-1+ is well-recognized as a marker that identifies early cardiac stem cells. However, according to Fuentes et al. Isl-1+ expressing cells are most abundant in neonatal tissue (Fuentes et al., 2013). Isl- 1+ is a transcription factor of the LIM-homeodomain family. Isl-1+ CPCs generate cells of the three cardiac lineages in vitro and in vivo: cardiomyocytes, vascular smooth muscle, and endothelial cells (Fuentes et al., 2014; Gao et al., 2019). The transcription factor Isl-1+ is required early in development for the survival, proliferation, and migration of cardiac progenitors into the primordial heart. In one study, Isl-1+

deficient mice lacked the outflow tract, right ventricle, and most of the atria (Weinberger et al., 2012). Fate-mapping has identified Isl-1+ cells in the atria, ventricles, and epicardium of the heart. Isl-1+ knockout mice exhibited growth retardation on day 9.5 in development and died on day 10.5 (Weinberger et al., 2012). Isl-1+ is transiently expressed in the second heart field (SHF) progenitor cells before their differentiation and integration into the heart tube (Weinberger et al., 2012; Barreto et al., 2019). Isl-1+ functions during heart development by shaping chromatin and creating a gene regulatory network (GRN) that drives cardiac development and defines cardiomyocyte identity (Gao et al., 2019). Specifically, Isl-1+ binds to and regulates the expression of transcription factors, epigenetic modifiers, and closed chromatin working with a complex (Brg1-Baf60c) to induce alterations in the chromatin landscape that enables the activation of genes that define cardiomyocytes (Gao et al., 2019). Gao et al., found that Isl-1+ target genes such as WNT5, JAG1, SMAD6, TBX3, TBX5, and TBX20 play a role in development of the atrioventricular canal and outflow tract morphogenesis. Isl-1+ expression is predominantly but not exclusively found in SHF progenitors (Ma et al., 2008).

Isl-1+ cardiac progenitor cells have also been studied for their use in cardiac repair (Ren et al., 2021). Multipotent Isl-1+ progenitors appear early in mesodermal differentiation due to co-expression of pluripotency genes but provide evidence of ventricular progenitor activity *in vivo* as shown by grafting studies (Foo et al., 2018). Foo et al., generated human ventricular progenitors isolated on the basis of Isl-1+ that were able to form a large ventricular muscle

patch on a murine kidney (Foo et al., 2018). Additionally, the patch was able to engraft into uninjured and injured murine hearts. Bartulos et al., isolated Isl-1+ CPCs from a murine embryonic stem cell (ESC) line and generated cardiospheroids (Bartulos et al., 2016). The Isl-1+ cardiospheroids showed enhanced differentiation capacity and effectively engrafted in the infarcted heart 4 weeks after injection, reducing scar size and increasing blood vessel formation. Another study transplanted Isl-1+ CPC patches that were generated using small intestinal submucosa extracellular matrix (SIS-ECM) which was then placed in mice following a myocardial infarction (MI) (Wang et al., 2017). The Isl-1+ CPCs were derived from mouse ESCs and were shown to differentiate in the SIS-ECM which served as a biodegradable scaffold. The SIS-ECM patches seeded with Isl-1+ CPCs and transplanted into the MI heart *in vivo* showed an increase in left ventricular ejection fraction (LVEF) from 33.49 to 62.16 (Wang et al., 2017). LVEF is defined as the amount of blood the left ventricle pumps out with each contraction. The LVEF in the heart is normally within the range of 55% to 70%. A LVEF less than 35% is considered severe heart failure in which the right and left ventricle do not pump in unison (Cleveland Clinic). These studies have led to clinical advances in heart regenerative therapy.

The ESCORT trial led by Menasche et al., is the first clinical trial using Isl-1+ CPCs. Menasche et al., developed a population of hESC-derived CPC expressing Isl-1+ and Stage-Specific Embryonic Antigen (SSEA-1) (Menasche et al., 2015). SSEA-1 is a surface marker that labels cells in a differentiation pathway and induces Isl-1+ expression. In this study, SSEA-1 was used to

identify a pure population of Isl-1+ CPCs with high levels of SSEA-1. Impure cell populations can cause undesirable effects such as variable engraftment, tumor formation and spontaneous differentiation (Fuerstenau-Sharp et al., 2015). Purity of a sample is especially important when isolating a cell population that is differentiating and intended for cell-based repair. A small fraction of undifferentiated cells can cause teratoma formation. In the Menasche study, the early-stage cells were embedded into a fibrin scaffold in patients suffering from ischemic left ventricular dysfunction. After cell treatment, all patients symptomatically improved with an increase in LVEF at 3 months post-delivery. Specifically, LVEF increased from 26% to 36% with no arrythmias, tumor formation, or immunosuppression related events. While this trial shows promise in cardiac regenerative therapy, safety is still a major concern. Patients in the ESCORT trial developed alloimmunization and one patient died of heart failure after 22 months. According to Menasche et al., allowing the early-stage progenitor cells to differentiate *in vitro* would unravel their ability to actively contract. However, identifying an optimal cell whose phenotype closely matches that of the recipient tissue would be the most ideal candidate. The ESCORT trial shows that hESCs , if purified and isolated at any early stage of development, have potential benefits for cardiac regenerative therapy.

Pluripotent stem cells (PSCs) is a collective term for Embryonic Stem Cells (ESCs) and Induced Pluripotent Stem Cells (iPSCs). While using PSCs for clinical trials has shown promise, utilization of these cells remains arguable. The use of PSCs is invaluable for tracing the events of early-stage cardiovascular

differentiation using cells derived from humans. ESCs may be used for stem cell-based applications but their use has potential risk of immune rejection after transplantation, teratoma formation from residual pluripotent stem cells, graft associated arrythmias and ethical concerns (Pawani & Bhartiya, 2013). Additionally, hESC derived cells for heart repair often do not match their *in vivo* counterparts displaying differences in electrophysiological parameters, contraction, and gene expression profiles (Churko et al., 2018). In contrast to ESCs, iPSCs overcome some of the limitations of ESCs. For instance, iPSCs are derived from somatic cells which can be patient-specific and can be reprogrammed to a pluripotent state. While the application of PSCs for cell based regenerative therapy poses potential risks, murine and human PSCs have been valuable in studies designed to investigate cardiac development *in vivo*.

Pluripotent stem cells have the ability to self-organize into 3D structures called organoids resembling embryo-like tissue patterns *in vitro* (Drakhlis et al., 2021). For example, murine PSC-derived pre-cardiac organoids have been shown to generate two heart fields (FHF and SHF) that were highly similar to their *in vivo* counterparts, providing a model for studying the morphological aspects of heart development (Andersen et al., 2018). PSCs can also be differentiated down a specific lineage to study stages of development. Specifically, human PSCs have been differentiated into cardiac mesoderm cells (CMCs) expressing early and late cardiac transcription factors (Vahdat et al., 2019). Additionally, murine, and human PSCs have been differentiated down the

mesendoderm lineage to study genes that are highly expressed at this stage of CPC development.

