Differentiation of human atrial myocytes from endothelial progenitor cell-derived induced pluripotent stem cells

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List of Abbreviations

AP	Action potential
AMHC1	Atrial specific myosin heavy chain 1
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenic protein-4
CD31	Platelet endothelial cell adhesion molecule
CM	Cardiomyocyte
CMV	Cytomegalovirus
CNV	Copy number variations
cTNT	Cardiac Troponin T
Cx40	Connexin 40
DKK-1	Dickkof homolog1
Dox	Doxycycline
EB	Embryoid Body
EC	Embryonic carcinoma
E-cad	Epithelial Cadherin
ECM	Extra cellular matrix
E-EPC	Early-endothelial progenitor cells
EF1α	Elongation factor-1 alpha
EMT	Epithelial Mesenchymal Transition
END2	Visceral endoderm like cells
EOS	Early Transposon Promoters
ESC	Embryonic Stem Cells
FGF2	Fibroblast growth factor
FZD	Frizzled
GSK3	Glycogen synthase kinase 3
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
L-EPCs	Late endothelial progenitor cells
LTR	Long terminal repeat

MET	Mesenchymal-to-Epithelial Transition
MMLV	Molony murine leukemia virus
PG12	Prostaglandin 12
PI3K	Phosphatidylinositol 3-kinase
PSCs	Human pluripotent stem cells
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenases
rtTA	Reverse tetracycline activator
SCNT	Somatic cell nuclear transfer
SHH	Sonic Hedgehog
SR	Sarcoplasmic reticulum
Tet	Tetracycline
TetO	Tetracycline resistance operon
TGFβ	Transforming growth factor β
VPA	Valporic Acid

Abstract

Recent advances in cellular reprogramming have enabled the generation of embryonic-like cells from virtually any cell of the body. These inducible pluripotent stem cells (iPSCs) are capable of indefinite self-renewal while maintaining the ability to differentiate into all cell types. Nowhere will this technology have a greater impact than in the ability to generate disease and patient-specific cell lines. Here we explore the capacity of human iPSCs reprogrammed from peripheral blood endothelial progenitor cells lines to differentiate into atrial myocytes for the study of patient specific atrial physiology.

Methods and Results: Late outgrowth endothelial progenitor cells (EPCs) cultured from clinical blood samples provided a robust cell source for genetic reprogramming. Transcriptome analysis hinted that EPCs would be comparatively more amenable to pluripotent reprogramming than the traditional dermal fibroblast. After 6 passages, EPCs were transduced with a doxycycline inducible lentivirus system encoding human transcription factors OCT4, SOX2, KLF4 and Nanog to permit differentiation after removal of doxycycline. The high endogenous expression of key pluripotency transcripts enhanced the ease of iPSC generation as demonstrated by the rapid emergence of typical iPSC colonies. Following removal of doxycycline, genetically reprogrammed EPC-iPSC colonies displayed phenotypic characteristics identical to human embryonic stem cells and expressed high levels of the pluripotent markers SSEA-4, TRA1-60 and TRA1-81. After exposure to conditions known to favor atrial identity, EPC- iPSC differentiating into sheets of beating cardiomyocytes that expressed high levels of several atrial-specific expressed genes (CACNA1H, KCNA5, and MYL4).

Conclusions: EPCs provide a stable platform for genetic reprogramming into a pluripotent state using a doxycycline conditional expression system that avoids re-expression of oncogenic/pluripotent factors. Human EPC-derived iPSC can be differentiated into functional cardiomyocytes that express characteristic markers of atrial identity.

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1. Introduction

Recent advances in cellular reprogramming have now made it possible to generate embryonic-like cells from virtually any cell of the body. These iPSCs are capable of indefinite self-renewal while maintaining the ability to differentiate into all cell types. Nowhere will this technology have a greater impact than in the ability to generate disease and patient-specific cell lines. Here we explore the capacity of human iPSCs reprogrammed from peripheral blood endothelial progenitor cells lines to differentiate into atrial myocytes for the study of patient specific atrial physiology. This report will systematically explore techniques to generate pluripotent stem cells from somatic cells before focusing upon gene based reprogramming and the underlying fundamental mechanisms.

2. Why use pluripotent stem cells as a model of human disease?

The ultimate focus of this project is to provide *proof of concept* that streamlines and personalizes the development of *in vitro* models of human disease causing mutations [1]. This concept originates from the work of Evans, Kaufman and Martin who showed that genetically modified mouse embryonic stem cells (ESCs) could be used to generate transgenic mouse models of human disease [2,3]. While these models have significantly contributed to our understanding of multiple cardiac diseases, they cannot fully replicate the patient phenotype due to the basic physiological differences between mice and men [4-6]. For example, the *SCN1b* knockdown mouse that exhibits a loss of the beta sub-unit of the cardiac sodium channel (SCN5A) shows prolonged ventricular repolarization, a feature not seen in patients with SCN1B loss-of-function [7]. Similarly, for the same given mutation, variation in the genetic background of the transgenic mouse profoundly

affects phenotype. When examining the effect of the SCN5a-1798insD mutation in two strains of mice, Remme and colleagues noted that 129P2 mice have a more severe phenotype than FVB/N mice carrying the same mutation [8]. This observation echoes clinical findings that disease expression varies markedly even within the same family [9-11]. Finally, fundamental differences in the basic physiology of humans and rodents, such as heart rate, often overwhelm and mask the pro-arrhythmic consequences of channelopathies.

Accordingly, attention has shifted towards more appropriate methods to better model human diseases. Ethical, societal and technical issues have significantly impaired the genetic manipulation of human ESCs as models of disease [12]. iPSC technology promises to surpass these limitations as somatic gene transfer has generated several disease specific cell lines to date [13-15] As outlined below, this technology is still not well understood and this project will form a critical link towards the safe an effective implementation by exploring the capacity of human iPSCs to differentiate into atrial myocytes for the study of patient specific atrial physiology.

3. The discovery of induced pluripotent stem cells

During development, cells become increasingly more specialized and restricted in their developmental potential. Truly pluripotent stem cells, such as ESCs, are clonogenic, self-replicative and capable of differentiating *in vitro* and after delivery into animal models. The field of embryonic stem cells began in the early 1980's with establishment of pluripotent stem cell or embryonic carcinoma (EC) lines that expresses similar morphology and differentiating potential to the inner cell mass (ICM) of the early embryo [2,16]. ESCs were isolated from the ICM of human blastocysts by in vitro fertilization

(IVF) [17]. The potential use of ESCs for research is limited by ethical concerns about the destruction of the embryo during isolating the cells and the possibility of these cells to produce teratomas when injected to immunodeficient mice [16,18,19]. Although ESCs are regarded as a 'gold standard' for pluripotency, there is a need for alternate sources of pluripotent cells which can be easily accessible and possess the same developmental potential as ESC.

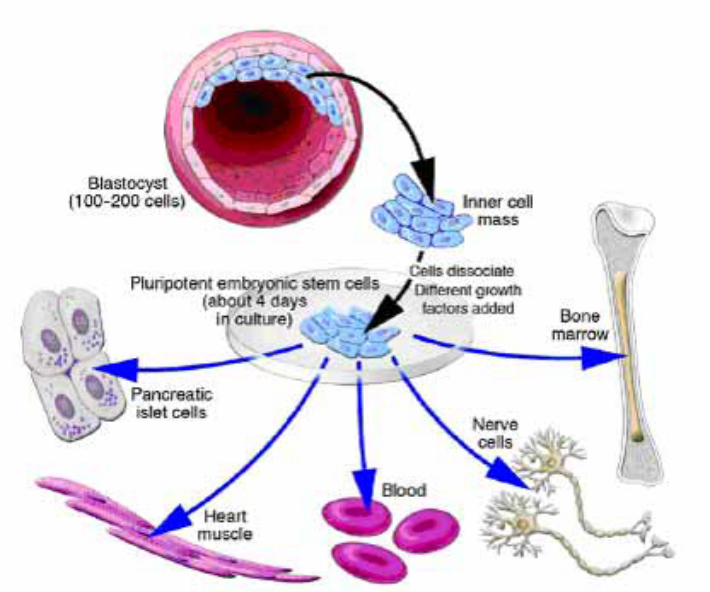

Figure 1. Pluripotent stem cells, isolated from the ICM in the blastocyst, have the ability to differentiate to all types of cells in the human body [20].

3.1 Genetic reprogramming by somatic cell nuclear transfer

Somatic cell nuclear transfer (SCNT) represented the first attempts to establish a truly pluripotent stem cell source. This technique is occurs when the nucleus from differentiated somatic cell is transferred into an enucleated oocyte leading to development of a live offspring, which contains an identical gene to the original somatic cell. Briggs and King first established the technique of SCNT, also known as "reproductive cloning" to show that the transfer of nuclei from embryo (blastula) frog

cells into enucleated oocyte developed normal swimming tadpoles [21]. Together with seminal experiments by Gurdon indicated that differentiated amphibian cells retain all of the genes necessary to support the generation of cloned organism, these transcriptional genes are present in the nucleus of the specialized cell and can be activated on exposure to intrinsic chemical environment present in the oocyte [22,23].

Mirroring this phenomenon in mammal, Wilmut and colleagues were the first to clone a normal adult sheep (Dolly) by transplanting mammary gland cell nuclei derived from an adult sheep to enucleated sheep eggs [24]. Subsequently, somatic cloning was successfully performed on other mammals including cats, cows, dogs, goats, mice, pigs and rabbits [25-34].

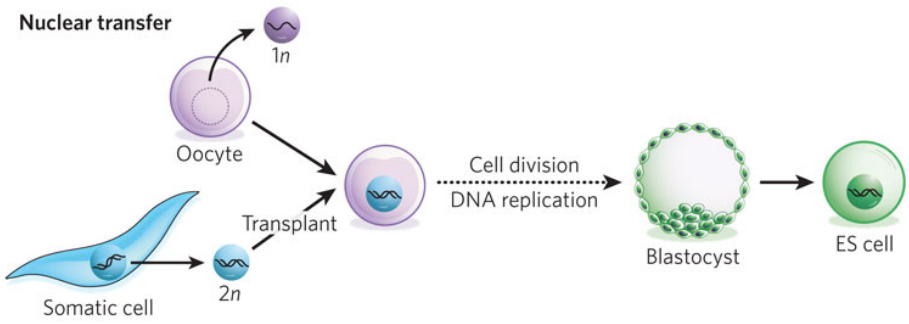

Figure 2: Schematic representation of the technique of somatic cell nuclear transfer: the nucleus of a somatic cell is transferred into an enucleated oocyte that reprogrammed to generate ESCs like. [35].

These landmark studies demonstrated that reversible (rather than irreversible) genomic alternations occur during normal development. Unfortunately, animals cloned using SCNT exhibit abnormal gene expression, telomere shortening, susceptibility to cancer and premature death; suggesting that SCNT results in faulty genomic

reprogramming and rationalizing studies to explore alternative techniques to genetically reprogram committed somatic cells [36-42].

3.2 Genetic reprogramming by cell fusion

Cell fusion between two different cell types was established by Harris and Waltkins [43]. As depicted in Figure 3, cell to cell fusion between pluripotent cells (i.e., teratocarcinoma) and somatic differentiation cells (i.e., thymocyte) generated hybrid cells referred to as a heterokayron which contains multiple genetically different nuclei. These hybrid cells morphologically resembled EC cells and represent pluripotent characteristics including down-regulation of differentiated genes, reactivation of pluripotency genes, differentiation potential to all three germ layers [44]. Several studies have since shown that fusion between ESC or embryonic germ cells and somatic cell sources (i.e., thymocyte) impart the characteristics of pluripotentcy [45-47]

Most importantly these cell fusion studies revealed the mechanism for somatic cell plasticity. This technique was noted to change the phenotype and the potency of somatic cells with hybrid cells expressing pluripotent related genes, which could differentiate into three germ layers [48,49]. In this study, fusion between the cytoplasms of ESCs and somatic cells did not demonstrate activation of pluripotency genes. In contrast, fusion between the nucleus of ESCs with somatic cells demonstrated the reactivation pluripotency genes within somatic cell [50,51]; suggesting that the cellular contents of ESCs nucleus contained transcriptional factors (e.g., Nanog and Oct4) which reverted the nucleus of somatic cells to a pluripotent state [52,54].

Although capable of genetically engineering pluripotent cells, these studies were limited by low efficiencies and marked difficulty eliminating residual ESC chromosomal

components, which results in genomic instability and the generation of abnormal tetraploid fusion cells.

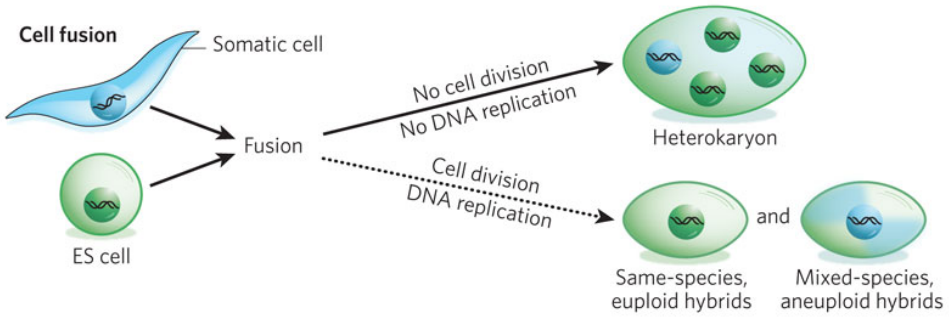

Figure 3. Schematic representation of reprogramming by cells fusion hybrids [35]. Two different cell types are fused to form a single cell that can be heterokaryons or hybrids cells. Heterokaryon cells are multinucleate and not dividing cells. In contrast, hybride cells form euploid hybrids if the fusion cells are derived from the same species, and form aneuploid hybrids if the fusion cells are derived from different species.

3.3 Genetic reprogramming by transcription-factor expression

The successful generation of pluripotent cells from somatic cells using ESCs fusion demonstrated that ESCs possess the key transcription factors that induce genetic reprogramming. These reprogramming factors also play critical roles in the maintenance of pluripotency. Based on these studies, Takahashi and Yamanaka devised a comprehensive screen for 24 transcriptional candidate genes related to pluripotency [55]. Some of these genes were thought to represent ESC specific genes (i.e., Oct4) while others were implicated solely in the long-term maintenance of ESC phenotype (i.e., Nanog) or rapid proliferation in culture (i.e., C-Myc). Retroviral–mediated transduction of all 24-transcription factors into mouse fibroblasts successfully induced the formation

of ESC-like colonies and was named "induced pluripotent stem cells" or iPSCs. High throughput selection of reprogrammed fibroblasts was attained through gene-based reactivation of the ESC specific gene Fbx15 driving neomycin resistance. Successive trials eliminated transcription factors to identify a core set of 4 pluripotency genes (Oct4, Sox2, Klf4 and C-Myc; abbreviated as OSKM) that drove pluripotent remodeling. These first generation iPSCs were similar to ESCs in that they expressed pluripotency markers (such as SSEA-1, Nanog, E-Ras, Dax1), generated teratomas when injected into nude mice and differentiated into all three germ layers. However genetic reprogramming was not complete as OSKM-driven reprogramming demonstrated differential pluripotent gene expression, incomplete DNA demethylation (when compared with ESCs) and the inability to produce live chimeras (viable only until day E13.5). Taken together, this landmark paper attained partial reprogramming and indicated the possibility of gene-based pluripotent remodeling [56-62].

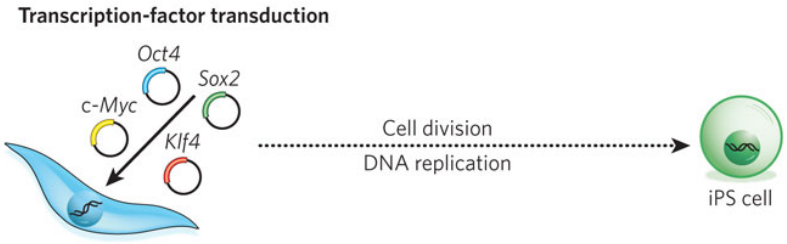

Figure 4. Schematic representation of reprogramming by transcription factor transduction:[35]. iPSCs can be generated from somatic cell through the introduction of four genes (Oct4, Sox2, Klf4 and c-Myc) by using retroviruses. iPSC have similar characteristics to ESC.