Mesendoderm is a transient stage in CPC development that differentiates toward the mesoderm and definitive endoderm lineage (Figure 1). Carpenedo et al., differentiated hPSCs toward the mesendoderm lineage to identify which genes are most highly expressed at the mesendoderm stage of cardiac differentiation (Carpenedo et al., 2019). RNA was collected from each stage of differentiation for RNA-Seq application to identify the expression of genes at each stage. Because the mesendoderm stage is transient, the Tian group identified transcription factor WHSC1 in murine PSCs and found that it is required for efficient pluripotency exit and mesendoderm specification. A small hairpin lentiviral construct was used to knockdown WHSC1 expression revealing elevated expression levels of pluripotency genes such as, REX1, OCT4 (POU5F1), Nanog and Lin28a (Tian et al., 2019). Additionally, WHSC1 depleted ESCs showed a strongly reduced expression of mesendoderm markers such as Gata4, T, SOX17 and KDR. Differentiation events identified using hPSCs are typically marked by the downregulation of pluripotency markers such as OCT-4, SOX2 and Nanog in conjunction with the activation of lineage-specific gene expression (Muhr & Ackerman, 2021). Signaling pathways such as canonical WNT, FGF, and BMP initiate the activation of transcription factors that regulate genes associated with a cardiac phenotype such as, NKX2 – 5, Gata 4, TBX5 (Witman et al., 2020). These early stages of differentiation are not clearly defined but are currently being studied using transcriptomics in an effort to characterize

early-stage cardiovascular progenitors as they differentiate along the mesodermal lineage. Mesendoderm stage cells have been used for cell-based repair with promising results and represent a population of interest for investigators in the field of cardiovascular stem cell research (Boumelhem et al., 2017).

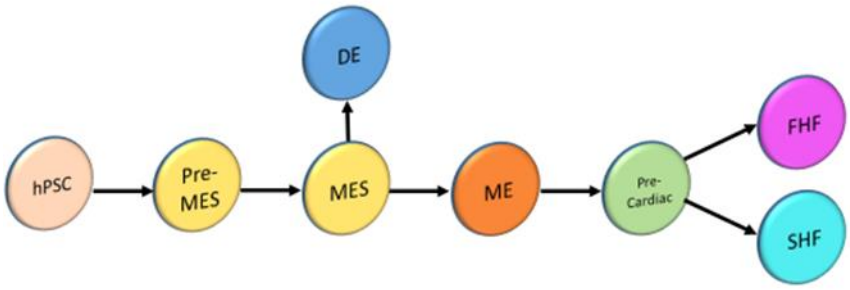


Figure 1: Representation of *in vitro* CPC differentiation from a pluripotent (hPSC) cell state. Cell stages: hPSC, pre-mesendoderm (Pre-MES), mesendoderm (MES), definitive endoderm (DE), mesoderm (ME), pre-cardiac mesoderm, first heart field (FHF) and second heart field (SHF).

Transcriptomics is an innovative technology that enables researchers to understand the heterogeneity and regulation of diverse cell phenotypes (Xiong et al., 2020). The transcriptome is the entire assembly of RNA transcripts in a given cell type. RNA-Seq transcriptomic analysis is a recently developed technology that uses high-throughput sequencing approaches to determine the sequence of all RNA transcripts in a given specimen (Reuther et al., 2019). Transcriptomics is a valuable approach that can be used to identify early-stage cardiac progenitor cells which would lower the risk of immune rejection, effectively engraft into the injection site, and have the ability to differentiate *in vivo* into all cells of the

cardiovascular lineage. The use of RNA-Seq transcriptomics on hPSCs committed to a cardiac fate elucidates transcripts that are present at specific stages of cardiac development. For example, Churko et al., performed a time course RNA-Seq analysis and used single cell populations of iPSCs that were differentiated along the cardiovascular lineage up to day 90. They collected RNA for RNA-Seq at various time points which corresponded to a defined stage of cardiac differentiation. This data helps researchers to understand the transcripts that are expressed at different stages of cardiomyocyte development. Similarly, Den Hartogh et al., differentiated hESCs using WNT pathway inhibitor Xav939 and collected RNA at specific time points to study the temporal expression of key molecules involved in cardiac lineage commitment upon differentiation (Den Hartogh et al., 2016). Time points were similar to those defined by Churko et al. These studies allow for the identification of early-stage cardiac progenitors by highlighting transcripts that are expressed early in CPC development (Table 1).

SALL4 is one example of a stem cell gene required for ESC pluripotency and early embryonic development (Xiong, 2014). Koshiba-Takeuchi et al., found that SALL4 and TBX5 ensure precise patterning of embryonic limb and heart (Koshiba-Takeuchi et al., 2006) while Pantier et al., reported that loss of pluripotency is triggered by downregulation of SALL4 (Pantier et al., 2021). SALL4 plays a role in embryonic stem cell self-renewal (Tatetsu et al., 2016). By binding to the regulatory region of genes such as OCT4 and SOX2, SALL4 regulates the pluripotency transcriptional network and controls the stemness gene EpCAM to maintain pluripotency (Tatetsu et al., 2016). SALL4 is

predominantly an enhancer binding protein. According to Miller et al., SALL4 occupancy of enhancer sequences along with pluripotency-associated transcription factors can enhance or interfere with transcription depending upon the target gene (Miller et al., 2016). It has been reported that SALL4 is capable of enhancing bone marrow regeneration and dramatically accelerates the expansion of hematopoietic stem cells from peripheral blood (Xiong et al., 2020). Tahara et al., found that SALL4 is essential for maintenance of neuromesodermal progenitors and promotes mesoderm commitment while inhibiting neural commitment (Tahara et al., 2019). The key role of SALL4 in self-renewal and stemness, suggests that this transcription factor may be expressed in early-stage cardiovascular progenitors isolated from neonates.

The search for the “ideal” cell type for heart repair has been an ongoing quest (Menasche, 2020). Cardiac progenitor cells appear early in embryonic development as demonstrated by genetic lineage tracing. While there are a number of cardiac progenitor populations for cell-based heart repair, research shows application of such cells to have limited efficacy. Isl-1+ marks an early-stage CPC and has been used to isolate clonal populations of early-stage cardiovascular progenitor cells in our laboratory. Specifically, neonatal Isl-1+ cardiovascular progenitor cells have been the focus of research in our laboratory due to their enhanced regenerative ability. Additionally, select mesendodermal and pre-cardiac markers have previously been characterized in our lab. According to Belair et al., SALL4 is necessary for mesendoderm specification and differentiation (Belair et al., 2020).

The aim of my project is to determine whether or not SALL4 may be used as a tool to isolate and identify the earliest stage CPC from human patients. These early-stage cells may then be of value for use in regenerative therapy. Results from this project will elucidate markers expressed at various developmental stages of CPC isolated from neonatal patient samples. The objective is to subsequently identify an early-stage CPC with a great degree of multipotentiality and cardiac repair potential.

Developmental Stage (*in vitro*)	**Markers**	**Human iPSCs**	**Human ESCs**
Pluripotent	SALL4	D3	D0-D10
	SOX2	D0-D1	D0-D5
	EPCAM	D1	D0
	TBX3	D2-D9	X
	POU5F1	D0	D0
Mesendoderm	MESP1	D3	D3
	T	D1-D3	X
	EOMES	D2	D3
	GSC	D1-D2	D3
	SOX17	D3	D3,D7
	GATA4	D1-D90	D3-D14
	MIXL1	D2	D3
FHF	HCN4	D9-D14	X
	NKX2-5	D7	D7-D14
	TBX5	D4-D90	X
	HAND1	D3-D90	X
SHF	ISL1	D6	D7-D10
	MEF2C	D6	D7-D10
	FGF10	D6	X
Epicardial	TBX18	X	D7-D14
	WT1	D30	D7-D14

Table 1: Genes which are highly expressed at different stages of CPC development. Genes are identified from published RNA-Seq based transcriptomic analyses based on *in vitro* differentiation of hiPSCS (Churko et al., 2018) and hESCs (Den Hartogh et al., 2016) toward a cardiomyocyte fate. (X=not represented in transcriptomic data)

CHAPTER TWO

METHODS

Ethics Statement and Maintenance of Cardiac Progenitor Cells

Human cardiac progenitor cell clones were previously isolated and were available for use in this project. 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.

Cardiac Progenitor Cell Expansion

CPC clones were isolated from cardiac tissue of five neonatal (1 day-1 month) and five adults (57-72 years) patients undergoing cardiovascular surgery (Fuentes et al., 2013). Twenty previously isolated clones were expanded for use in the experiments described here. Briefly, atrial tissue was cut into small clumps (~1.0 mm3) then enzymatically digested using collagenase at a working concentration of 1.0mg/mL for 2 hours at 37 degrees Celsius. The resulting solution was then passed through a 40-um cell strainer. Cells were cloned in a 96 well plate by limiting dilution to a final concentration of 0.8 cells per well to create populations for expansion. Clones were screened for the expression of Isl-1+ and supplemented with growth media comprised of 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 100 µg/mL Penicillin-Streptomycin (Life Technologies, Carlsbad, CA), 1.0% minimum essential medium non-essential amino acids

solution (Cat no. 11120052, Life Technologies, Carlsbad, CA), and 22% endothelial cell growth media (Lonza, Basel, Switzerland) in Medium 199 (Life Technologies, Carlsbad, CA) (Fuentes et al., 2013).

RNA Sequencing

RNA samples isolated from neonatal and adult Isl-1+ CPC clones were previously sent to PrimBio Research Institute for RNA-Sequencing (Exton, PA, USA). rRNA was removed from the total RNA sample using an rRNA removal kit from Illumina (San Diego, CA, USA) (cat# MRZG12324). Sequencing libraries were assembled with an Ion Total RNA-Seq Kit v2 from Thermo Fisher (Waltham, MA, USA) (Cat# 4479789). Nucleic acid binding beads from Ambion (Austin, TX, USA) were used to purify the cDNA library (Cat# 4479681) prior to PCR amplification. Agilent dsDNA High Sensitivity kit was used to determine the quality of the library (Agilent, Santa Clara, CA, USA). The samples were enriched via an Ion OneTouch ES instrument and an Ion PI™ Hi-Q™ OT2 200 Kit (Thermo Fisher, Waltham, MA, USA). Sequencing was performed using an Ion Proton sequencer (Thermo Fisher, Waltham, MA, USA) and a species-specific protocol for our samples. Next, sequence files were aligned to the human genome and quality was determined using the Strand NGS program. Normalization and quantification of the aligned reads were performed using the Deseq algorithm within the Strand NGS program. The Audic–Claverie test and the Benjamini–Hochberg correction test were used for statistical analysis.

Significance was determined using a 2.0-fold change minimum cutoff. (Camberos et al., 2021)

Transcriptomic Analysis

To analyze transcriptomic data, Isl-1+ neonatal (N=3) and adult (N=3) pooled CPC gene transcripts were uploaded to Ingenuity Pathway Analysis (IPA) (Qiagen, Valencia, CA, USA). Core analysis was performed with specific parameters set for human cells (Camberos et al., 2021). Categories specific to cancer and viral disease were removed and categories with a p-value < 0.05 were reported. IPA's upstream regulator analysis tool was used to visualize significant networks generated from uploaded transcriptomic data. Lastly, transcripts were uploaded to StemChecker (http://stemchecker.sysbiolab.eu/as) to determine the expression profile of the neonatal Isl-1+ CPC pool versus the adult Isl-1+ CPC pool. StemChecker refines the uploaded gene lists by identifying overlap with published literature in which stem cell gene sets (stemness signatures) are reported (Pinto et al., 2015). Stemness signatures are further categorized into expression profiles which are comprised of nine stem cell types: Hematopoietic Stem Cells (HSC), Mesenchymal Stem Cells (MSC), Embryonal Carcinoma (EC), Mammary Stem Cells (MaSC), Neural Stem Cells (NSC), Intestinal Stem Cells (ISC), induced Pluripotent Stem Cells (iPSC) and Spermatogonial Stem Cells (SSC).

Purification of Total RNA

Total RNA was purified from individual CPC clones stored in RNA Protect (Qiagen, Valencia, CA) and Qiazol Reagent (Qiagen, Valencia, CA). 1 ml of RNA protect or 700 ul of Qiazol was used to lyse the cells before being centrifuged though the provided RNeasy spin column followed by a series of washes with provided buffers. RNA was eluted with RNAse-free water and optical density of RNA was read using the Nanodrop 2000 to get the concentration of RNA. RNA was run on a 1% agarose gel to assess purity (Park et al., 1996). First Strand cDNA synthesis was performed from 2ug total RNA using Superscript III Reverse Transcriptase (Invitrogen).

RT-PCR Analysis

RT-PCR was performed using the RT^2 SYBR Green qPCR Master mix and the plate was loaded into the iQ5 RT-PCR Cycler. PCR plates were run under the following conditions: 94 °C for 10 min, 94 °C for 15 s, 58-60 °C (depending on primer) for 1 min, 72 °C for 30 s, repeated for 45 cycles. Primers for genes of interest are listed in table 2. RT-PCR products were visualized using 1%-2% agarose gel electrophoresis and a low mass DNA ladder was used to assess the size of the PCR product. Fold changes were calculated using the delta-delta Ct method (Schmittgen & Livak, 2008).

Human Gene	Forward Sequence	Reverse Sequence
SALL4	CACAAGTGTCGGAGCAGTCT	CCGTCCGTACCTAACAGAGC
SOX2	AACCAGCGCATGGACAGTTA	GACTTGACCACCGAACCCAT
EpCAM	TGGGGAACAACTGGATCTGG	CCCACGCACACACATTTGTAA
TBX5	CTCAGTCCCCCGGAACAAC	CACGTACCTCCCAGCTCAAG
Tfap2c	TGGTTGGTTTTTGTGTCCGC	TTGCTTCGTGCCTACCCTTT
Actin	TTTGAATGATGAGCCTTCGTCCCC	GGTCTCAAGTCAGTGTACAGGTAAGC

Table 2: Gene specific primer pairs created with NCBI Primer-Blast were used in the Reverse Transcription Polymerase Chain Reaction (Reported 5' to 3').

Statistics

Data was processed using Microsoft Excel and GraphPad Prism 7.02.

Transcriptomic data was used to generate heatmaps using GraphPad Prism 7.02.

CHAPTER THREE

RESULTS

Transcriptomic Analysis Elucidates Age-Related Differences in Stemness-Expression Profiles and Mechanistic Networks

Stem cells present a unique regenerative ability offering potential for treatment of a variety of diseases. There are a number of stem cell types each having a distinct gene expression profile. RNA-Seq based transcriptomics was used to identify the expression profile and stemness features within the neonatal and adult Isl-1+ CPC population. Using StemChecker, the Isl-1+ neonatal CPC population (N=3) presented a gene expression profile most similar to induced pluripotent stem cells (P-value=0.024). Specifically, of the nine expression profiles curated by StemChecker, the transcriptome of the Isl-1+ neonatal CPC population contained a higher percentage (forty-three percent) of transcripts associated with iPSCs. In contrast, the transcriptome of the Isl-1+ adult CPC population (N=3) presented a gene expression profile (forty-two percent) most similar to mesenchymal stem cells (MSC) (P-value 2.01E-6) (Figure 2). Transcripts associated with iPSCs and MSCs represent multipotentiality, self-renewal ability, and proliferation. Both are promising cell sources for autologous cell therapy and facilitate post-infarct recovery (Neef et al., 2022).