In a similar paper published weeks later, Yu and Thomson selected 14 other candidate-reprogramming factors on the basis of enriched expression in human ESCs and the involvement in the establishment/maintenance of pluripotency [63]. Combinations of these genes were cloned into fibroblasts using lentiviral vector and iPSCs colonies were selected based on Oct4-induced neomycin-resistance. In a manner similar to the original Takahashi and Yamanaka paper, selective depletion of transduced genes identified a core set of 4 genes (Oct4, Sox2, Nanog and Lin28; abbreviated as OSNL) that were sufficient to reprogram somatic cells into iPSCs.

Like ESCs, iPSCs are capable of differentiating into any cell type of the human body and provide a suitable autologous source of personalized somatic cells. This innovation opens the possibility for personalized drug therapy, genetic correction and cell transplantation [17]. Additionally, this technology avoids many of the ethical, destruction of fertilized eggs and immune-mediated concerns inherent in the use of human ESCs [18].

4. Molecular mechanisms underlying induction of pluripotency

Reprogramming somatic cells to a pluripotent fate is a stepwise process. Imaging analysis has shown that the first response after introduction of exogenous factors into somatic cells is an increase in cell division rate and a decrease in cell size [64]. Thus, crosstalk between ectopic gene expression and molecular control of cell morphology plays a critical role in cell fate determination and attaining a pluripotent state. These changes are mirrored by strong expression of cell cycle genes (such as Cyclins, ccnd1 and ccnd2) within reprogrammed cells [65]. Flattened elongated fibroblasts then morphologically change to produce small tightly packed clusters with remodeled cell to cell matrix interactions that represent a gain of epithelial cells features (or reprogramming

induced mesenchymal-to-epithelial transition; MET)) [66-69].

These remodeled iPSCs are further characterized by tight intercellular contacts and the expression of epithelial genes (such as Epithelial Cadherin (E-cad)). E-cad is a transmembrane protein located at adherent junctions that plays an essential role in cell–cell contact formation [70]. In early mammalian development, E-cad is involved in embryonic blastocyst integrity and intracellular contact signaling essential in embryo formation [71,73]. A time series microarray study of reprogrammed fibroblasts confirms initiation of MET with early down-regulation of mesenchymal genes (i.e., Snail, Twist, Zeb1) and up-regulation of epithelial markers (i.e., Cdh1, Epcam [73]). Interestingly, Wang et al has shown that partially reprogrammed iPSCs (i.e., not successfully transduced with all 4 reprogramming factors) demonstrated incomplete MET with a phenotype that demonstrated partial expression of both fibroblast and iPSCs characteristics [74]. Thus initiation of MET is a crucial early process during cell reprogramming as the morphological changes and expression of epithelial genes occurs before complete repression of pluripotency genes [66,68].

Interestingly, recent studies have also demonstrated that adult epithelial cells (such as keratinocyte, hepatocyte) or organ-based progenitor cells do not need to pass through MET while being reprogrammed into iPSCs [75,76]. This may be partially related to incomplete expression of epithelial characteristics or low-level expression of critical pluripotency genes. Only a fraction of transduced cells express all 4 requisite ectopic transcription factors (i.e., OSKM or OSNL) to undergo genetic reprogramming to an iPSC fate. The remaining (partially transduced) cells undergo apoptosis or cell cycle arrest/senescence through accumulation of DNA damage and oxidative stress [77,78].

The latter process is thought to be a barrier to reprogramming as several studies have demonstrated that repressing apoptosis or cell cycle arrest results in higher reprogramming efficiency [79-83]. For example, the p53 pathway is activated by DNA damage and induces senescence and apoptosis through the Bax/Bc12 pathway [84]. Knockdown of the p53 pathway or a main downstream target of the p53 pathway (i.e., p21) enhances the efficiency of iPSCs generation [78, 80,83-86]. The factor most likely responsible for success or failure to genetically reprogram somatic cells to an iPSC fate is likely c-Myc as it induces the production of oxidative stress radical oxygen species, which results in marked DNA damage. Thus, culturing the cells in low oxygen culture condition [87] or in vitamin C supplement [88] improves iPSCs generating by inhibiting the up-regulation of p53 [84].

Epigenetic regulators also play a crucial role in the control of gene expression by methylation of promoters within DNA and posttranslational modifications of histone proteins within chromatin [89,90]. DNA methylation takes place at cytosine of CpG dinucleotides that is modified by DNA methyltransferases, which maintains methylation and enhance *de novo* methylation [91-93]. Pluripotent stem cells highly express demethylation marks in transcription gene Oct4 and Nanog whereas the differentiation cells suppress these genes via DNA methylation. During reprogramming, a number of pluripotency genes are demethylated by an enzyme known as demethylase or by substitution of methylated with un-methylated cytosine. Thus, removing methylation during reprogramming enables the reactivation of the endogenous pluripotency genes [94,95]. Accordingly, several studies have indicated that small molecules including

RG108 and 5-azacytidine enhance reprogramming efficiency through inhibition of DNA methyltransferase [96,97].

The histone ocatemer is subjected to genome-activity modulating modifications that include histone acetylation and methylation via N-terminal tails of histones. Histone acetylation H3K4Ac and histone methylation H3K4me3 represent a transcriptionally active mark, while H3K27me3 and H3K9me2/3 are transcriptionally less active [98]. In pluripotent states, the "bivalent domain" of H3K4me3 actives transcription while high expression of H3K27me3 represses transcription the silence genes responsible for differentiation and activate those responsible for pluripotency [65, 99-101]. Polycomb group proteins also play an important role in maintaining of the pluripotent state and have been shown to suppress genes that bind to histones containing H3K27me3 [102-104]. Thus, pluripotent cell chromatin is transcriptionally more permissive while differentiation is accompanied by a transition to chromatin that is transcriptionally less active. It follows that, several studies have shown that histone deacetylase and demethylase inhibitors (such as Valporic Acid (VPA) and vitamin C) promote efficient iPSC reprogramming and can even be used to substitute for transduction of the c-Myc gene [88,97, 105,106].

The dynamic regulation of gene expression is directed by specific transcription factors. A transcription factor is a DNA binding protein that acts as an activator or repressor at gene regulatory elements to modulate gene expression [107-109]. Re-establishing pluripotency in a somatic cell is initiated by re-activation of an ESC-specific transcription network. Genome wide analysis has shown that Oct4, Sox2 and Nanog bind to their own promoters and the promoters of the other genes; thus forming an auto-regulatory circuitry [100,101]. This auto-regulatory loop is the master regulator of cell

states and serves to activate their own genes while enhancing the stability of gene expression to maintain the pluripotent state [110,111].

Oct4, Sox2 and Nanog co-occupy common genomic targets promoters of transcriptionally active and inactive genes, which serve as the core transcriptional network. These core transcriptions factors maintain the pluripotency by promoting the cascade of pluripotency genes (such as Sall4, Dax1, Essrb, Tbx3, Tcl1, Rif1, Nac1 and Zfp281) while simultaneously suppressing the activity of developmental genes (such as Gata6, Myf5 and Hoxb1) and the recruitment of epigenetic regulators complexes (such as Polycomb) to the promoters of target genes [99,103,104,112-114]. Pereira et al demonstrated the role of Polycomb group proteins in epigenetic remodeling by studying the role in fusion between ESCs and lymphocytes and demonstrated that ESCs failed to remodel somatic cell genome in deficient Polycomb group proteins [115].

Mechanically, iPSC reprogramming is initiated by the overexpression of the combination viruses encoding OSKM (or OSNL) that integrate into the somatic cell genomes. Once these factors enter the cytosol, viral promoters induce transgene expression to form all four proteins that activate accessible gene promotes within the nucleus. These processes enable the epigenetic machinery to remodel chromatin through histone and DNA methylation. In essence, pluripotency genes are re-activated by transcription factors and maintain expression through chromatin remodeling. Conversely, differentiation genes are repressed by epigenetic modulators and kept silent through reprogramming. The late stage of the iPSC reprogramming involves silencing of the exogenous genomes carrying the transcription initiators. As such, iPSCs function indistinguishably from ESCs derived from blastocysts.

In conclusion, these studies indicated that overcoming of senescence and apoptosis by reprogramming factors is essential to initiate reprogramming. While the maintenance of iPSC proliferation without increasing the risks of tumor formation, and epigenetic rearrangements while establishing a self-sustaining transcription factor network are required for successful reprogramming to a pluripotent state. This study will leverage these findings by applying VPA prior to genetic reprogramming to make the somatic cell target chromatin is transcriptionally more permissive in an effort to enhance the efficiency of cellular reprogramming.

5. Cytokine signaling pathways regulating pluripotency

The stimulation of signaling pathways plays an essential role throughout embryonic development and markedly influence stem cell fate. Moreover, signaling pathways have been closely involved in regulating pluripotency [116-119]. Recently, several studies have shown that signaling pathways also play an essential part in the improvement of the reprogramming efficiency.

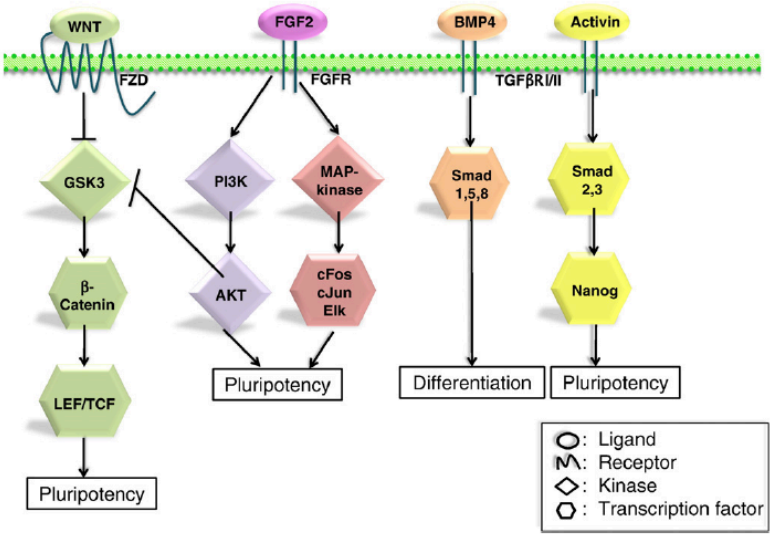

Figure 5. Signaling pathway of pluripotency cells [119].

Fibroblast growth factor-2 (FGF-2) has been demonstrated to maintain the pluripotent cell state in the presence of the feeder cells or matrix complex (such as Matrigel) [17,120]. Indeed, ESCs which themselves produce FGF-2 activate the MAPK-ERK pathway via the receptor tyrosine kinase FGFR1 that stimulates downstream expression of MAPK genes (such as Fos, Jun and Elk) that help maintain a pluripotent state [120,122]. In contrast, inhibition of MAPK-ERK signaling induces differentiation [121,123]. FGF-2 has shown to maintain the pluripotent stem cell state through activation of phosphatidylinositol 3-kinase (PI3K) signaling pathway. PI3K maintains pluripotent cells by stimulating a downstream effector of AKT (such as GSK3 and mTOR). Inhibition of PI3K results in significant downregulation of pluripotent genes (such as Oct3/4, Nanog, and Sox2) [124,125].

Nodal and Activin also help to regulate early embryonic development and suppress the differentiation of human ESCs [126-128]. These cytokines activate the transcription factors smad2 and smad3 that bind to stimulate the Nanog promoter and help maintain the pluripotency phenotype of human ESCs [129, 130]. Blockade of the Smad2 and Smad3 signaling by bone morphogenic protein-4 (BMP4) showed rapidly down-regulates Nanog expression and drives the differentiation of ESCs [130]. Human ESC culture with Activin alone also induces the expression of FGF-2 in serum free medium [131]. Collectively, activation of MAPK and PI3K by FGF-2 along with activation of Smad2 and Smad3 by Activin regulates the pluripotency and self-renewal of ESCs to prevent spontaneous differentiation while also suppressing BMP4 expression [132,133].

Finally, canonical Wnt signaling pathway through Wnt glycoproteins and their receptor frizzled (FZD) play contradictory roles in the fate of ESC. The binding of Wnt

ligand to a FZD receptor inhibits the activity of glycogen synthase kinase 3 (GSK3) through phosphorylation of the protein Disheveled to decrease the activity of the destruction complex and results in accumulation of β-catenin within the cell. Thus, stable β-catenin translocates into the nucleus and activates the transcription of target genes through Tcf DNA-binding factor [134]. Specifically, the receptor FZD7 plays a critical role in human ESC pluripotency as FZD7 knockdown decreased the expression of pluripotent genes including Oct4 and Nanog and promoted the differentiation of human ESCs [135]. Direct post receptor pathway stimulation also plays an important role in mediating Wnt effects as shown by studies were ESCs were treating with the synthetic inhibitor 6-bromoindirubin-3'- oxime to directly inhibit GSK3 and promote the maintenance of a pluripotent phenotype [136].

However, Dravid et al., proposed a different the role of Wnt signaling in pluripotency of ESCs [137]. This report indicated that in the absence of other pluripotent stimulation, application of Wnt ligands to ESCs promotes the differentiation of ESCs to dictate stem cell fate and expansion; thus strongly suggesting that other signaling pathways are also required to maintain the pluripotent state of human ESCs. Recent work has suggested that FGF-2 mediated PI3K signaling may play an important role in potentiating the effects of Wnt stimulation by synergistically phosphorylating GSK3a to help Wnt maintain the pluripotency of ESC [138-140].

These studies demonstrated that extracellular cues (i.e., transforming growth factor b (TGFb) and Wnt) have a crucial impact on the reprogramming of somatic cells and maintenance of a pluripotent state. Manipulating both extrinsic and intrinsic signaling to control the initiation and maintenance of a pluripotent state is needed to efficiently

reprogram somatic cells to a pluripotent phenotype.

6. Technical considerations underlying induction of pluripotency

6.1 Origin of somatic cells

The efficiency of iPSCs generation and methods used to deliver reprogramming factors are dependent on the cell types being genetically reprogrammed. Cell sources typically include: dermal fibroblasts, amniotic fluid-derived cells, skin keratinocytes, ESC-derived fibroblasts, CD34 blood cells, mesenchymal stem cells, adipose stem cells, umbilical cord blood cells, dental stem cells, and oral mucosa fibroblasts [55,76,141-147].

The most common cell type used for iPSCs generation is skin derived dermal fibroblasts. However, these cell required surgical excision and reprogram with a low efficiency [55]. Furthermore, recent studies have shown that the dermal fibroblast reprogramming using retroviral methods expressed a detrimental high level of copy number variations (CNV; a DNA segment that presents at a variable copy number in comparison with a reference genome) [148,149]. Therefore, alternative cells lines to generate iPSCs are required to minimize the risk for additional mutation, less invasive procedures and greater amenability to genetic reprogramming.

A peripheral blood is an attractive alternative starting cell to generate iPSCs because it is easily accessible, minimally invasive and has less exposure to environmental mutagens/toxins [141]. Several studies have successfully reprogramed blood cells such as T cells and B cells to iPSCs but genetic reprogramming is limited by a low capacity of these cells to expand in culture, modest reprogramming efficiency and the presence of DNA rearrangements in their receptor loci by generation of antigen specific surface immunoglobulins [150-152]. In addition, one study paradoxically demonstrated that B

cell derived iPSCs failed to differentiate into B cells [151]. A finding that was attributed to generation of a defective in B cell gene (i.e., Pax5 gene) during iPSC reprogramming that is required during B cell differentiation. This latter study stresses the importance of choosing a starting cell type that lacks DNA rearrangements to avoid later problems differentiating iPSC to a target phenotype.