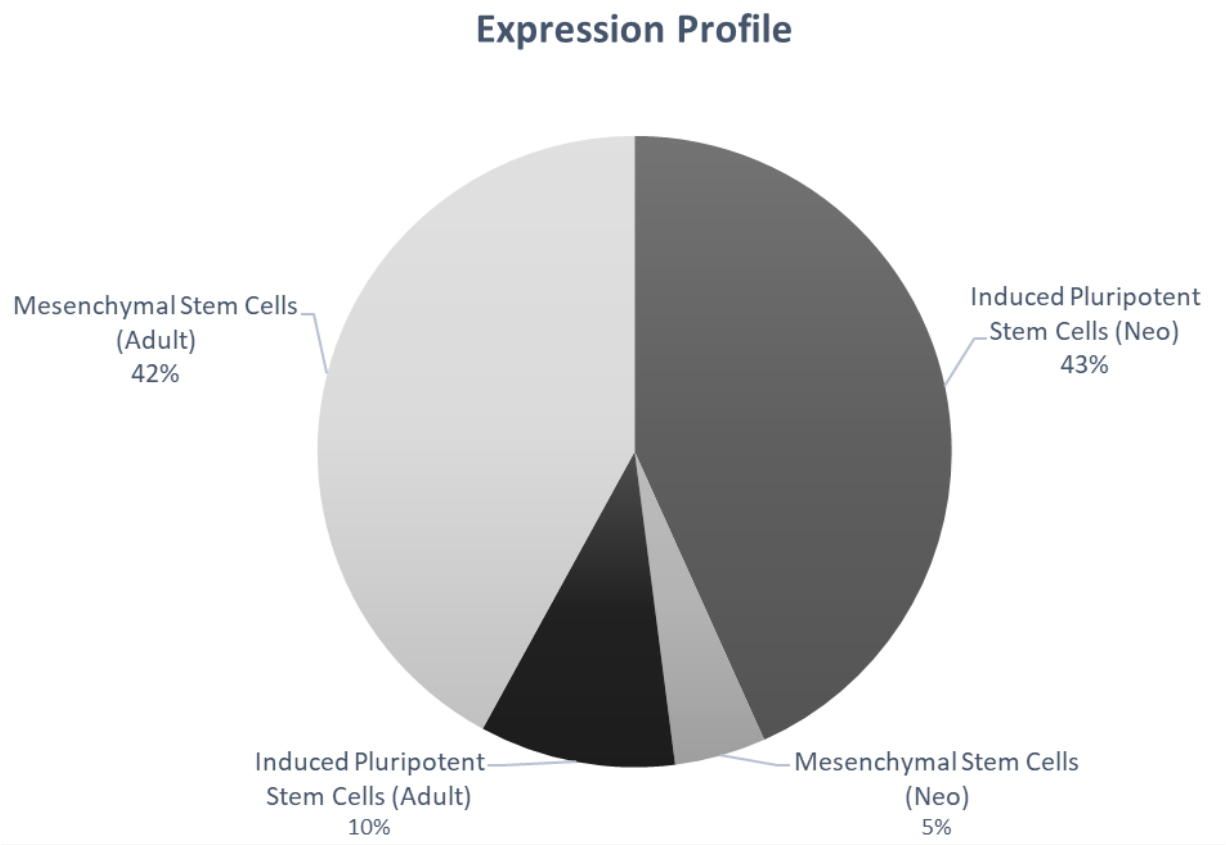
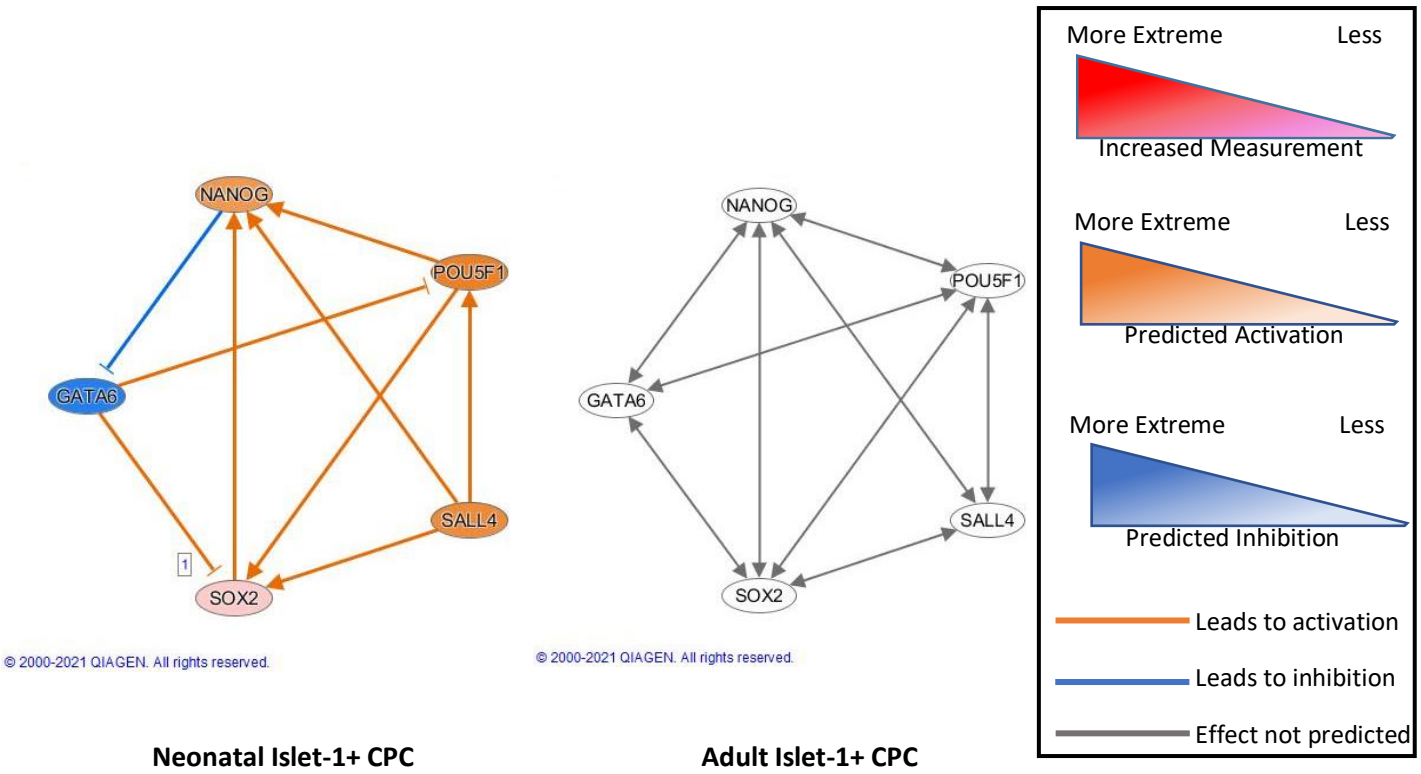


Figure 2: StemChecker analysis comparing the iPSC and MSC expression profile for Isl-1+ neonatal and adult CPCs. Isl-1+ neonatal CPC (N=3) transcriptome had the greatest number of transcripts corresponding to iPSCs. Isl-1+ adult CPC (N=3) transcriptome had the greatest number of transcripts corresponding to MSCs.

Using RNA sequencing, gene expression profiles of the Isl-1+ neonatal and adult CPC clonal populations were compared to one another. A fold change cutoff of 2.0 was applied to focus on statistically significant gene expression changes when comparing each age group. The neonatal population (N=3) consisted of 2673 transcripts and the adult population (N=3) consisted of 1381 transcripts. Transcript sequence information was uploaded to Ingenuity Pathway Analysis (IPA) software to identify transcripts implicated in mechanistic networks (Figure 3). Mechanistic networks are signaling cascades that connect upstream

regulators to visualize how they may work together to elicit gene expression changes observed in experimental data (Kramer et al., 2014). A mechanistic network was identified in the neonatal population data containing key stemness genes: SOX2, OCT4, and Nanog. In addition, Gata 6, an early-stage differentiation gene was found in the network (Fisher et al., 2017). This mechanistic network was not identified in the adult population data. Stemness gene, SALL4 was added to the network using Ingenuity Pathway Analysis grow tool. IPA path explorer tool was used to determine the connection between SALL4 and the stemness genes. The connections are based on the IPA knowledge base which is curated from published experimental data. IPA's overlay tool allows analyses from different dataset uploads to be applied to a network or pathway. The overlay tool was used to determine if the transcriptome of the adult CPC population contains mRNAs implicated in the network. In addition, IPA's molecule activity predictor function was applied to the network to identify predicted activation and inhibition of the molecules as determined by the z-score. Figure 3 shows that the mechanistic network is predicted to be activated in the neonatal population while the network shows no significant activation or inhibition for the adult population. Within the network, genes highlighted in red were identified within the transcriptomic dataset. The color of the lines indicates the expected directional effect between two molecules based on published literature.



Neonatal Islet-1+ CPC **Adult Islet-1+ CPC**

Figure 3: Mechanistic network of upstream regulators and their predicted regulators generated by IPA. Neonatal Isl-1+ CPCs (N=3) (left) show predicted activation and/or inhibition of genes using IPA tool: molecule activity predictor. Adult Isl-1+ CPCs (N=3) (right) shows insignificant prediction activation and/or inhibition of genes using IPA tool: molecule activity predictor.

Individual Islet-1 + Neonatal CPCs Exhibit Pathway Specific Transcriptomic Differences

Using the comparative transcriptomic data of three individuals patient-derived Isl-1+ neonatal CPC clones, all fold change values were entered into IPA software to identify the differences and similarities within the neonatal population. The cardiovascular stem cell clone expanded from an 8 day old patient (neonate N1) showed enhanced expression of genes activated for calcium signaling, transcriptional regulatory network in embryonic stem cells and factors promoting cardiogenesis when compared with the clone that was isolated from neonate 3 (N3). Additionally, each Isl-1+ neonatal clone showed differences in pathway-specific transcript expression levels when comparing neonate 1, neonate 2 and neonate 3. SALL4 was found to be most highly expressed in the clone isolated from the 8 day old patient. Although transcripts identified within the Transcriptional Regulation of Pluripotent Stem Cell pathway were identified in each of the three Isl-1+ neonatal CPC clones, neonate 1 expressed the highest levels (Figure 4).

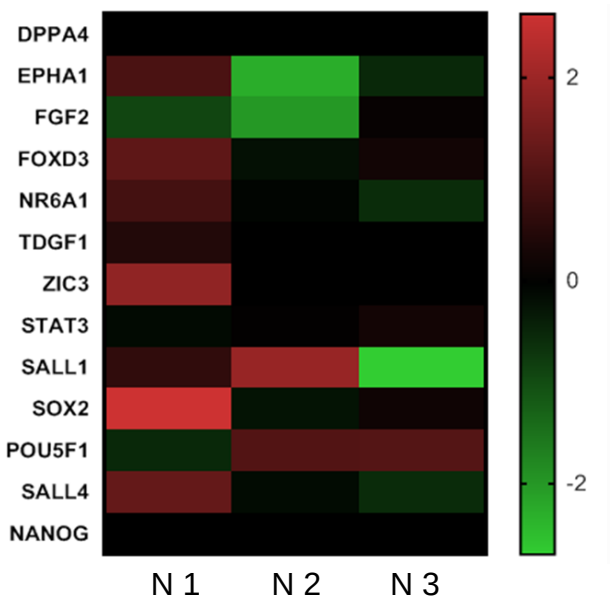


Figure 4: Heatmap of transcripts in the Transcriptional Regulation of Pluripotent Stem Cells pathway in the three-patient derived Isl-1+ neonatal CPC clones (N1, N2 and N3).

Neonatal Islet-1+ CPCs Exhibit Stage Specific Transcriptomic Differences

Cardiogenesis begins at the start of gastrulation. Specifically, epiblast cells ingress through the primitive streak to form the mesendoderm and subsequently mesoderm. Stage specific gene expression during cardiogenesis is used to identify differentiation steps in development. Comparing previously published stage-specific RNA-seq transcriptomic data to our RNA-seq Isl-1+ neonatal CPC transcriptomic data yields stage specific differences in gene expression within the Isl-1+ neonatal CPC clones (Figure 5). Stages of CPC development are: Pluripotent/epiblast, pre-mesendoderm, mesendoderm, FHF and SHF (Maher & Xu, 2013). Each individual Isl-1+ neonatal clone showed differences in expression level. Specifically, the heatmap shows that N1 expresses the highest number of genes correlated to all CPC developmental stages as opposed to N2 and N3.

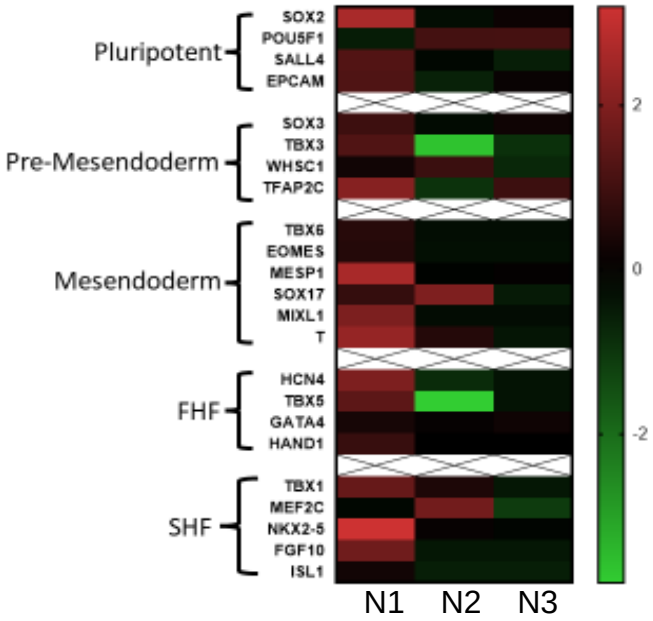


Figure 5: Heatmap of markers that identify stages (Pluripotent, pre-mesendoderm, mesendoderm, FHF, SHF) of CPC development between the individual neonatal Isl-1+ CPC clones.

Transcriptomic Analysis Reveals a Correlation between SALL4, SOX2, EpCAM and TBX5

IPA allows the search of individual genes to identify the predicted role of SALL4 and shows interactions between genes based on experimental and literature references. According to IPA, SALL4 interacts with SOX2, EpCAM and TBX5 (Figure 6A). SALL4 increases the expression of SOX2 and vice versa by interaction with their enhancer proteins (Yang et al., 2012). SALL4 and TBX5 also have a protein-protein interaction that impacts their expression (Koshiba-Takeuchi et al., 2006). Activation of SALL4 is involved in upregulation of EpCAM mRNA as shown in Figure 6A (Zeng et al., 2014). Neonate 1 shows the greatest correlation of genes associated with SALL4 activation/function compared to neonate 2 and 3 (Figure 6B).