It makes teleological sense that cell sources naturally expressing endogenously low levels of the key reprogramming factors (OSKM or OSNL) may require less genetic manipulation to become iPSCs. Neural stem cells have a high expression level of endogenous pluripotent genes sox2 and c-Myc. These cells can be reprogrammed to iPSCs with only small number of reprogramming factors (i.e., Oct4 and Klf4) [153]. Investigators have also reported successful reprogramming of primary CD34+ hematopoietic progenitor cells to iPSCs [144,154]. However, the low amount of CD34+ cells in peripheral blood (estimated less than 0.1%) is a key factor limiting the availability of these cells for reprogramming. To further this approach towards clinical application, Loh and colleagues treated patients with granulocyte-colony stimulating factor, a process that can only be applied if the donor is in good medical condition, to expand the number of CD34+ cells in circulating peripheral blood and generated iPSCs from these cells [154].

Culture guided endothelial progenitor cells (EPCs) represent a further refinement of this approach. In 1997, Asahara et al. reported that EPCs could be isolated from human peripheral blood and expressed endothelial surface markers (such as CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2)) [155]. In this method, mononuclear cells of either adult peripheral blood or human umbilical cord blood were cultured on

fibronectin-coated dishes. Two days later, non-adherent cells were re-plated on fibronectin-coated dishes until colonies appeared which contain round cells with spindle-shaped cells in 5 – 9 days. These colonies referred to as colony-forming unit-ECs (CFU-ECs) or early endothelial growth (early EPC). This report indicated that a heterogeneous mononuclear cell population, which enriched for the stem cell marker CD34 or vascular endothelial growth factor receptor-2 (VEGFR-2) expression could proliferate to produce early EPCs. A similar approach was later developed in which whole unfractioned mononuclear cells were cultured in defined media for 4 days. After removal of the non-adherent cells, the adherent cell population displayed similar features suggestive of an endothelial phenotype and function [156]. These cells have since been shown to bind endothelial-specific markers such as lectin Ulex Europeus Agglutinin-1 (UEA-1) and uptake of endothelial-specific ligands such as acetylated low-density liproprotein (acLDL) [157]. Further these culture guided cells express surface markers such as von Willebrand factor (vWF), platelet-endothelial cell adhesion molecule (PECAM-1 or CD31), VEGFR-2, vascular endothelial cadherin (VE-cadherin or CD144), Tie-2/TEK (angiopoietin-1 receptor precursor or tunica intima endothelial cell kinase) [158]. Late endothelial progenitor cells (L-EPCs), referred to as the 'endothelial colony-forming cells' (ECFCs), can be isolated using the same method as E-EPCs [155,160]. In this method, mononuclear cells are extracted from either adult peripheral blood or human umbilical cord blood and plated onto fibronectin plates in endothelial growth media and non-adherent cells are removed during washing steps [160,161]. L-EPCs colonies emerge between 10–21 days after plating and display cobblestone morphology [161,162]. These cells express endothelial marker proteins including von Willebrand factor, CD31 and

VEGFR2, but not the progenitor marker CD133 [163-165]. Recent studies have identified that L-EPC are derived from CD34+ CD45- cells that express VEGFR2, but do not derived from CD133 fraction cells [166-170]. L-EPCs have a high proliferative potential and can be induced to form endothelial tubes *in vitro* [161,166,171].

Recently, L-EPCs have been used for iPSCs generation [182,183]. In these studies, iPSCs were derived from L-EPCs using a traditional retrovirus method encoding Oct4, Sox2, Klf4 and C-Myc. L-EPCs-iPSCs had a normal karyotype, expressed pluripotent surface markers (e.g., Tra-1-60) and differentiated into all three germ layers. In contrast to fibroblast-iPSCs, L-EPC-iPSCs have fewer CNVs compared with their parent L-EPC line; indicating a greater degree of genetic stability in culture. L-EPCs-iPSCs were also able to recapitulate a mutation in the gene encoding the bone morphogenetic protein type II receptor from patients with primary pulmonary hypertension.

Collectively, these studies indicated that cell type of origin is a valuable tool for iPSC generation and subsequent differentiation to somatic targets. Thus, rationalizing the development of techniques using cell sources that endogenously expressing the key reprogramming factors (such as OSKM or OSNL). As such, this study will use L-EPCs as the cell source to genetically reprogram patient blood samples to an iPSCs fate.

6.2 Gene delivery

Several of different methods have been optimized for the delivery of exogenous genes into somatic cells that enhance reprogramming efficiency and iPSC quality. The constitutively active Molony murine leukemia virus (MMLV)-based retroviral vector was first used by the Yamanaka group in the first generation of iPSCs to over-express Oct4, Sox2, Klf4 and C-Myc within dermal fibroblast cells [55]. Transgene expression was

driven by a 5'MMLV long terminal repeat (LTR) promoter that eventually lead to silencing of ectopic expression and permitted the reactivation of endogenous gene expression. Further work has demonstrated that the MMLV LTR promoter often generates partial reprogramming that is highly dependent upon exogenous expression with a marked failure to drive forward the re-activation of endogenous genes [55,65,75,184-187]. Most importantly, several studies have shown that constitutively activation of c-Myc by MMLV LTR may escape repression and result in the formation of c-Myc driven tumors in chimeric animals [74,188,189].

As a result of these concern, investigators explore the capacity of lentiviral mediated somatic gene transfer to express the key pluripotent genes. Lentiviral vectors are a subclass of retroviruses, which can infect both dividing and non-dividing cells [190]. The first such report of these vectors demonstrated that human fibroblast can be reprogrammed into iPSC using lentiviruses encoding Oct4, Sox2, Nanog and Lin-28 genes under the control of the constitutive human elongation factor-1 alpha (EF1a) promoter which was subsequently silenced during iPSC proliferation [63]. Reprogramming efficiency of human iPSCs generation by lentiviral system was enhanced up to 70 fold when Oct4, Sox2, Klf4 and c-Myc were combined with the SV40 large T antigen under the control of the EF1a promoter [191]. Zhao et al. have further shown that lentiviral reprogramming using the cytomegalovirus (CMV) promoter increases reprogramming efficiency of human iPSC generation by combination of Oct4, Sox2, Klf4 and c-Myc with two additional factors (i.e., p53 siRNA and UTF1) [85]. Most interesting and despite the random nature of lentiviral transgene insertion, a recent study has shown

that the lentiviral integration occurs at a lower frequency in cancer-related genes; thus limiting the prospect of lentiviral-associated oncogene activation [192].

To overcome the difficulties inherent in reactivation or incomplete suppression of lentiviral-transduced transgenes, an inducible drug lentiviral system has been recently developed. This high efficient tool promises to provide a powerful means of temporally controlling gene expression and has been successfully used to reprogram somatic cells into iPSCs [193]. This method eliminates the risk of constitutively active gene expression to reduce the threat of tumor formation. It also allows for selection of fully iPSC colonies as withdrawal of the induction drug Dox during the reprogramming stops proliferation of the incompletely reprogrammed cells that depend fully on the exogenous factors [193,194]. The Tetracycline (Tet) system is a prokaryotic inducible promoter system that has been adapted in mammalian cells [195-197]. The Tet system depends on incorporation of two separate plasmids, namely the response and regulator plasmid. The response component composed of tetracycline resistance operon (tetO) and a transcription factor inserted downstream of tetO that all fused within a minimal CMV promoter. The regulator plasmid is composed of the reverse tetracycline activator (rtTA) embedded to the herpes simplex virus transactivator protein VP16. Binding of rtTA to tetO in the presence of Dox leads to induction of gene expression (Tet-On system; Fig. 6) [195-198]. To provide temporal control of transgene expression, these vectors are contained within an inducible expression system tetO. As shown in Fig. 6, each transgene has a tetracycline responsive element-binding site after the constitutive promoter CMV.

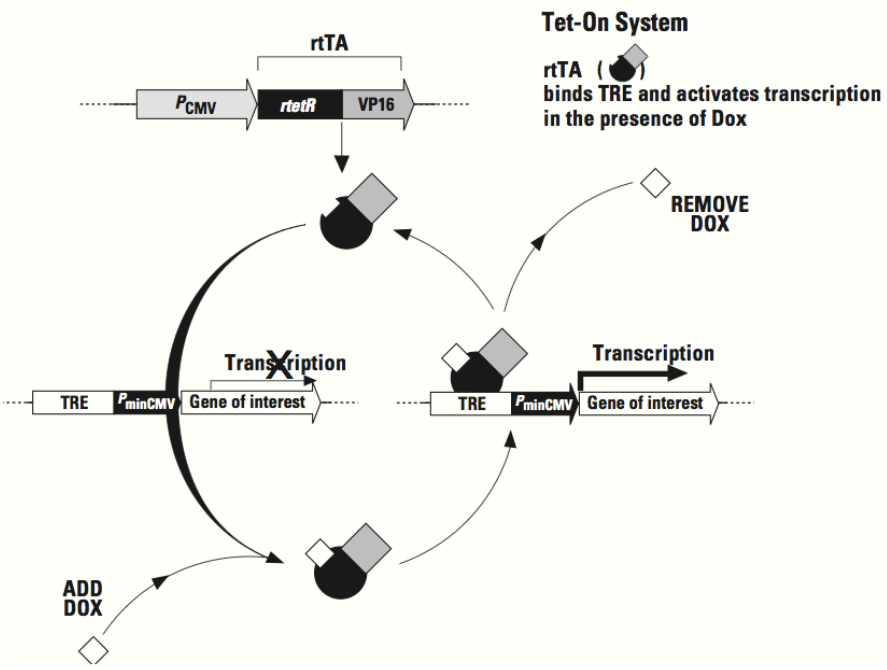

Figure 6. Schematic of gene regulation in the Tet-On Systems. [199]

In application of doxycycline, co-transduction of the cells with the CMV-reverse tetracycline controlled rtTA results in expression of the tetracycline-responsive element binding to the tetO site and express of the transgene. By withdrawal of Dox after minimum of 10-15 days results in rtTA dissociation and represses expression of exogenous transgene and endogenous genes initiate to be expressed.

The Tet-On regulatory systems are represent a widely used system for cellular reprogramming that provides temporal gene expression within reprogrammed cells while limiting the potential for tumor formation. This system will be used in this study to tightly control the expression and repression of key reprogramming genes.

6.3 Identification of iPSC colonies

Akin to a needle in a haystack, the low efficiency of iPSCs reprograming hinders the recognition of truly reprogrammed clones from partially reprogrammed or transformed colonies. To overcome this limitation, various groups have developed drug selection methods that depend on the reactivation of endogenous pluripotency genes (such as Fbxo15, Nanog or Oct4) that drive cell resistance to toxic drugs. However these approaches have limitations. For example, activation of Fbxo15 gene initiates at very early stages of reprogramming process while most of the cells are not yet fully reprogrammed resulting in positive selection of partially reprogrammed cells [55]. Moreover, a drug selection method requires genetic engineering of somatic cells. An alternative approach entails the use of an Early Transposon Promoters with Oct4/Sox2 enhancer (EOS) to drive the expression of fluorescent proteins. Thus activation of EOS promoter generates the emergence of iPSCs colonies with fluorescence protein to enable iPSC isolation but still requires further genetic manipulation of cells [200,201].

As an alternative, several groups have developed live cell staining methods involving the expression of human iPSCs surface markers (e.g., TRA-1-81, TRA-1-60 and SSEA4) to select iPSCs colonies [202,203]. Early work by Chan et al., has shown that a combination of surface marker expression with a retrovirus expressing fluorescence reporter can accurately indentify fully reprogrammed iPSCs [204]. While recent work has dispensed with viral reporter to identify high quality iPSCs using live cell staining alone [205-207].

Thus in summary, the combination of somatic source cells expressing low levels of critical reprogramming factors (L-EPCs; section 6.1) with doxycycline inducible

reprogramming vector (Dox-inducible, section 6.2) and live cell identification of reprogrammed iPSCs (section 6.3) will be used in this study. This is the first study of it's kind to combine all three techniques and will likely be referenced as a benchmark technique to efficiently generate high quality iPSCs for trouble-free differentiation to target phenotypes.

7.0 Cardiac differentiation of pluripotent stem cells

7.1 Introduction

Development of the mammalian heart involves a series of morphogenetic processes that are controlled in response to extracellular signaling proteins that guide the differentiation of pluripotent stem cells toward the cardiac fate [208]. The understanding of the molecular mechanism in human cardiogenesis was obtained from early cardiac development in animal models and ESCs differentiation [209-211]. In 2001 Kehat et al was first reported the generation of CM from ESC [212,213]. This work was soon followed by several studies that improved the capacity of pluripotent cells to generate CM cells by using differentiation methods [211,213].

The capacity of iPSCs to recreate cardiac tissue CM was first demonstrated in the landmark Yamanaka paper [55]. In this work, differentiated iPSCs exhibited spontaneous contracting foci and expressed cardiac-specific markers. Over the years that followed, a series of optimization studies examined the capacity of first generation iPSC lines to differentiate into CM lineages [214,215]. These approaches include Embryoid Body formation (EB) [216-219] co-culture with stromal layer [220] and direct cytokine-guided differentiation [221].

7.2 Embryoid Body Formation

Embryoid bodies are three-dimensional aggregates of pluripotent stem cells that undergo differentiation and cell specification into all the three-germ layers including CM in a manner similar to the early embryo [216-219]. In this technique, small clumps of pluripotent stem cell are dispersed in a suspension culture containing serum media to form spherical aggregates. These spheres or "embryoid bodies" (EB) are replanted onto matrix-coated dish and the rhythmically contracting outgrowth of EB is observed after several days in culture [212]. The EB technique is widely used, as it is simple and inexpensive approach. The efficiency of CM generation from this method is less than 10% and is highly dependent on cell type of origin. Several studies have been optimized the generation of highly throughput EB formation mediated cardiac differentiation by developing define media, addition of growth factors and controlling the EB size [222-226].

Studies have demonstrated the heterogeneity nature of EBs has adverse effects on the reproducibility and synchronicity of differentiation [210,222,223]. To overcome these limitations, a protocol was optimized in which specific numbers of pluripotent stem cells were aggregated into low attachment V-bottomed wells by centrifugation, resulting in identical size of EBs in each well [216]. This study indicated that the manipulating cell number could control the size of the EBs. The optimal size for cardiogenesis is in between 250 to 300 um in diameter on the third day of cardiac differentiation. Forced aggregation methods provide a high efficiency differentiation when culture with growth factors without specialized apparatus. A silicon wafer–based microfabrication technology that produces several numbers of EBs has markedly enhanced the efficiency of EB

generation by forced aggregation [224]. Other methods have been optimized to obtain uniform size of EBs including microwells and micropattering approach [225,226]. Microwells are microfibricated of defined size wells coated with extracellular matrix such as Matrigel that maintain undifferentiation cell in a definite size. Removal of the colonies from the microwell can be used for EB based differentiation. This technology generates an optimal size for cardiac differentiation, which range from 100 to 300 um X-Y dimension and 120 um Z dimension. Although this technology provides uniform-size colonies of pluripotent stem cells with less background differentiation, this technology is not currently commercially available. Finally, micropattern EB techniques have been shown to provide an optimal size for mesoderm generation and cardiac differentiation. In this technique, single cell suspension of pluripotent stem cells are plated into micropattern extra cellular matrix (ECM) island that provide microenvironment niches for undifferentiating cells. The size of the colonies depends on the size of Matrigel Island. Transferring the colonies into suspension culture generates homogenously uniform size EB than the traditional method. However, this technology is not in widespread use because it requires custom microcontact-printing equipment.

These studies suggest that heterogeneity EB colonies could limit the potential and efficiency of cardiac differentiation potential to preclude a uniform population of cells for phenotype testing; thus rationalizing attempts to explore other methods of cardiac differentiation.

7.3 Co-culture with END2 cells:

An alternative method for cardiac differentiation is based on the role endoderm cells play to provide signals to adjacent mesoderm cell for during embryonic development [227-230]. Visceral endoderm like cells (END2), which derive from mouse P19 embryonal carcinoma, have been designed for cardiac induction. Cardiac differentiation was induced by co-culture of pluripotent stem cells with END2 cells and results in the formation of beating cells with characteristics similar to CMs [231-233]. In this protocol, pluripotent stem cells co-culture with END-2 adopts a cardiac fate within 12 days while the majority of the cells resemble human fetal ventricular CM based on morphological and electrophysiological analysis [231].