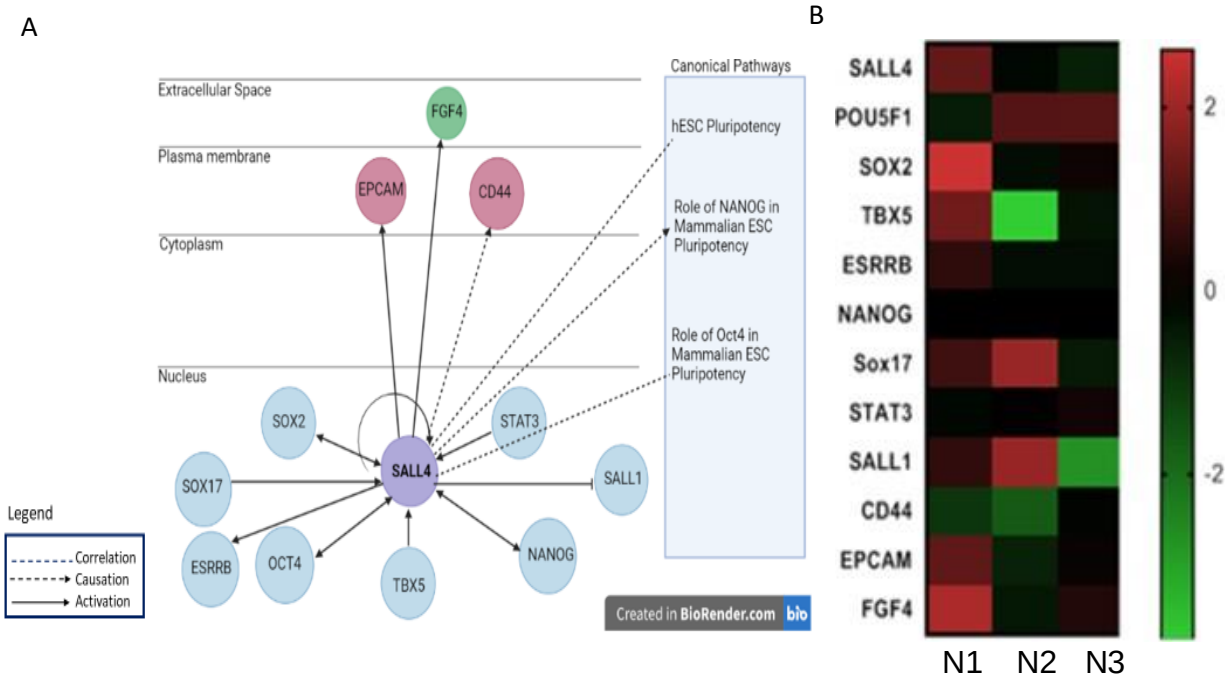


Figure 6: Genes and canonical pathways associated with SALL4 (A). Heatmap of genes associated with SALL4 that are expressed in neonatal CPC clones N1, N2 and N3 (B).

SALL4 is Expressed in Individual Islet-1+ Neonatal Cardiac Progenitor Cell Clones

Clonal populations of human neonatal CPCs expressing Isl-1+ were previously isolated and characterized for further use in the laboratory (Fuentes et al., 2013). Additionally, the CPCs have previously been selected for expression of early developmental markers such as Mesp1, PDGFRa, and SSEA1 (Baio et al., 2018). SALL4 expression in Isl-1+ neonatal CPCs was therefore analyzed by RT-PCR to confirm RNA-Seq analysis in the three sequenced clones and to determine whether additional neonatal CPC clones express SALL4. Ten Isl-1+ neonatal CPC clones were screened for expression of SALL4 mRNA. Figure 7A shows that SALL4 is expressed in 9 of the 10 Isl-1+ neonatal CPC clones, confirming the RNA-seq data and identifying one neonatal CPC clone that did not express SALL4. Figure 7B shows the corresponding actin expression for each clone.

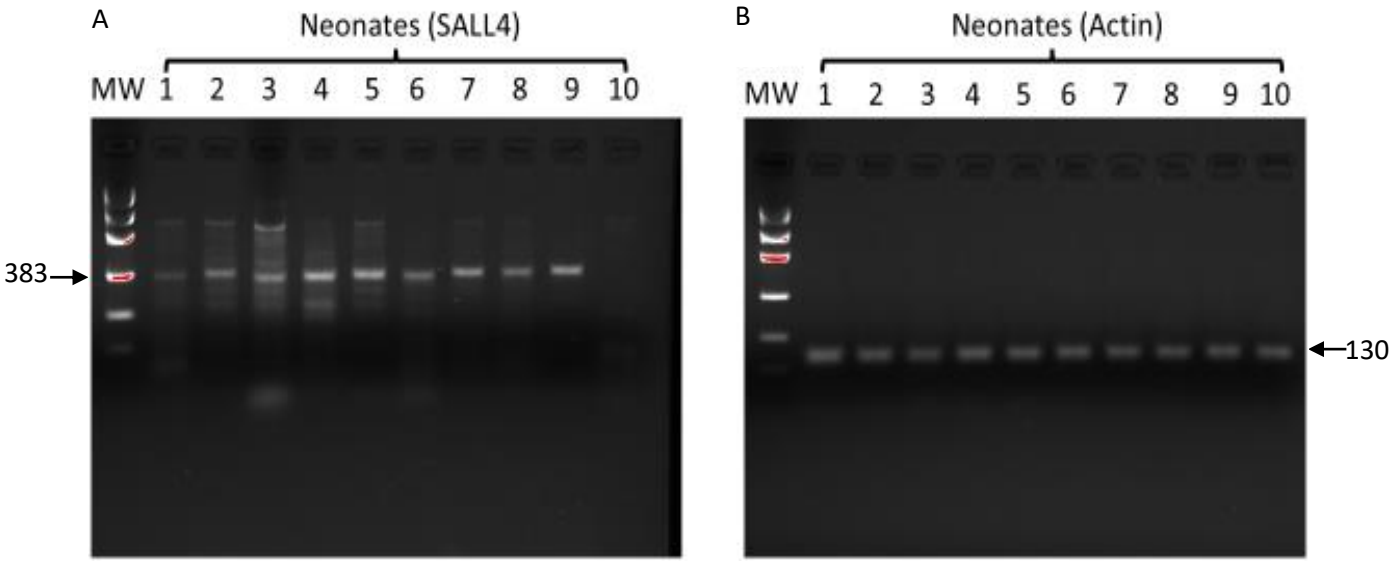


Figure 7: Gel Image of SALL4 (383 bp) expression in 10 Isl-1+ neonatal CPCs (A) and corresponding actin (130 bp) (B) as detected by RT-PCR.

SALL4 is Co-Expressed with SOX2, EpCAM and TBX5 in Neonate 1

We next used gene specific primers to assess expression of SOX2, EpCAM and TBX5 via RT-PCR. Neonate 1 expresses SOX2, EpCAM, and TBX5 by RNA-Seq based transcriptomics (Figure 8). To confirm transcriptomic data, neonate 1 was tested for expression of SOX2, EpCAM, and TBX5 by RT-PCR. Figure 9 confirms the expression of these transcripts in neonate 1.

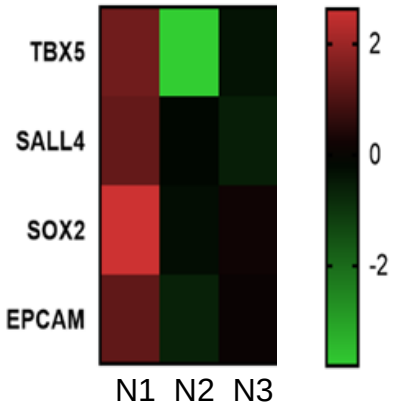


Figure 8: Heatmap visualization generated from Isl-1+ neonatal RNA-Seq data showing differentially expressed genes in neonate 1, neonate 2 and neonate 3 CPC clones. Red indicates a high gene expression level while green indicates a low gene expression level.