Interestingly, cell to cell contact between pluripotent stem cells and END-2 cell does not appear to be necessary for cardiac differentiation as several studies have shown that exposure of ESCs to END2 conditioned media results in efficient CM generation [234-236]. Studies showed that END-2 cells produce soluble cardiac inductive factors and cytokine inhibitory factors. These inhibitory factors (i.e., prostaglandin 12 (PG12)) deplete insulin, which acts as a potent inhibitor of cardiogenesis and stimulator of neuroectoderm differentiation [235,236]. Thus CM can be derived in media condition containing insulin- , serum-free supplemented with PG12.

Ultimately, the efficiency of cardiac differentiation from END-2 co-culture is low as CM are estimated to comprise only 2-3% of all cells in culture. Moreover, cardiac differentiation from co-culture system may increase the risk of tumorigenic co-culture cells; thus limiting their potential for clinical purposes and high fidelity disease modeling.

7.4 Culture guided differentiation

Several growth factors recapitulate the cardiogenesis in cell culture by stimulating the formation of mesoderm from pluripotent stem cells. Cardiac differentiation from pluripotent stem cells can be derived by guided differentiation in adherent culture involving serum free medium supplemented with growth factors Activin A and BMP4 [55,221-239]. These growth factors are a member of the TGF b family that plays roles in embryonic development including induction of gastrulation-like events, meso-endoderm development and induction of cardiogenesis [240-246].

In these methods, pluripotent stem cells are cultured in a feeder cell-free system using a media supplemented with specific cytokines. Cardiac differentiation is induced by Activin A and BMP4. Exogenous growth factors are then removed and the cells are maintained in serum free media for 2-3 weeks. Cardiac beating cells and progenitor cells (defined by the presence of Nkx2.5) are detected 6 days after Activin A treatment. The percentage of cardiac progenitor cells increases over time such that they comprise almost 30% of the final culture.[221].

The canonical Wnt signaling also promotes cardiac differentiation from pluripotent stem cells. This role is biphasic depending upon the timing of Wnt stimulation [247,248]. Early exposure of EB to Wnt3a induces cardiac differentiation by induction mesoderm formation. This effect is further enhanced by adding Activin A to produce approximately 50% CM [249]. Other studies have demonstrated that the addition of a canonical Wnt inhibitor (Dickkof homolog 1(DKK-1)) at late stage of differentiation enhances cardiac production [250]. Current protocols expose pluripotent cells to Activin A, BMP4 and basic fibroblast growth factor (bFGF) for the first 4 days of differentiation to induce the

mesoderm formation. After this, cardiac induction is accelerated by the addition of DKK-1 and VEGF from days 4 to 8 [250,251]. Finally, addition of a canonical Wnt signaling inhibitor (such as IWP-4 or IWR-1) at later stages of cardiac differentiation enhance the expression of cardiac progenitor marker such as Isl1, Nkx2.5 and cardiac myosin heavy chain, resulted in generating up to 98% functional human CM from pluripotent stem cells [252-255].

A recent matrix sandwich protocol has been developed to further enhance the efficiency of CM generation by combining cardiac induction cytokines and ECM [256]. ECM plays a crucial role in development by provides a signaling crosstalk between ECM receptor and cytokine receptors. In this report, single cells of human pluripotent stem cells (PSCs) were cultured onto Matrigel coated dish in defined media. When the cells reached 90% confluence, a matrix overlay of Matrigel was added to the monolayer cells. Once cells attained 100% confluence, Activin A supplemented media was added for 1 day followed by BMP4 and bFGF for 4 days. CMs were observed between 10-15 days in culture that represent high purity (40%-90%) and high yield (4-11 CM per input).

Thus, the matrix sandwich protocol provides a robust cardiac differentiation for ESC and iPSCs lines that generate high purity and yield of CM. This study will extend the use of matrix sandwich technology to the culture guided differentiation of patient-derived L-EPC-iPSCs towards a CM fate for the study of patient disease states.

8. Signaling pathways underlying cardiac differentiation

The early stages of cardiac differentiation represent an Epithelial to Mesenchymal Transition (EMT) that is controlled by several signaling pathway including Nodal/ActivinA, BMP, canonical Wnt, Notch and Sonic Hedgehog signaling pathway.

In vertebrates, Nodal/Activin signaling regulates generation of the mesoderm and endoderm germ layer that provide source cells for cardiogenesis [255]. Nodal/Activin are required for the first two days of cardiac differentiation and the absence of these signals direct the differentiation toward neuroectodermal lineage [257]. Nodal signaling pathway is initiated by the phosphorylation of the Smad2 pathway that forms a complex with Smad4, which translocates into the nucleus to activate the expression of Nodal and Lefty genes [257-259].

The other member of TGF-β family that impacts on cardiac differentiation is the BMP4 signaling pathway. BMP4 directs pluripotent of stem cells into a mesodermal fate rather than neuroectodermal fate. A recent study has shown that BMP4 represses the neuroectodermal marker PAX6 to activate the mesoderm marker Brachyury T^+ in a dosage and time dependent manner [260]. BMP4 signaling acts through Smad2/Smad3 signaling to mediate mesodermal specification [261]. Several studies have demonstrated that combination of both Activin A and BMP4 play a significant role during the first stage of cardiac differentiation [261-263]. Nodal signaling synergizes with TGFβ expression to induce the formation of cardiogenic precursor such as KDR^+. However suppression of Nodal signaling and persistence of TGFβ promotes the formation of vascular smooth muscle and endothelial cells [264]. The canonical Wnt pathway is also

involved at the first stage of cardiac induction. b-catenin was showed to induce EMT in ESCs and expression of mesodermal markers [221,265,266].

The P38-MAPK pathway also regulates the expression of cardiac progenitor cell makers (MEF2C, GATA2 and ATF2) through the activation of the transcription factor AP1 [267]. Inhibition of P38-MAPK pathway directs the differentiation into neural lineage instead of CM differentiation [268]. However, inhibition of P38-MAPK pathway is required during the later stage of differentiation of cardiac progenitor cells toward immature CMs. Study have shown that inhibition of this pathway 12 days after starting EB cardiac differentiation improves cardiomyogenesis with up to 80% beating cells [269, 270]. P38 was shown to regulate the mitosis gene expression such as cyclin A and cyclin B in CM that correlate with the cardiac growth during the development. Thus, P38-MAPK inhibition could induce the proliferation of adult CM in combination with addition of basic fibroblast growth factor type 1 [271].

Sonic Hedgehog (SHH) and Notch signaling pathway have also been shown to regulate the differentiation from cardiac mesodermal cells to cardiac progenitor cells. SHH plays a critical role during embryogenesis including the cardiomyogenesis specification [272]. Blocking SHH pathway in mouse delay the induction of cardiac differentiation and the expression of cardiac progenitor NKX2.5. In aggregate P19 cells, SHH expression acts through GLI1 and GLI2 to specify mesodermal cells into cardiac lineage [272].

Retinoic acid (RA) is a metabolite product of vitamin A that is produced through a sequential of oxidative reactions mediated by enzymes (such as alcohol dehydrogenases (ADHs) and retinaldehyde dehydrogenases (RALDHs)). The responsible enzyme for RA

synthesis in early cardiac development is retinaldehyde dehydrogenase 2 (RALDH2), thus the presence of RA is indicated by the expression of RALDH2 [273,274]. RA receptors composed of RA receptor (such as RARα, β, and γ) and retinoid X receptor (such as RXRα, β, and γ) that acts as a ligand-activated transcription factor [275, 276]. Upon activation of these receptors by RA, they form heterodimers (i.e., RAR-RXR) or homodimer (i.e., RXR-RXR) that bind to a sequence of DNA (known as the RA response element (RARE)) for activation or repression transcription factor of target gene. The primarily receptors for RA activation in the embryonic heart are RARα and RARβ receptor [277].

Retinoic acid is known as a morphogen that control anterior posterior polarity to the heart [278]. During early cardiogenesis, RALDH2 is expressed in relation to the sino-atrial precursors expression, indicating that RA may be synthesis along the anterior–posterior axis of the heart tube [278,279]. Inhibition of the RALDH2 in mouse embryos showed abnormally distribution of second heart field genes (such as Isl1, Tbx1, Fgf10, and Fgf8*)* in a posterior direction [280,281]. In avian cardiac morphogenesis, the expression of RALDH2 in the posterior region is coinciding to the activation of atrial specific myosin heavy chain 1 (AMHC1) gene, a marker of atrial phenotype [282]. Treatment of explant cardiogenic tissue with RA at stages 5-6 and 7-8 activates the expression of AMHC1 in the anterior myocardium fated to develop ventricular outflow track [283]. To confirm this study, Patwardhan and colleagues have rotated the normal cardiac anteroposterior polarity 180° at stage 8, generating hearts with anterior region expressing atrial marker (i.e. AMHC1) and posterior region expressing ventricular marker (i.e. VMHC1) [284]. Inhibition of RA signaling in specific time interval during

mouse and chicken development (i.e. stages 10-11) generates embryos with heart defect (i.e., large ventricles and smaller atria), however the addition of RA rescue the phenotype [285]. Furthermore, in mouse embryonic stem cell, RA accelerates the differentiation of atrial and pacemaker cell [286].

Collectively, these studies demonstrated that manipulating the signaling pathways that control cardiac development boosts the capacity of cardiac differentiation protocols to generate high purity CM for disease phenotyping.

9. Characterization of iPSCs derived cardiomyocytes

Characterization the cardiac differentiation of cells derived from ESC and iPSC is important to identify the capacity of these protocols to replicate the gene expression profile, structural properties and functionality of the human heart. After the initiation of cardiac differentiation, pluripotency cell markers such as Oct4 and Sox2 are downregulated [67,215]. The appearance of contracting areas within the plated cells is the first visual sign of successfully cardiac differentiation [227] but the developmental stages of CMs within the embryo can be divide into multiple stages characterized by the sequential generation of: 1) mesoderm (T or Brachyury homolog, FOXC1, DKK1), 2) cardiogenic mesoderm (MESP1, ISL1, KDR), 3) cardiac mesoderm (NKX2.5, GATA4, TBX5, MEF2C, HAND1/2) and 4) early CM (ACTN1, MYH6, TNNT2) [252]. Later cardiac-specific transcription factors (e.g. Nkx2.5, GATA4, MEF2c, Tbx-5, and Tbx-20), sarcomeric proteins (e.g. α-actinin, troponins, sarcomere myosin heavy chain, atrial and ventricular myosin light chains and tropomyosin), gap junction proteins and other cardiac-specific proteins (e.g. atrial natriuretic peptide, creatinine kinase-MB and myoglobin) are expressed in both ESC-CM and iPSC-CMs [212,221,297]. At an ultra-

structural level, iPSCs derived CMs show clearly identifiable sarcomeres and intercalated disks [219,287]. The maturity of CM varies among different cells line that could be affected by cell lines, differentiation time, cocultured cells, and culture conditions [214,215,231,288].

Several studies have demonstrated that the structures of human PSC-CM are similar to fetal CM and important differences are observed when compared to adult CM [212, 287]. During the initiation of contracting cells in early PSC-CM, the beating cells are small and vary in shape from round to slightly elongated shapes [288,289]. Late PSC-CM which are grown in culture more than 30 days, develop a more elongated structure that remains small compared to adult CM [290]. These cells are mononuclear and lack of t-tubule network of adult CMs [291,292]. Finally the functional profiling has demonstrated that the Ca+ handling in PSC-CM shows an immature sarcoplasmic reticulum capacity (SR) to store and release calcium ions. Thus, calcium ions enter the cells through the sarcolemma instead of being released by the SR and, ultimately result in slower of excitation-contraction coupling than adult CM [293-295]. Early stages of iPSC-CM demonstrate a proliferation rate similar to embryonic or fetal cardiomyocytes which is markedly decreased from the pluripotent state [296-297]. BrdU and Ki-67 staining demonstrates that proliferation gradually decreases to <1% of the early PSC-CM rate in later stages CM differentiation such that it is similar that seen in fetal cardiac development [296].

Finally, iPSC-CM contract spontaneously and synchronously after 10 days of differentiation and can be maintained for 1 year in culture [298]. Electrophysiological studies revealed that iPSC-CM display heterogeneous culture including atrial-, nodal-,

and ventricular-like action potential (AP) [299,300] that also show variable AP characteristics between studies and within studies with different cell lines [214], differentiation methods [266], and time in culture [301].

10. Directed culture of iPSCs towards an atrial or ventricular lineage

Obviously, to better understand a discreet patient phenotype iPSC-CM need to be directed towards a specific cardiac cell type (i.e., atrial or ventricular). The sole work in the literature demonstrating this attempt was Zhang et al who demonstrated direct differentiation of ESC toward atrial and ventricular myocyte fate [302]. In this report, cardiac differentiation was induced by BMP4, bFGF and Activin A. Noggin was shown to reduce the downstream activities of BMP4 pathway and promote cardiogenesis [303]. Thus, treatment the cell with Noggin between days 4 and 5 increases the cardiac differentiation efficiency. Addition of retinoic acid or retinoid acid inhibitor (i.e. BMS-189453) to the culture in parallel experiment promoted cardiac differentiation toward atrial or ventricular fate respectively. Treatment the cells with retinoic acid from days 6 to 8 directed the differentiation into atrial myocyte fate as confirmed by the characteristic atrial AP, immunohistochemistry, Ca^{+} spark analysis. Conversely, ventricular differentiation was observed when a retinoid inhibitor (i.e. BMS-189453) was added to the culture media. Despite cTNT expression level was similar in retinoic acid and retinoic acid inhibitor, BMS-189453 increased the expression of specific ventricular gene (such as IRX4, MLC-2V) as confirmed by the characteristic atrial AP, indicating that the majority of the cardiomyocyte in the retinoic acid inhibitor treated culture are ventricular like myocyte while the treated culture with retinoic acid specified CM differentiation to atrial like myocyte. The extent to which this guided approach applied to iPSCs is not known

but provides a template for this study to develop a guided differentiation protocol to model atrial cardiomyocytes using iPSCs.

11. Previous work to replicate cardiac diseases using iPSC cell modeling

The first efforts to replicate human cardiac diseases was performed by Carvajal-Vergara and colleagues who generated cardiomyocytes from two human iPSC lines with a heterozygous T468M substitution in PTPN11 (protein tyrosine phosphate, non-receptor type 11) [304]. This mutation is characterized clinically as LEOPARD (Lentigines, Electrocardiographic abnormalities, Ocular hypertelorism, Pulmonary valve stenosis, Abnormal genitalia, Retardation of growth and Deafness) syndrome, an autosomal dominant developmental disorder of multiple organ systems resulting from a missense mutation in PTPN11 gene. The authors estimated hypertrophy based on cell size, sarcomeric organization and nuclear localization of NFATC4 (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4), which gave a measure of patient phenotype.

The second report by Moretti et al. [305] investigated the effect of an ion channel mutation in patients with documented Long-QT syndrome type 1, an autosomal dominant diseases characterized by KCNQ1 gene mutation and prolongation of the QT interval on an electrocardiogram. The KCNQ1 gene encodes IKs, the ion channel controlling the slow component of the delayed rectifier K+ current, partially responsible for the re-polarizing phase of the action potential. The authors were able to differentiate iPSC into cardiomyocyte and recapitulate the electrophysiological phenotype in culture using a whole-cell patch-clamp electrophysiology method. Soon after the report by Moretti et al,

iPSC technology was used to replicate single cell models from individuals with LQT and catecholaminergic polymorphic ventricular tachycardia type (CPVT).

As can be seen in Table 1, a variety of disease states have been characterized using iPSCs-based profiling. The majority of studies utilized retroviral induction to an iPSCs phenotype with subsequent a EB-based approach to differentiate cells. As previously outlined this provides a mixed cell phenotype with the possibility of transgene re-expression.

This study promises to refine these methods to develop a robust protocol to generate and differentiate patient iPSCs to a CM phenotype using: 1) small molecule (VPA) to precondition somatic cells into a transcriptionally more permissive state (section 4), 2) a cell source with endogenously high levels of key reprogramming factors (L-EPCs; section 6.1), 3) drug controlled expression of reprograming factors (dox-inducible, section 6.2), 4) high efficiency guided generation of CM (matrix sandwhich differentiation protocol; section 7.4) and 5) guided differentiation to an atrial phenotype (retinoic acid; section 10).