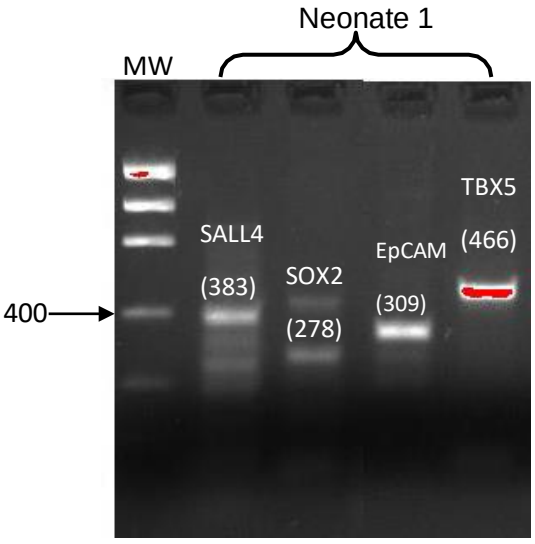


Figure 9: Gel image of RT-PCR products: SALL4, SOX2, EpCAM and TBX5 mRNAs are expressed in neonate 1 as detected by RT-PCR.

SALL4 Expressing Islet-1+ CPCs are Sub-Staged by TFAP2C

A gene specific primer for transcription factor TFAP2C was used to further define the developmental stage of the SALL4, SOX2, TBX5, and EpCAM expressing Isl-1+ CPCs. The gene expression profile of Neonate 1, obtained from the 8 day old patient, was consistent with that of an early mesendodermal stage cell. Therefore, we sought to determine whether or not all day 8 CPC clones have a gene expression profile similar to neonate 1. We compared three independent CPC clones identified from different 8 day old neonates and found that they all expressed transcripts encoding SALL4, SOX2, TBX5, and EpCAM . According to Churko et al., TFAP2C is expressed on day 2 of directed cardiomyocyte differentiation, with day 2 corresponding to early mesoderm. Additionally, TFAP2C has been shown to drive mesendoderm competence and shift the developmental trajectory toward the pluripotent state (Pastor et al., 2018). We therefore performed a PCR experiment designed to determine whether or not TFAP2C was expressed in any or all of these day 8 CPC clones. Figure 10 shows that neonate 1(A) expresses TFAP2C while neonate 1(B) and neonate 1(C) clonal samples do not, thereby confirming that the day 8 Isl-1+ neonatal CPCs are not all similar and can be sub-staged. Figure 11 shows a heatmap derived from RT-PCR confirming the gel image in figure 10. Additionally, TFAP2C expression confirms that neonate 1(A) represents the earliest cardiac progenitor cell that can be isolated from neonatal human patient samples.

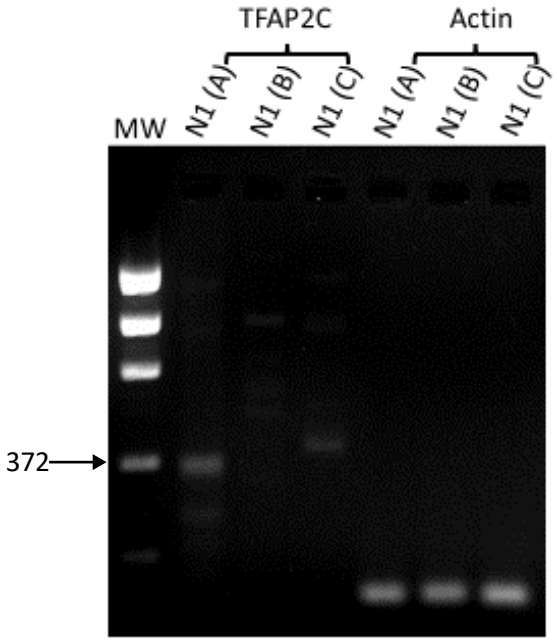


Figure 10: Gel image of TFAP2C (372 bp) mRNA expression in three 8-day old Isl-1+ neonatal CPC clones which were derived from different patients represented as neonate 1 (A), N (B) and N (C) and corresponding Actin (130 bp) expression as detected by RT-PCR. TFAP2C was detected in neonate 1 (A).

Figure 11: Heatmap visualization of RT-PCR analysis of TFAP2C mRNA levels in the 8-day old neonatal CPC clones represented as neonate 1 (A), N (B) and N (C). RT-PCR was performed on cDNA using gene specific primers. Relative quantification of each gene expression level was normalized according to actin gene expression. Red indicates a high gene expression level while green indicates a low gene expression level.

CHAPTER FOUR

DISCUSSION

In the present study, clonal populations of Isl-1+ neonatal CPCs isolated from the human heart were utilized to identify an early-stage CPC subpopulation. The results show that SALL4 identifies a subpopulation at the pre-mesendodermal stage of development. The identification of pre-mesendodermal cells in neonatal Isl-1+ cardiovascular tissue using transcriptomics further validates the neonate as a source of cells with enhanced stemness. Mesendodermal stage cells can differentiate toward both mesoderm and endoderm, rendering them precursors for numerous stem cell- based applications. The new information reported here identifies markers that allow the substaging of pre-mesendoderm/mesendoderm population of cardiovascular progenitors in cardiovascular tissue derived from human patient samples. This study identifies the earliest pre-mesendodermal cells that reside in, and can be isolated from, the neonatal heart.

Currently there is limited evidence identifying the functional effect stage specific cardiac progenitor cells on regeneration and/or the superiority of one cell type over the other. Previous studies demonstrate the effectiveness of using early-stage cells. For example, Blin et al., utilized SSEA-1+/Isl-1+ CPCs derived from Rhesus ESCs in an allograft model of myocardial infarction. This preclinical study revealed that upon transplantation of SSEA-1+/Isl-1+ progenitors scar tissue size was reduced, cells differentiated to ventricular

myocytes and no teratoma was formed (Blin et al., 2010). These cells are characterized as very early CPCs, segregating into the FHF and SHF. This population is derived from Rhesus ESCs and goes through the mesendodermal stage of development giving rise to the 3 main cardiac lineages of the heart under induced cardiac differentiation. The absence of inappropriate differentiation *in vivo* suggests that early-stage cells take cues from the cardiac environment to achieve lineage specific differentiation.

Lineage tracing studies in mice showed that development of the heart and hindlimb is regulated by Isl-1. Therefore, Isl-1+ may mark a subset of early-stage progenitors that differentiate along a common heart/hindlimb pathway of development (Yang et al., 2006). According to Akiyama et al., SALL4 plays a critical role at early steps of limb development (Akiyama et al., 2015). Specifically, inactivation of SALL4 in the mesendoderm before limb outgrowth causes defects of proximal-anterior skeletal elements specifically in the hindlimbs (Akiyama et al., 2015). While Isl-1+ has been elucidated in cardiac and hindlimb progenitor cells, SALL4 has yet to be examined in CPCs.