Authors	Disease state	Cell Source	Induction method	Differentiation Protocol	Phenotype characterized using
Carvajal-Vergara, X., et al. 2010 [304]	LEOPARD	Dermal Fibroblast	Retrovirus OSKM	EB	CM morphometry
Moretti et al., 2010 [305]	LQT1-KCNQ1	Dermal Fibroblasts	Retrovirus OSKM	EB	CM morphometry
Egashira et al., 2012 [306]	LQT1-KCNQ1	Dermal Fibroblast	Retrovirus OSKM	EB	CM morphometry
Itzhaki et al., 2011 [307]	LQT2-KCNH2	Dermal Fibroblast	Retrovirus OSK+VPA	EB	CM morphometry
Matsa et al., 2011 [308]	LQT2-KCNH2	Dermal Fibroblast	Lentivirus OSLN	EB	CM morphometry
Lahti et al., 2012 [309]	LQT2-KCNH2	Dermal Fibroblast	Retrovirus OSKM	EB	CM morphometry
Malan et al., 2011 [310]	LQT3- SCN5A	Dermal Fibroblast	Retrovirus OSKM	EB	CM morphometry
Terrenoire et al., 2013 [311]	LQT3-SCN5A	Dermal Fibroblast	Lentivirus OSKM	EB	CM morphometry
Yazawa et al., 2011 [312]	LQT8-CACNA1C	Dermal Fibroblast	Retrovirus OSKM	EB	CM morphometry
Davis et al., 2012 [313]	Overlap S.-SCN5A	Dermal Fibroblast	Lentivirus OSKM	END-2	CM morphometry
Fatima et al., 2012 [314]	CPVT1-RYR2	Dermal Fibroblast	Retrovirus OSKM	END-2	CM morphometry
Itzhaki et al., 2012 [315]	CPVT1-RYR2	Dermal Fibroblast	Retrovirus OSK+VPA	EB	CM morphometry
Jung et al., 2012 [316]	CPVT1-RYR2	Dermal Fibroblast	Retrovirus OSKM	EB	CM morphometry
Kujala et al., 2012 [317]	CPVT1-RYR2	Dermal Fibroblast	Retrovirus OSKM	END-2	CM morphometry
Novak et al., 2012 [318]	CPVT2-CASQ2	Dermal Fibroblast	Lentivirus OSKM	EB	CM morphometry
Ma et al., 2013 [319]	ARVC-PKP2	Dermal Fibroblast	Retrovirus OSKM	EB	CM morphometry

Table 1- Summary of published studies characterizing human disease states using iPSCs-derived CMs.

12. Future Directions:

iPSC technology has a greater impact than in the ability to generate disease and patient specific cell lines. This technology promises the ultimate approach to understand the physiological consequences of human mutations as it obviates the need for artificial manipulation of gene expression in heterologous models while avoiding the ethical issues inherent in the genetic manipulation of human ESCs. Furthermore, this natural recapitulation of the human diseases avoids artificial gene expression stoichiometry that may distort other relevant systems and physiology.

iPSC-based tissue specific models of disease will help to better understand patient specific mutations at a fundamental level and will open the gateway for truly personalized medicine by providing a stable platform for individualized therapies. Despite the lack of standardization and optimization in method of production of atrial cardiac myocyte, this thesis is designed to provide the proof of concept that stable in-vitro iPSCs are capable of generating high fidelity milieus to enable the development of atrial modeling disease and novel therapies. In recent years, familial forms of atrial fibrillation have been explored using sophisticated molecular approaches. One such study demonstrated a causative role mutation in the potassium channels responsible for the slow re-polarization of atrial myocytes (KCNQ1, KCNE2, KCNJ2) [320-322]. However, the vast majority of atrial fibrillation is sporadic and non-familial. As such genes are infrequently identified in these cases. Thus providing impetus for the prevailing dogma that genetic mutants are a rare cause of the arrhythmia in sporadic atrial fibrillation [26].

In June 2006, our research team published, in the New England Journal of Medicine, the novel finding of genetic mutations in the Cx40 gene (GJA5) in patients

with atrial fibrillation and normal structural hearts ('idiopathic' atrial fibrillation). This discovery identified a critical role for Cx40 in the pathogenesis of atrial fibrillation. Subsequently, we identified two novel germ line mutations in Cx40 (Ala96Ser and Gly311Ser) that impair gap junction conductance and increase vulnerability to atrial arrhythmias [324]. These discoveries mark years of diligent work and emerge from the extensive use of costly resources to validate disease-causing mutations. The usage of such resource methodologies would be cost prohibitive for clinical translation and, taking into account the emergence of the $1000 genome, procedures that expand, strengthen and refine the characterization of these mutations are desirable.

We will develop this theme by using Cx40 mutant derived cardiomyocytes to study the effect of enhance gap junction conductance using a novel class of pharmacophores. Rotigaptide (ZIP123, AAP10) is a well-studied hexapeptide that enhances Cx43 gap-junction conductance [325]. While phase 1 trials demonstrated the compound is safe and well tolerated, phase IIa were completed but not published [326]. Experimental studies demonstrated that atrial fibrillation duration is significantly reduced in ischemia-induced atrial fibrillation but unchanged in models of heart failure- or tachycardia-induced atrial fibrillation [327]. This finding likely reflects the ability of rotigaptide to inhibit the ischemia-induced dephosphorylation of Cx43 (e.g. at Ser-368) in the ischemic areas, while there was almost no effect of rotigaptide in the non-ischaemic areas [328]. While this data supports the notion that enhanced gap junction conductance is antiarrhythmic, the lack of effect in non-ischemic conditions limits the utility in atrial fibrillation or gene based diseases.

Recently, a new class of pharmacophores (RXP-E) have been developed that binds

the carboxyl terminal domain of Cx43 (Cx43CT) and prevents cardiac gap junction closure and action potential propagation block [329,330]. While these compounds do not alter Cx40 conductance, we postulate that enhanced Cx43 conductance will overcome the effect of disease causing mutations in Cx40 and furthermore will demonstrate the capacity of iPSC based disease models for drug-discovery. Once demonstrated, this platform could conceivably be transitioned to high-throughput screening of candidate compound libraries for novel therapeutic discovery.

13. Aim and Hypotheses

Here, we explore the capacity iPSCs reprogrammed from peripheral blood EPC lines to differentiate into atrial myocytes for the study of patient specific atrial physiology

Aim: To determine wither multipotent L-EPCs provide a suitable platform for genetic reprogramming into hiPSCs using viral vector and differentiate them into functional cardiomyocyte

Hypotheses:

1- L-EPCs provide a hardy and highly proliferative multipotent stem cell source that express the key genetic transcripts underlying cellular reprogramming to a pluripotent fate.

2- Genetic reprogramming of L-EPCs using viral vectors enhances the expression of pluripotent stem cell markers.

3- Guided culture of L-EPC-hiPSCs can promote the expression of markers indicative of a cardiac fate.

14. Material and Methods

14.1 Cell culture

After informed consent under an research protocol approved by the University of Ottawa Heart Institute Research Ethics Board, human EPCs were cultured from blood samples obtained from healthy volunteers or patients undergoing clinically indicated coronary angiograms. Mononuclear cells were obtained from 80 ml peripheral blood and separated by Histopaque-1077 density gradient (Sigma) and cultured in fibronectin (BD Biosciences) coated plate in Endothelial cell basal growth medium-2 (EBM-2, B3156; Lonza, Basel, Switzerland) with microvascular endothelial growth medium (EGM-2-MV) supplements (Lonza, CC-4147), 20% Fetal Bovine Serum (FBS), Epidermal Growth Factor (EGF), Insulin Like Growth Factor (R3-IGF-1), Vascular Endothelial Growth Factor (VEGF), Gentamycin Sulfate (GA-100), Ascorbic Acid, and Hydrocortisone. The medium was changed every 2 days. Late-EPCs appeared approximately between 12 to 20 days in culture. Late-EPCs exhibited a high proliferative capacity and had a cobblestone appearance.

HES H9 and iPSC were maintained on Matrigel in E8 medium (StemCell Technologies, Vancouver, Canada; cat. 05850). ESC-medium was changed every day and cells were passaged every 5-7 days. EPC-iPSC or ESCs were collected using gentle cell dissociation reagent (StemCell Technologies).

14.2 Lentivirus Production and hiPSC generation

A second generation with three-plasmid vectors system of human FU-tet-O-hOct4, -Sox2, -Klf4, and -Nanog lentiviral vectors were used for L-EPCs reprogramming. To provide temporal control of transgene expression, these vectors are contained within an inducible expression system (Tet-on). Application of Dox results in rtTA binding and transgene expression.

To generate an inducible system for factor-mediated reprogramming of Late-EPC into iPSCs, HEK293 cells cultured on 10-cm dishes were co-transfected at 60-70% confluence with a three-plasmid vectors system, 10 μg of envelope plasmid pMD2.G (Addgene), 15 μg of packaging plasmid psPAX2 (Addgene) and 25 μg of each iPSC factor expression plasmid FU-tet-O-hOct4, FU-tet-O-hSox2, FU-tet-O-hKlf4, FU-tet-O-hNanog and reverse tetracycline transactivator FUdeltaGW-rtTA (Addgene) using Lipofectamine 2000 (Invetrogen). Transfection mixes were removed from the cells after 12 h and medium was replaced to virus media (EGM-2-MV + 30% iFBS). Virus-containing supernatant was collected 60–72 hr post-transfection and filtered through a 0.45 mmfilter. Virus-containing supernatants were pooled for 4 factor infections and supplemented with rtTA virus with a ratio (1 of each factor: 2 rtTA) in the presence of 6 mg/ml of polybrene. Human L-EPCs were seeded at density $5x10^4$ in Fibronectin coated dish and induced with 5mg VPA (Sigma) 2h before the transduction. Five consecutive infections were spin at 500g/2h and performed over a period of 48 hr. After transduction, L-EPCs were maintained in complete EGM-2MV medium for 5 days and re-plated at different densities between 5×10^3 and 5×10^4 cells on Matrigel-coated dishes (BD Biosciences). To induce reprogramming, culture medium was replaced 2 days later to

hESC conditions E8 (STEMCELL Technologies) and EGM-2-MV (1:1) supplemented with Dox (Sigma-Aldrich; 2 mg/ml) for 5 days and continued with E8 supplemented with Dox for another 10 days. Human iPSCs colonies were mechanically picked based on morphology between 20-25 days after Dox induction and replated onto matrigel. iPSCs were mechanically dissociated for a few passages and then adapted to gentle dissociation passaging (STEMCELL Technologies)**.**

14.3 Quantitative PCR

Total RNA was isolated and purified using RNeasy Micro RNA extraction kit (Qiagen) followed by DNase treatment to avoid DNA contamination. Quantitative PCR was performed for expression of pluripotency markers (Oct4, Sox2, Klf4 and Nanog) and cardiac markers (gap junction proteins; Cx40 and Cx43, and cardiac marker, cTNT, atrial markers; CACNA1H, KCNA5 and MYL4) using qPCR master mix on a Roche Thermocycler**.** The expression level of the transcript in each sample was measured using custom primers followed by normalization to GAPDH expression.

14.4 Cell Proliferation assay

Cell proliferation was measured with Cell Counting Kit-8 (Dojindo). Late-EPC were plated at 96-well plates at approximately $1x10^4$ cells per well and cultured in the growth medium. After 24h and 48h, each well were added with 10 μl of the Cell Counting Kit-8 solution and then incubated at 37 ºC for 2 h. Cell number assays were performed in a 96-well format plate reader by measuring the absorbance at a wavelength of 450 nm (OD450).

14.5 Flow cytometry analysis

Single cells were trypsinized with TrypLE (Invitrogen) for 5 minutes at 37°C followed by neutralization by adding equal volume of EGM-2-MV medium. The dissociated cells were centrifuged, washed and filtered through a 40 um cell strainer (BD Pharmingen). appropriate volume of staining buffer (PBS supplemented with 0.1% FBS) to obtain a cell density of 0.5 $x10^6$ cell/ 50 ul. Cells were then dispensed into 1.5 ml micro centrifuge tubes (50 ul/tube) and stained with isotype control or antigen-specific antibodies diluted in the staining buffer. Cell-surface antigen expression was detected using fluorescently conjugated antigen-specific antibodies (direct staining); ; APC anti-human CD31 Antibody (BioLegend, 303115), Human VEGF R2/KDR (R&D, FAB357P), CD34 and CD45 (BioLegend) by incubating at 4°C for 30 minutes. The cells were washed 2-times with and 1 ml of Cell Wash. Unstained samples were used as a negative control. The cell suspension was transferred into the FACS tubes and data was acquired on on a Guava flow cytometer (Millipore). Cells were gated on forward and side scatter dot plots. 10,000 events per sample were acquired.

14.6 Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and washed in PBS. Samples were then permeabilized with 1% Triton-X100 in PBS for 10 minutes and blocked with 5% normal goat serum in 1% PBS-BSA for 1 hour at RT followed by 2 washes with PBS. Primary antibodies in 1% PBS-BSA were used for 1hour at RT. Samples were washed with 0.2% Tween 20 in PBS twice and 1X PBS twice. Secondary antibodies specific to the primary IgG isotype were diluted in PBS and incubated at room

temperature for 1 hour in the dark at RT. DAPI was used to visualize nuclei at a concentration of 10 mg/ml in PBS.

The following antibodies were used: mouse anti-TRA-1-60 (1:100 dilution, Millipore), mouse anti-TRA-1-81(1:100 dilution, Millipore), mouse anti-SSEA4 (1:100 dilution, Millipore), Alexa Fluor 488 (1:1000 dilution, Invetrogen) labeled secondary antibodies. Negative controls were stained without the use of primary antibodies. Fluorescence images were acquired with a Zeiss AxioCam Camera .

14.7 Telomerase Activity

L-EPC and iPSCs were trypsinized and washed once with PBS. The pellet cells were re-suspended in 200 µl of 1× Lysis Buffer /10^5-10^6 cells and incubated on ice for 30 minutes. The cells were spin in a microcentrifuge at 12,000x g for 30 minutes at 4°C.

Telomerase activity was quantified within L-EPC and iPSCs by real time PCR detection of supernatant-mediated addition of TTAGGG telomeric repeats (MT3011, Allied Biotech Inc).

14.8 Differentiation of human iPSC into atrial myocytes

Prior to differentiation, stem cell colonies were passaged onto matrigel-coated dishes in E8-medium. EPC-iPSC or ESCs were collected using gentle cell dissociation reagent (StemCell Technologies). The cell aggregates were re-suspended in E8 medium and Rho kinase (ROCK)-inhibitor Y-27362 (Sigma, 10µM) was added to prevent cell death.[19] Small clumps were cultured on matrigel-coated plates and the medium was changed daily with E8 medium until the cells were 90% confluent, a thin layer of matrigel was overlaid by freshly mixing matrigel 0.5 mg in 15 ml ice cold E8 medium and replacing the medium in each well of a 6-well plate with 2.5 ml of matrigel

containing E8 until the cells were 100% confluent. To initiate differentiation, the medium was replaced with Roswell Park Memorial Institute medium (RPMI1640; Invitrogen) with B27 (no insulin) and Activin A (100 ng/mL; R&D Systems), BMP4 (40 ng/mL; R&D Systems) and bFGF (10 ng/mL, Invitrogen) for 4 days without medium change. Five days later, Noggin (250 ng/ml, R&D Systems) was added followed by retinoic acid (1 μM, Sigma) and DKK1 (200 ng/ml, R&D Systems) for 2 days. Two days later, retinoic acid was removed and DKK1 was continued for another 2 days. The medium was changed every 3 days after day 11 with RPMI1640 B27 with insulin. Spontaneous beating clusters were typically observed on 15 days after starting the differentiation protocol. Beating areas were counted and their morphology evaluated using a regular light microscope.

14.9 Identification and evaluation of beating areas

Cells were monitored at day 15 from the start of differentiation and beating areas were counted and their morphology evaluated using a regular light microscope. A beating area on the plate is an aggregate of cardiac cells differentiated from stem cells. The size, shape and location of beating area may vary even between cells of the same line and with the same growth medium.