The mesendoderm stage has been established in models such as mouse and hPSCs. However, a homogenous niche of progenitors can segregate by various mechanisms to form subpopulations with different fates. Transcriptomics can give insight into the way we study cellular heterogeneity in vivo. Characterization of different cell populations forming an embryo allows one to make predictions about their ontogeny and showcase sets of genes that interact

with each other to control specific cell functions (Sendra et al., 2021). This is referred to as a gene regulatory network (GRN). An RNA-Seq analysis found that in mESCs, the MesEndoderm Transcriptional Enhancer Organizing Region (*meteor*) enhancer was found to be indispensable and hardwired in pluripotency and a key determinant of lineage specification during development (Alexanian et al., 2017). Alexaninan et al., sorted mesendodermal progenitors from differentiating ESCs according to Eomes expression and found that enhancer usage is coordinated with mesendoderm-specific expression of key lineage determining factors. Induction of the *meteor* transcription at a pluripotent stage is sufficient to promote mesendoderm specification and stimulate cardiogenic differentiation. Stavish et al., found that the exit from pluripotency involves intermediates that retain pluripotency while simultaneously exhibiting lineage-bias (Stavish et al., 2020). They found that a substate of human PSCs expressed the early endoderm marker GATA 6. GATA 6 positive cells were able to regenerate long-term pluripotent cultures and differentiate toward the endodermal lineage. This explains that substates that co-express pluripotency and differentiation markers represent differentiation intermediates, and these may exist as transient states during development. A study by Mononen et al., used trajectory mapping and RNA seq analysis to reveal branch points in hESC cardiac differentiation which resulted in a continuous pluripotent to cardiomyocyte differentiation trajectory (Mononen et al., 2020). This trajectory revealed that multiple lineages branch off of the pluripotent cluster of cells such as: cardiac mesoderm, endoderm-like cells, and fibroblast like cells. Interestingly, a global

transcriptional dynamics analysis across time showed that gene expression differed significantly between days 3 and 6 suggesting that up until day 6, cells rapidly progress through transient and distinct cellular states until day 7 (Mononen et al., 2020). Upon analysis of Mononen et al., published transcriptomic data: the pluripotent cluster, cardiac mesoderm cluster, and endoderm cluster all expressed SALL4. Taken together, these studies support the concept that the expression of markers of mesendoderm as well as pluripotency represents an intermediate cell stage that is very early along that cardiovascular path.

Using transcriptomic analysis, we found that select Isl-1+ neonatal CPCs expressed cardiac lineage and pluripotency markers. Additionally, transcriptomics revealed that human neonatal Isl-1+ CPCs have a gene expression profile similar to iPSCs. Alternatively, adult Isl-1+ CPCs expressed a gene profile similar to that of MSCs. The adult Isl-1+ CPCs did not express stemness genes indicating that neonates have enhanced stemness properties. This demonstrates that neonatal Isl-1+ CPCs derived from human heart tissue are at a very early stage along the path to becoming cardiovascular cells. Transcriptomic analysis showed that Isl-1+ neonatal CPC clones have genes involved in the transcriptional regulation of pluripotency pathway which includes transcription factor SALL4 (Figure 4). This pathway activates expression of other pluripotency associated factors while repressing lineage specific genes and activating their own gene expression (Yeo & Ng, 2013). This model accounts for how cells can sustain self-renewal and pluripotency while remaining poised for

differentiation. While SALL4 was found to be prevalent in the Isl-1+ CPC clones tested, transcriptomic analysis of Isl-1+ neonatal CPCs using IPA revealed that SALL4 interacts with TBX5, EpCAM and SOX2.

SALL4 co-expressing Isl-1+ neonatal CPCs expressed transcripts which are involved early in embryonic development such as TBX5, SOX2, and EpCAM. A subpopulation co-expressing these genes suggests that this subpopulation may reside in a stage of development earlier than the mesendoderm. Using a microarray analysis, Tanimura et al., found that SALL4 and SOX2 bind to the promoter region of the same genes in ESCs. This suggests that the SALL4 and SOX2 overlapping gene set is enriched for genes involved in maintaining pluripotency (Tanimura et al., 2013). Next, SALL4 plays a role in various tissues and organs during embryonic development. Disruption of SALL4 in ESCs results in early embryo defects and lethality during peri-implantation (Yang et al., 2018). Additionally, heterozygous disruption of the SALL4 allele leads to multi-organ malformations including limb and heart defects (Pantier et al., 2021). Specifically, TBX5 regulates SALL4 expression in the developing heart and forelimb and interacts with SALL4 to synergistically regulate downstream gene expression. According to Abboud et al., mesodermal and cardiac gene expression in OCT4-induced human mesendodermal, mesodermal and neighboring cells was monitored by lineage tracing analysis in the presence or absence of SALL4 (Abboud et al., 2015). Results showed that expression of genes that play a role in cardiac development such as, MESP1, MYOCD, GATA4, TBX5, Isl-1 and Mef2c were induced to a lesser extent in the absence of SALL4. This suggests

that SALL4/TBX5/ SOX2/EpCAM co-expressing Isl-1+ cardiac progenitors reside early in embryonic development. Given that SALL4, SOX2, EpCAM, and TBX5 expression was found to be prevalent in the Isl-1+ neonatal CPC clones, TFAP2C was identified to further sub-stage these cells. Transcriptomics data published by Churko et al., demonstrated that TFAP2C is expressed transiently at day 2 of directed cardiomyocyte differentiation from hiPSCs. Additionally, Valcourt et al., revealed that overexpression of TFAP2C hindered movement along the hESC developmental trajectory, shifting cells toward the pluripotent state while remaining poised for mesendoderm specification (Valcourt et al., 2021).

The present study sought to identify a subpopulation of Isl-1+ neonatal CPCs. Our findings demonstrate that SALL4 is co-expressed in select Isl-1+ CPC clones. This pre-mesendodermal CPC is associated with the early stages of embryonic development and preserves characteristics of an intermediate cell state between pluripotency and mesendoderm. Overall, SALL4 functions to maintain stemness, plays a role in cell expansion and early embryonic and cardiac development. Risks of stem cell transplantation includes immune rejection and teratoma. A study by Cao et al., utilized multilineage differentiating stress enduring cells (MUSE Cells) for MI (Cao et al., 2020). These cells express pluripotency and linage biased markers and do not reject after transplantation. The MUSE cells express HLA-G which has been shown to promote graft tolerance in heart transplantation which protects MUSE cells from immunologic attack (Young, 2018). Furthermore, while iPSCs have been proven to be

beneficial for cardiac repair, they possess some degree of variation between cell lines and typically match the genomic profile of the donor. Studies have shown that stem cell derived cardiomyocytes have immature calcium handling (Satin et al., 2008) and lack maturity or metabolic characteristics of mature CM found in the healthy heart (Veerman et al., 2015). However, a study by Maxwell et al., revealed that application of mechanical and electrical stimuli to early-stage cells can induce morphological and functional maturation of hiPSC-CMs to adult like human cardiac tissue. Early-stage intensity trained cardiac tissue increased expression of genes associated with adult-like conduction, maturation, energetics, and calcium handling. Maxwell et al., shows that intensity training may allow for the achievement of a more advanced level of maturation in vivo (Maxwell & Xu, 2018). Another feature of early-stage cells is their cellular plasticity which has been reported *in vivo* after primitive heart tube formation (Meilhac & Buckingham, 2018). This reveals that the differentiation of CPCs is not fixed and can change upon positional cues or intrinsic signaling. To date, not a single cell type has been approved by the FDA for treatment of any form of heart disease. The results reported here provide new insight into the various stages of neonatal Isl-1+ CPCs which can be isolated from human tissue. The ability to isolate and expand these early-stage cells for experimental and clinical applications may prove to be valuable as the search for an optimal cell source for cardiac regenerative therapy continues.

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