14.10 Statistical analysis

All data is presented as mean ± SEM. To determine if differences existed within groups, data was analyzed by a one-way ANOVA or two-way ANOVA, as appropriate. If such differences existed, Bonferroni's corrected t-test was used to determine the group(s) with the difference(s) (Prism 5.00; GraphPad Software, Inc.). Differences in categorical

measures were analyzed using a Chi Square test. A final value of $P \leq 0.05$ was considered significant for all analyses.

15. Results

15.1 Generation of endothelial progenitor cells from human blood samples

Peripheral blood was obtained from 4 patients (66±12 years old; 100% male) undergoing routine coronary angiograms and 5 healthy volunteers (29±5 years old, 65% male) after informed consent. Patients were recruited from Drs. Froschl, Labinaz and So's clinical practice at the time of coronary angiography.

Early-EPCs emerged from plated mononuclear cells within 10 days. These cells were small and consisted of multiple thin, flat or spindle-shaped cells that emerged from a central cluster of rounded cells (Figure 7). Left untended, these cells demonstrated peak growth after 2 weeks in culture and senescence by 4 weeks of culture. Late EPCs grew from sub-cultured early EPCs by 15-20 days after re-plating and formed between 2 and 7 highly proliferative colonies that were expanded for subsequent experimentation. These late EPCs demonstrated typical cobblestone morphology and underwent multiple sub-cultures without appreciable senescence. [331].

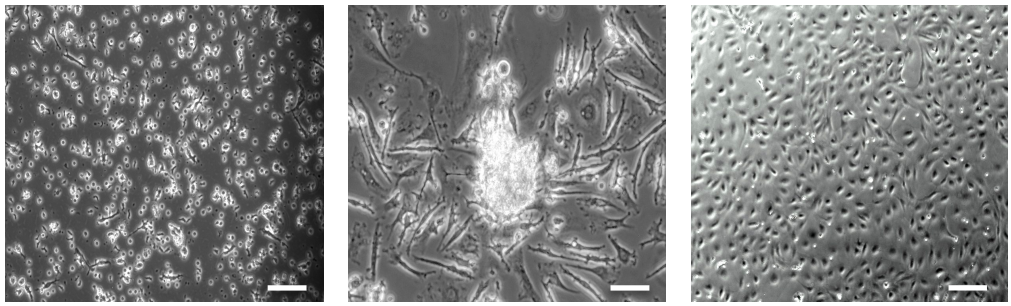

Figure 7: Morphological characterization of EPCs. For morphological assessment, "Early" and "Late" EPC from peripheral blood were directly imaged by phase-contrast light microscopy. Bar indicates 40um.

To characterize L-EPCs surface marker expression, the expression of endothelial cell lineage markers on L-EPCs was quantified using flowcytometry. It has been shown that cell surface marker examination of late-EPCs have characteristic expression of CD31+/VEGFR2+/CD14–/CD45– [332]. As shown in (Figure 8), passage 6 L-EPCs expressed markers characteristic for L-EPCs including platelet endothelial cell adhesion molecule (CD31, 98±1%,) vascular endothelial growth factor receptor 2 (VEGFR2; 75±6%) and CD34 (71±2.8%) [333]. In consistent with the finding that L-EPC generating precursors are derived from CD34+ CD45- cell fraction, but not to the CD34+ CD45+ hematopoietic fraction, these culture guided cell did not express hematopoietic cells specific surface antigen such as CD45 [170,333].

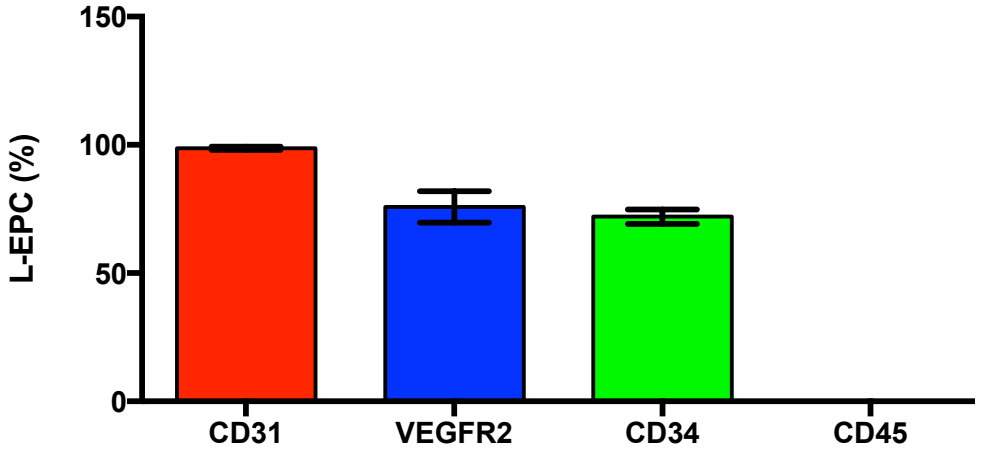

Figure 8: L-EPCs express endothelial cell surface markers (n=3 cell lines with three technical replicates performed per cell line). Flow cytometric analysis demonstrated that 98±1%, 75±6% and 71±2.8% of cells at passage 6 expressed CD31 and VEGFR2 and CD34, respectively but do not express CD45.

L-EPCs were also highly proliferative. These cells could be cryogenically stored and recovered without impact on viability or cell phenotype (Figure 9). Cell growth was

assessed using a commercial CCK-8 viability assay and demonstrated that L-EPCs grew to more than 10^6 cells by passage 6 with a population doubling every 24.2±3.2 hours. These cells could be cryogenically stored and recovered without impact on viability or cell phenotype (population doubling time: 21.3±3.6 hours, p=0.69; n=5).

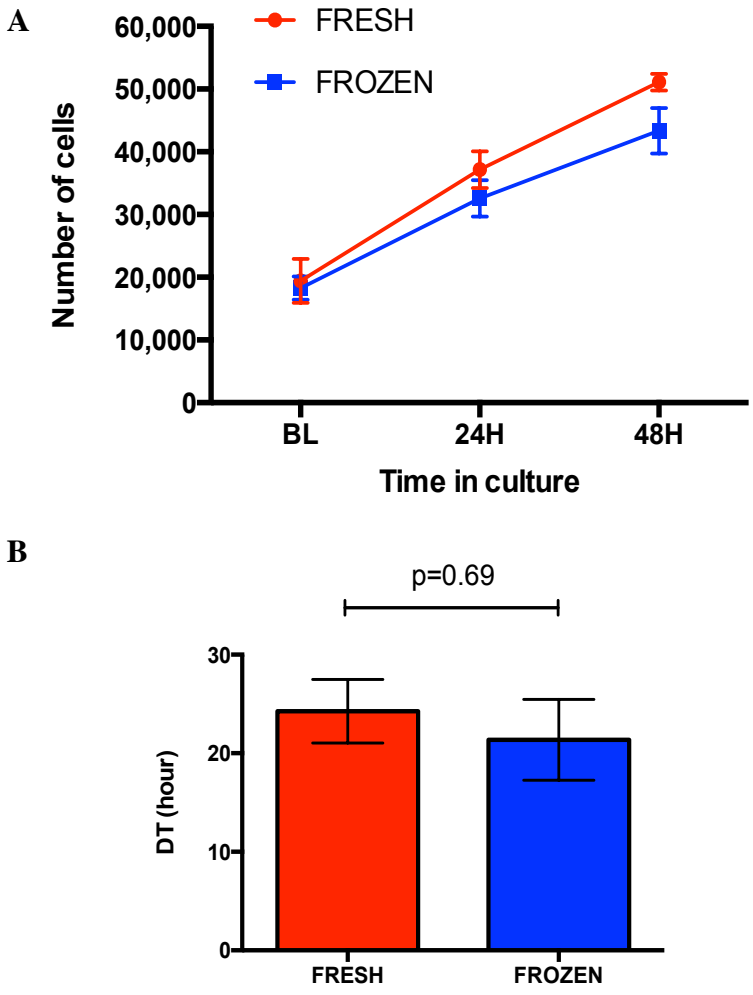

Figure 9: Growth curve (A) and doubling time of L-EPCs (n=5). Cell growth was assessed using a commercial CCK-8 viability assay that demonstrated that passage 6 and 7 L-EPCs doubled every 24.2±3.2 hours. These cells could be cryogenically stored and recovered without impact on viability or cell phenotype (doubling time: 21.3±3.6 hours (p=0.69)

Hochedlinger et al showed that hematopoietic stem and progenitor cells generate higher iPSC reprogramming efficiency than terminally differentiated B and T cells [25]. Moreover human neural stem cells with high endogenous levels of pluripotency gene such c-Myc and Sox2 have been able to generate iPSCs with only one factor Oct 4 [153]. To rationalize the use of a more permissive cell source for genetic remodeling, qPCR was performed and demonstrated high expression in the pluripotent markers Oct4, Sox2 and Nanog as compared to normal human dermal fibroblasts. These data demonstrated a 10±1.0, 96±3.2 and 20±1.0 fold greater expression in the pluripotent markers Oct4 ($p=0.046$), Sox2 ($p\geq0.001$) and Nanog ($p=0.0215$) and as compared to normal human dermal fibroblasts (Figure 10). Taken together, this data confirms that primary cultured EPCs provide a robust and easily attained cell source for genetic remodeling while transcriptome analysis hints that EPCs will be comparatively more amenable to pluripotent reprogramming than the traditional dermal fibroblast

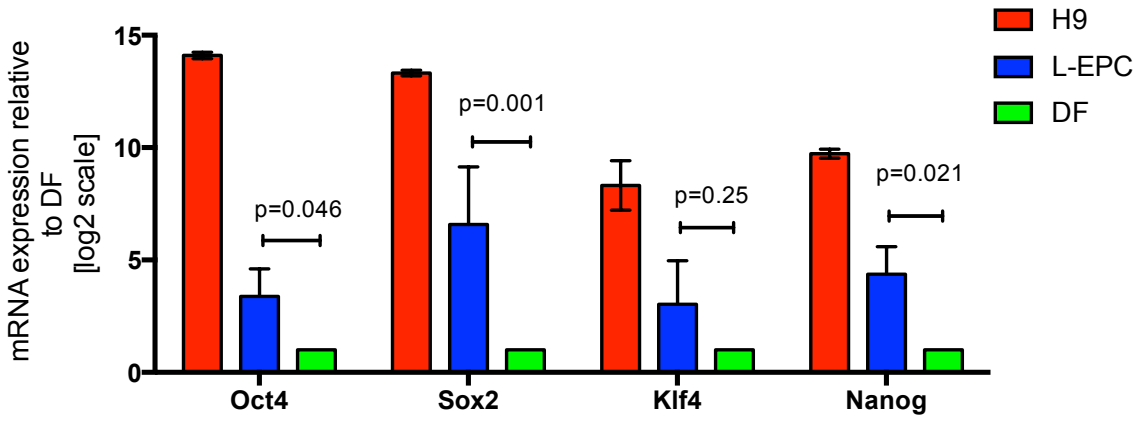

Figure 10: L-EPCs express pluripotency markers. Quantitative PCR was performed and demonstrated a 20, 10 and 96 fold greater expression in the pluripotent markers Nanog ($p=0.0215$) Oct4 ($p=0.047$) and Sox2 ($p\geq0.001$) as compared to normal human dermal fibroblasts. H9 cells represented as a positive control (n=3).

15.2 Generation and Characterization of L-EPC-iPSCs:

To generate iPSC from L-EPCs, we transduced L-EPC (without VPA pre-treatment) using inducible lentiviral-drug system method and no iPSCs colonies were formed over 30 days of observation (Figure 11).

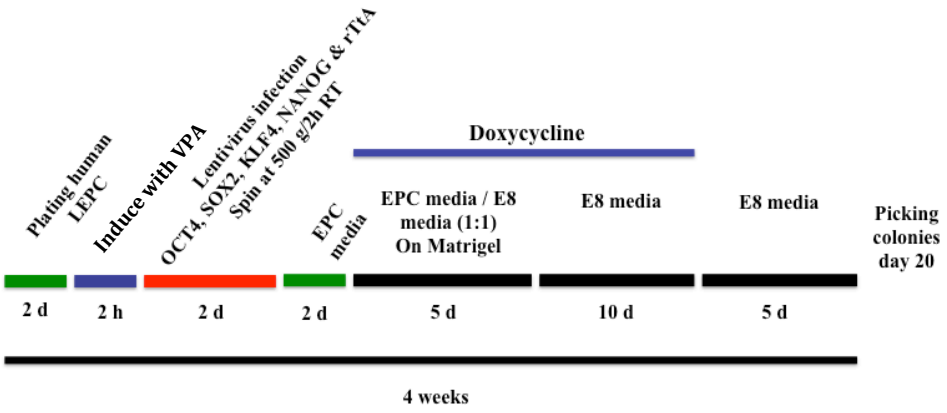

Figure 11: Schematic of the inducible lentiviral-drug system used to genetically reprogram L-EPC to iPSC. Also shown is VPA pre-treatment.

Quantitative PCR analysis confirmed that Oct4, Sox2, Klf4, Nanog transgenes were not highly expressed in lentiviral treated L-EPCs as compared to control L-EPCs (Figure 12).

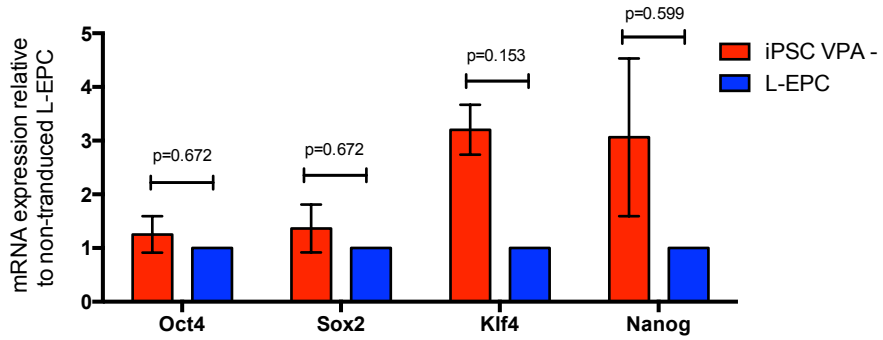

Figure 12: OSKN transgene expression without VPA pre-treatment. Quantitative PCR was performed on L-EPC-iPSCs 30 days after lentiviral transduction and demonstrated low transgene expression of Oct4 (p=0.672), Sox2 (p=0.672), Klf4 (p=0.153) and Nanog (p=0.599) compared to non-transduced L-EPCs (n=3 cell lines and 3 technical repeats).

Histone deacetylase inhibitors (such as VPA) enhance the efficiency of cellular reprogramming and limit the number of reprograming factors needed in dermal fibroblast cultures by making chromatin transcriptionally more permissive through histone deacetylase inhibition [97]. This intriguing study demonstrated that pre-treatment of dermal fibroblasts with VPA enhanced iPSC generation when using only three reprograming factors (OSK) rather than all four reprograming factors (OSKM); while VPA treatment alone was insufficient to reprogram cells [97]. In our study, treatment of human L-EPCs with VPA before the transduction was sufficient to enhance iPSC reprogramming. As shown in (Figure 13), VPA pre-treatment in combination with lentiviral transduction and doxycycline exposure increased the expression of Oct4, Sox2 and Klf4, by 11±1 (p=0.017 vs. EPCs transduced without VPA), 405±1 (p=0.001 vs. EPCs transduced without VPA) and 42±2 (p=0.042 vs. EPCs transduced without VPA) fold, respectively. Nanog showed a tendency towards greater expression but this did not attain significance (16±1 fold increase; p=0.108 vs. EPCs transduced without VPA).

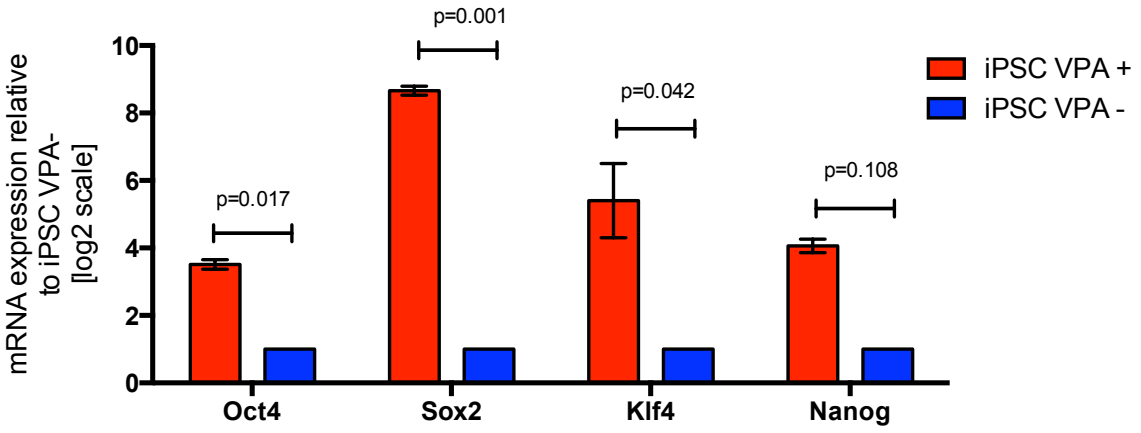

Figure 13: Effect of VPA on OSKN transgene expression. Reprogramming of L-EPCs after pre-treatment with VPA enhanced iPSC generation by increasing the expression of

Klf4, Oct4 and Sox2 compared to L-EPCs transduced without VPA pre-treatment (n=3 cell lines and 3 technical repeats).

Although pre-treatment of L-EPCs with VPA boosted the efficiency of iPSC generation from 0% to 50%, failure to effectively reprogram L-EPCs still occurred. This uniformly represented application of inadequate lentiviral titers ($<10^6$ IU). Therefore the protocol was further refined such that after treatment of L-EPC with VPA, five consecutive infections occurred during centrifugation at 500g/2h. This technical modification was introduced to increase the likelihood of virus integrating into the L-EPCs. To promote L-EPC survival this protocol was over a 48 hour period during which L-EPCs were maintained in EPC media. After lentiviral transduction, genetic reprogramming was induced by replacing EPC culture medium with ESC media supplemented with Dox (2 mg/ml) for 15 days [194].

Silencing of the retroviral OSKM genes during the reprogramming stage represents faithfully reprogrammed cells [57, 58, 206], whereas partially reprogrammed cells represent incomplete silencing of the viral genes and their expression persist to differentiation stages, resulting in tumor formation [55]. To test the activity and leakiness of inducible drug system in the generation of iPSC, we infected dermal fibroblast cells with lv-Oct 4 using the same protocol. Quantitative PCR demonstrated high expression of Oct4 after 10 days of Dox treatment 1991.9±0.1 fold greater than control dermal fibroblast cells ($p=0.001$ vs control DF cells; n=3 DF cell lines and 3 technical repeats). Withdrawal of Dox returned the expression of Oct4 back to the basal level when sampled 10 days ($p=0.9$ vs control DF cells; n=3 DF cell lines and 3 technical repeats). These results provide reassurance that removal of Dox results in lentiviral transgene repression

to permit the endogenous expression of pluripotent genes following the engagement of the pluripotent core transcriptional network [56, 62,194].

The first L-EPC-iPSCs colonies appeared on 5±1 days after Dox treatment; indicating a greater L-EPC reprogramming efficiency than terminally differentiated cells such dermal fibroblast that took taking 16±2 days to emerge in culture using a similar protocol (Chi square value 5.378, $p \leq 0.02$ vs. the expected frequency of iPSCs colonies). Colonies were picked at day 20 based on a human ESC like morphology that showed high nucleus-to-cytoplasm ratios and prominent nucleoli (Figure14). iPSC colonies were expanded and maintained in the absence of Dox for more than 30 passages. The baseline expression of pluripotent transgenes enhanced the efficiency of iPSC generation by 59±7.1 fold when compared to NHDF treated in parallel with a similar protocol (0.8±0.1 vs. 0.01±0.001x % iPSC pre plated cell, respectively; $p<0.001$). Human iPSCs colonies were mechanically picked based on morphology between 20-25 days after Dox induction and re-plated onto matrigel for differentiation. iPSCs were mechanically dissociated for a few passages and then adapted to gentle dissociation passaging [182,183].

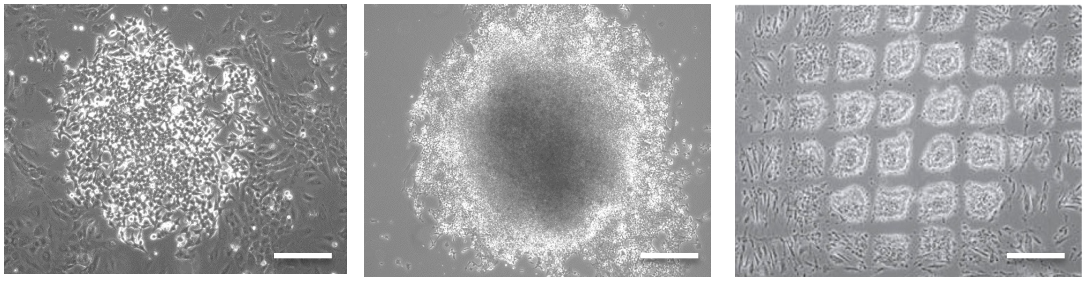

Figure 14: Characterization of L-EPC-iPSCs. Phase contrast images of L-EPC-iPSC at 5 and 20 days following application of Dox demonstrating the characteristic human ESC-like morphology with a high nucleus-to-cytoplasm ratio and prominent nucleoli. Bar indicates **40** um.

The pluripotent potential of iPSCs clones generated from L-EPC lines was confirmed using immunohistochemistry. This analysis demonstrated expression of human ESC-specific surface antigens Tra-1-61, Tra-1-81 and SSEA4 characteristic of ESC phenotype and indicating successful genetic reprogramming (Figure 15).

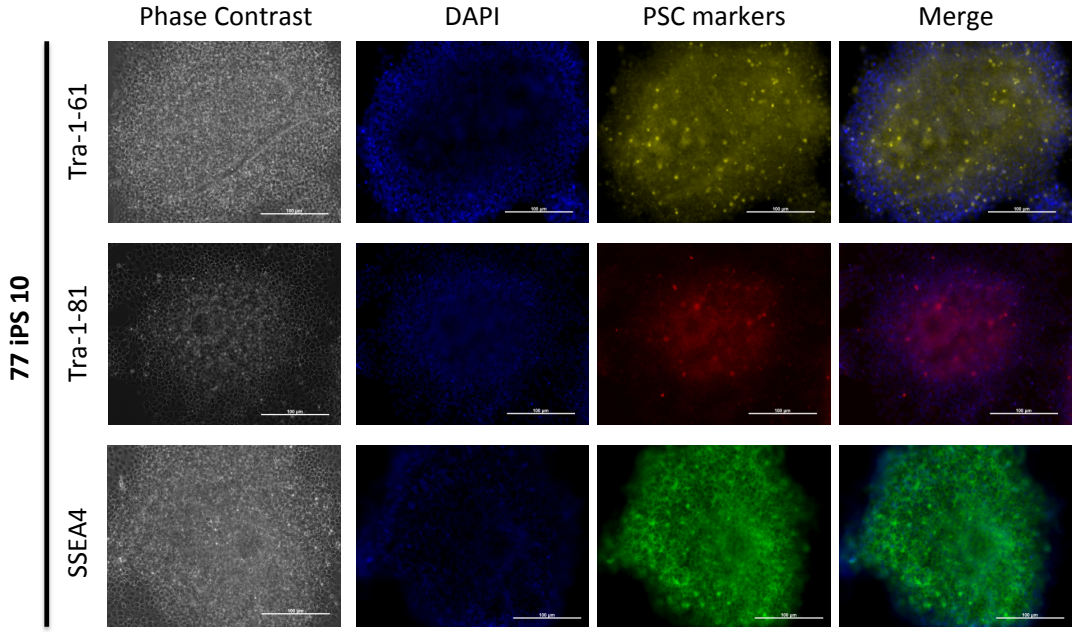

Figure 15: Immunohistochemical characterization of L-EPC-iPSCs. Immunostaining of iPSCs clones generated from L-EPC lines demonstrated expression of ESC-specific surface antigens Tra-1-61, Tra-1-81 and SSEA4 characteristic of embryonic stem cells phenotype- indicating successful genetic reprogramming. Bar indicates 40 um.

Further characterization of L-EPC-iPSCs was performed using qPCR analysis after 10 passages in the absence of Dox treatment. This data demonstrated the expression of Klf4, Oct4, nanog and Sox2 remained elevated within L-EPC-iPSCs by 13.2±1.47 ($p=0.047$ vs. non-transduced L-EPCs), 9.88±1.33 ($p=0.001$ vs. non-transduced L-EPCs),

51±1.37 (p=0.16 vs. non-transduced L-EPCs), 340±1.72 (p=0.001 vs. non-transduced L-EPCs) fold, respectively (Figure 16).

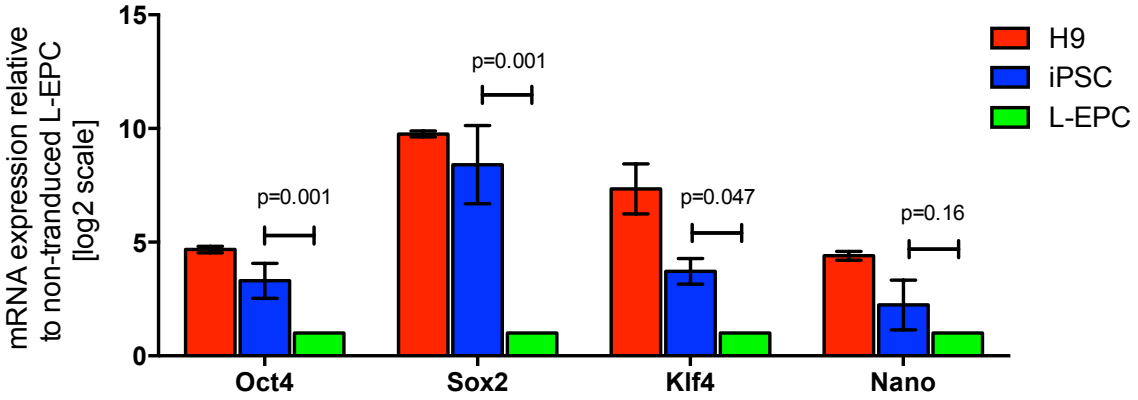

Figure 16: L-EPC-iPSCs express pluripotency markers following extensive subculture and withdrawal of doxycycline. Quantitative PCR analysis demonstrated high expression of pluripotency marker genes Oct4, Sox2, klf4 and Nanog within L-EPC-iPSCs as compared to unmodified L-EPCs.

Telomere length and telomerase activity also regulate the growth of stem cells by protecting DNA lost after cell division. In the absence of telomerase activity, telomeres undergo a progressive shortening with each cell division leading to cellular senescence and eventual apoptosis [334]. In a manner consistent with successful genetic reprogramming, passage 10 L-EPC-iPSCs demonstrated a 51-fold increase in telomerase activity when compared to L-EPCs ($p \leq 0.01$). When compared to the gold standard H9 ESCs, EPC-iPSCs tended to demonstrate lower expression of Klf4 (162.2±1.3 fold, p=0.09), Nanog (21±1.3 fold, p=0.65), Oct4 (25.5±1.2 fold, p=0.49) and Sox2 (867±0.44fold, p=0.005)- which may represent partial reprogramming or, more likely, contamination from non-transduced EPCs during mechanical isolation. This data

indicates that transduction of EPCs with doxycycline-responsive transgenes results in highly efficient reprogramming but necessitates pre-treatment with VPA to ensure adequate activation of the core pluripotent transcriptional network and maintenance of the iPSCs phenotype.

15.3 Directed differentiation of L-EPC-iPSCs into cardiomyocytes

Differentiation of iPSCs into CM involves sequential induction of mesendoderm, cardiogenic mesoderm, cardiovascular precursor cell and cardiomyocyte transgenes. The matrix sandwich monolayer protocol was chosen for investigation as it generates high purity and CM yields by combining Matrigel with cardio-inductive cytokines [256]. In this protocol, iPSCs were seeded on Matrigel coated dish within mTeSR1 media. When cells reach 90% confluence, Matrigel with mTeSR1 media was applied on the monolayer cells for another 1 to 2 days while the cells attained 100% confluency. Given the *in situ* myocardium is composed of CM surrounded by ECM that both maintains structural integrity and provides inductive contact stimuli, the matrigel overlay on monolayer culture cells creates a sandwich of ECM that polarizes the phenotype of undifferentiated epithelial cells to promote an EMT transmission and the generation of CM [335,336].

Based on several preliminary experiments and to further promote cardiovascular differentiation by L-EPC-iPSCs, we adapted the classical matrix sandwich technique by introducing Activin A, bFGF and BMP4 early within the differentiation protocol (Figure 17) [256].

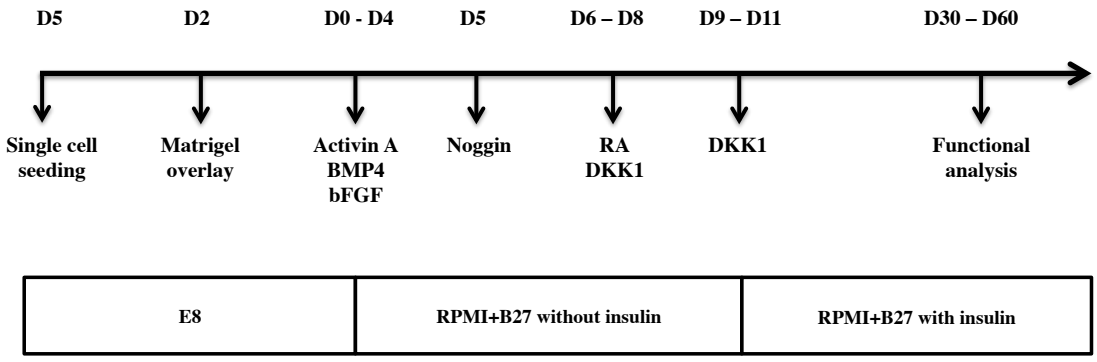

Figure 17: Schematic of the cell culture protocol used to successfully differentiate EPV-iPSCs into atrial CM

Clumps of beating cells appeared between 10 to 15 days after the induction of differentiation and ranged from 4 to 11 CMs per iPSC. The beating area markedly expanded by day 30, at which time undifferentiated cells began to detach from the plate. The morphology of beating areas varied in their shape but was uniformly circular. The average rate of beating areas varied between 30-85 beats/min. There was considerable variability in the beating frequency within the same plate and long pauses (approaching 10 seconds) were not infrequently observed beating cycles. When a beating area exhibited pauses, these frequently were observed as clusters of 8-10 short-coupled beats (8.1±0.91 ms CL) followed by a significant pause. (Figure 18)

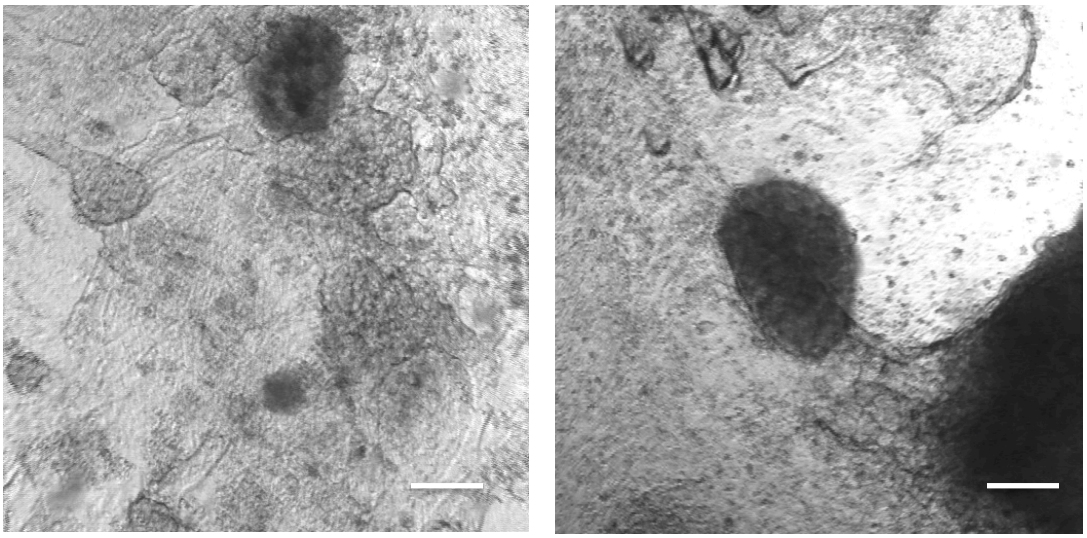

Figure 18: Morphology of beating areas within L-EPC-iPSCs derived CMs. Phase contrast images of beating areas after 15 days of differentiating within a modified Matrigel sandwich protocol. Bar indicates 40 um.

Quantitative PCR demonstrated that EPC-iPSCs derived CMs expressed cTNT after 30 days of differentiation (72±16 fold greater than control H9 cells; p=0.003; Figure 19). The extent to which the modified matrix sandwich method promoted an atrial phenotype was evaluated by profiling mRNA expression of the atrial marker Connexin 40 (Cx40). After 30 days of culture in the modified matrix, Cx40 expression remained negligible (1.5±0.3 fold greater in L-EPC-iPSCs derived CMs as compared to H9 cells (p=0.144)).

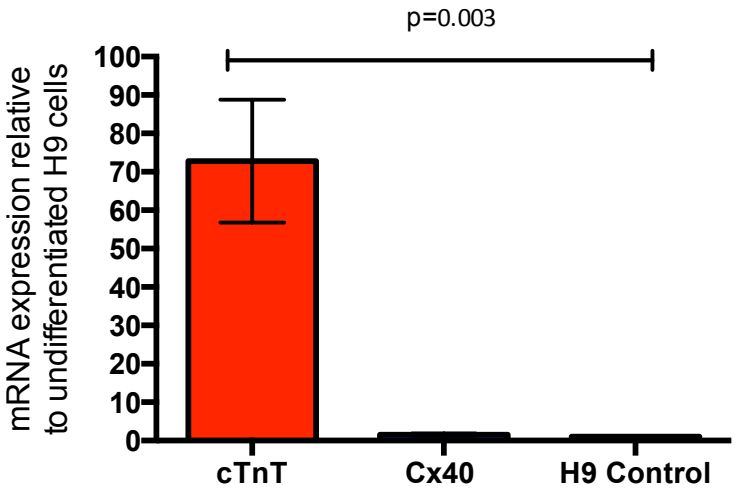

Figure 19: Directed differentiation of pluripotent stem cell sources into atrial cells phenotype. After 15 days in guided cardiogenic culture, qPCR analysis indicated that iPSC-CM cells expressed characteristic cardiomyocyte markers, such as cTnTc (72±16 fold, p=0.003 vs. primitive H9 cells) but only low level expression of atrial specific markers such as Cx 40 (Cx40;1.5±0.3 fold, p=0.144 vs undifferentiated H9 cells). (n=3 cell lines with 3 technical repeats)

This data demonstrates that L-EPC-iPSC can be efficiently guided towards attaining a cardiogenic phenotype but that current protocols do not suffice to guide L-EPC-iPSCs towards the atrial phenotype needed to personalize the study of atrial arrhythmias.

15.4 Directed differentiation of L-EPC-iPSCs into atrial myocytes

Based on these findings, the capacity of inductive retinoic acid culture to direct the fate of L-EPC-iPSCs within a modified matrix sandwich was investigated. This approach was founded on evidence that retinoic acid regulates anterior posterior-polarization of the heart and expression of atrial specific genes [302,278,286]. In a

manner consistent with the notion that EPC-iPSCs represent an immature model of human atria, the expression of Cx40 was markedly reduced when compared inferior to transcript expression within positive control atrial appendage mRNA harvested from patients at the time of clinically indicated cardiac surgery (228±4 fold reduced, p=0.001, n=3). As shown in Figure 20, expression of Cx40 was increased by 56±13 fold (p=0.001 vs. H9) during prolonged culture (60 days) in cardiogenic media. In consistence with study that indicates the assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. This study reported that 60 days of CM differentiation increase the amount and degree of organization of the sarcomeres within the cytoplasm [338]. Thus, optimization experiments demonstrated progressive expression of Cx40 within the newly described protocol and confirmed that 60 days of cardiogenic induction was the ideal time for full atrial maturation of L-EPC-iPSCs.

To confirm the ability of matrix sandwich differentiated EPC-iPSCs to an atrial phenotype, we profiled the expression of several atrial-specific expressed genes (CACNA1H, KCNA5, and MYL4) and Cx43 (abundant in both atria and ventricles) transcripts within EPC-iPSCs CMs after prolonged culture. [339,341]. As demonstrated in Figure 20, expression of the voltage-gated calcium channel transcript CACNA1H and potassium channel transcript KCNA5 was increased by 1382± 1.1 (p=0.001 vs. H9) and 72.00 ± 1.5 (p=0.328 vs. H9) fold respectively within retinoic acid treated L-EPC-iPSCs CMs after 60 days of culture. This expression profile compared favorably with positive control atrial appendage mRNA (p=0.558 and 0.781 for CACNA1H and KCNA5, respectively). In accordance with the notion that prolonged culture in presence of retinoic acid promotes atrial differentiation, the atrial specific transcript MYL4 that

encodes the atrial myosin alkali light chain was increased by 6225±2 fold as compared to undifferentiated cells (p=0.001). This compares favorably with positive control atrial appendage mRNA (35.45±4.01 fold reduced, p=0.416). Finally, the fidelity of prolonged differentiated L-EPC-iPSC CMs to an atrial phenotype was confirmed using qPCR analysis indicating non-significant expression of the ventricular specific transcript Cx43 (p=0.497 vs. H9).

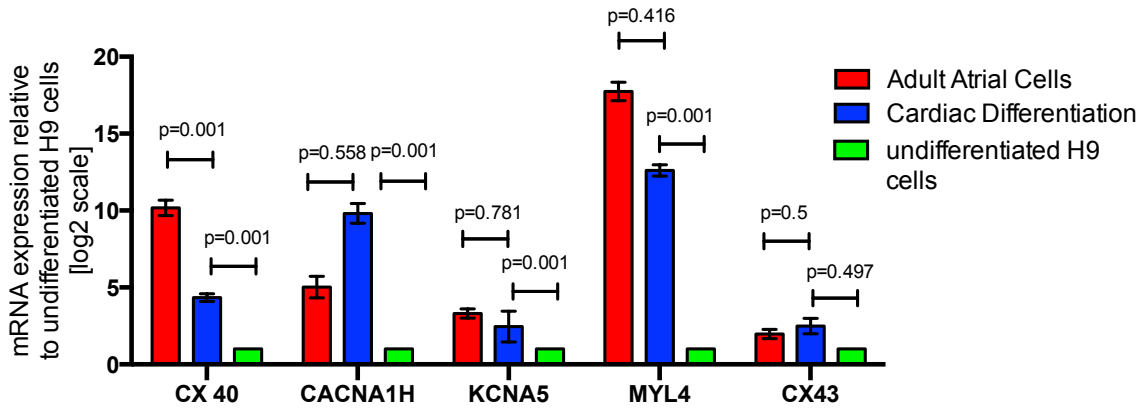

Figure 20: Directed differentiation of pluripotent stem cell sources into atrial cells phenotype. A. After 60 days in cardiogenic guided culture, the expression of atrial specific markers (Cx40 and MYL4) were increased indicating that prolonged culture is needed to guide the expression of this atrial specific marker.

This data illustrates the ability of prolonged L-EPC-iPSCs within a modified matrigel sandwich to guide the expression of atrial specific transcripts while avoid contamination by cells of other cardiac identity.

16. DISCUSSION

This project has demonstrated that L-EPCs are a highly proliferative, homogeneous population that can be cryogenically frozen and thawed without loosing in their characteristics. In contrast to the fibroblasts that need biopsy samples to derive patient-specific iPSCs, the ability to culture iPSCs from a simple clinical blood sample enables the study of a wide range of diseases without risking major trauma or infection. Application to the study of atrial arrhythmias specifically demonstrates that retinoic acid guided differentiation under a prolonged culture protocol is needed to attain atrial phenotype. We are demonstrating the use of primary culture blood derived stem cells to replicate an atrial myocyte phenotype. Success using this protocol provides a template approach to approach to understand the physiological consequences of human mutations as it obviates the need for artificial manipulation of gene expression in heterologous models and avoids the ethical issues inherent in the genetic manipulation of human ESCs. Furthermore, this natural recapitulation of the human diseases avoids artificial gene expression stoichiometry that may distort other relevant systems and physiology.

Efficient generation of iPSC lines from clinical blood samples

The efficiency of iPSCs generation and methods used to deliver reprogramming factors are dependent on the cell type being genetically reprogrammed. Although dermal fibroblasts are commonly used for iPSCs generation, these cells require surgical excision, inefficiently reprogram,[55] and express a high level of detrimental copy number variations creating a heterogeneous clonal population.[148,149] Therefore, alternative cell lines to generate iPSCs are needed to minimize the risk of additional mutations and enhance genetic reprogramming while avoiding invasive procedures. Peripheral blood

provides an attractive alternative starting cell because it is easily accessible, minimally invasive and has less exposure to environmental mutagens/toxins.[154] It follows that several studies have successfully reprogramed blood cells such as T cells and B cells to iPSCs but reprogramming is limited by the modest expansion in culture, low reprogramming efficiency and the presence of DNA rearrangements resulting from the generation of antigen specific surface immunoglobulins.[150–152] Additionally, one study paradoxically demonstrated that B cell derived iPSCs failed to differentiate into B cells- a finding attributed to the generation of defective Pax5 during iPSC reprogramming.[151] It also makes teleological sense that cell sources naturally expressing endogenously low levels of the key reprogramming factors may require less genetic manipulation to become iPSCs. While investigators have reported successful reprogramming of primary CD34+ hematopoietic progenitor cells to iPSCs, the low amount of CD34+ cells in peripheral blood (estimated less than 0.1%) is a key factor limiting the availability of these cells for reprogramming.[154] As such, highly proliferative and robust culture guided EPCs have been used for iPSCs generation.[182,183] In these studies, EPC-iPSCs demonstrated a normal karyotype, expressed pluripotent surface markers (e.g., Tra-1-60) and differentiated into all three germ layers. In contrast to DF-iPSCs, EPC-iPSCs had fewer CNVs as compared with their parent late EPC line (indicating a greater degree of genetic stability in culture) while recapitulating a mutation in the gene encoding the bone morphogenetic protein type II receptor from patients with primary pulmonary hypertension. Collectively, these studies and our data indicate that EPCs provide a valuable tool for iPSCs generation with the capacity to differentiate into somatic targets.

In his seminal paper, Yamanaka cultured iPSCs using retrovirus encoding Oct4, Sox2, Klf4, and C-Myc [55]. It was indicated that retroviral transduction causes genomic insertion of transgenes, which may trigger tumor formation. The re-expression of C-Myc transgene is the major cause for tumor formation that observed in iPSCs [57]. In contrast to the previously published papers that used a traditional retrovirus method for reprogramming, we have used a doxycycline-inducible lentiviral system encoding four reprogramming factors Oct4, and Sox2, Kl4 and Nanog to provide the temporal control for exogenous factor expression to produce iPSCs. iPSCs produced with this system have a pluripotent profile similar to ESCs. While transgene "leak" has been demonstrated using the Dox system, [205] we did not detect partial reprogramming or transcript leak after Dox removal [194]. The advantages of the inducible drug system over a constitutive expression system are that ''self-selection'' of reprogrammed cells in the absence of drug reduces the risk of oncogenic transformation. After 15 days, Dox was removed and stable program while unstable reprogramming transformed colonies revert to a differentiated phenotype. Therefore, L-EPCs genetically reprogrammed using the Dox inducible system display high reprogramming efficiencies taking just 5 days to appear in culture and forming iPSC colonies similar to ESC including morphology, proliferation, feeder dependence, surface markers, gene expression, telomerase activities indicating that these cells are efficiently reprogrammed and do not depend on continuous expression of the transgenes for self renewal. Thus our data are consistent with the notion that progenitors cells are more amenable to transcription factor-induced remodeling, thus more efficiently reprogrammed.

Differentiation of human EPC-iPSC into cardiac chamber-specific cardiomyocytes

Using human ESCs in the differentiation has its ethical concerns that can be avoided by using human iPSCs, but this requires the development of better techniques. Development of the mammalian heart involves a series of morphogenetic processes that are controlled in response to extracellular signaling to guide the differentiation of pluripotent stem cells towards a cardiac fate.[208] Increasing understanding of this early cardiac development from animal models and ESCs has improved the ability to generate CMs from pluripotent cells using culture guided differentiation [211,213]. The cardiac differentiation protocols have become more refined and efficient with several iterations but these techniques provide low yield and immature cardiomyocytes. However, a recent matrix sandwich protocol has been developed to enhance CM generation by combining cardiac induction cytokines and contact mediated stimulation using extra-cellular matrix (ECM).[25]

To date, characterization of ESC and iPSC derived cardiomyocytes has focused upon the expression of cardiac-specific transcription factors (e.g. Nkx2.5, GATA4, MEF2c, Tbx-5, and Tbx-20), sarcomeric proteins (e.g. α-actinin, troponins, sarcomere myosin heavy chain, atrial and ventricular myosin light chains and tropomyosin), gap junction proteins and other cardiac-specific proteins (e.g. atrial natriuretic peptide, creatinine kinase-MB and myoglobin) without regard towards attaining chamber-specific cell type.[212,297] This distinction is crucial as the electrophysiological properties of many common arrhythmias are chamber specific- supporting the notion that iPSC CM need to be directed towards a specific cardiac cell type (i.e., atrial or ventricular) to fully understand the interplay between genetic changes and patient disease. Zhang et al

provide the only work supporting direct differentiation of established ESC lines toward atrial and ventricular fates [256]. In this report, the addition of retinoic acid promoted cardiac differentiation toward atrial or ventricular fate. Treatment the cells with retinoic acid from days 6 to 8 directed the differentiation into atrial myocyte fate while retinoid inhibitor prompted the adoption of a ventricular myocyte fate. This is a promising result, especially as the efficiency of the protocol is usually highly cell line dependent and same protocols do not always work in a similar manner with individual human ESC and human iPSC lines [337]. We have shown a timing application of cardiac induction by extracellular matrix and cytokine factors that results in the robust cardiac differentiation from L-EPC-iPSC. The overlay of monolayer-cultured PSCs with Matrigel along with cytokine treatment induces efficient generation of CMs. As indicated with previous work on cardiac differentiation protocols, these protocols exhibit heterogeneous CM cell types including nodal-like, atrial-like and ventricular-like CMs based on electrophysiological properties [215]. However, the majority of CMs are ventricular-like (which estimated more than 80%). Previous studies on animal model have shown that retinoid signaling specified sinoatrial cell fate, whereas ventricular fate is determined in the absence of retinoid acid [268]. Moreover, studies have suggested that the CMs maturation develop adult-like phenotype with time in culture [199].

The atrial myocyte fate was specified by exogenous retinoid acid treatment and exhibits progressive maturation in culture after 60 days in culture based on high expression of atrial gene Cx40. Profile expression of several cardiac (CACNA1H and KCNA5), atrial (MYL4) specific transcripts represented high expression level within L-EPC-iPSCs CMs. We indicated low expression of ventricle (Cx43), resulting in

specification of atrial myocyte within L-EPC-iPSCs CMs. Our results indicated that the activation of retinoid signals direct the atrial specification of cardiac differentiating. Conversely, a previous study on mouse ESCs represented that the specification of ventricular CM is enhanced by retinoid acid application [293]. This result showed differences between the differentiation culture systems (i.e., mouse ESCs vs human ESCs vs iPSCs), method of differentiation (i.e., EB vs monolayer method), and timing of application could affect on cardiac specification.

17. Conclusions

EPCs provide a stable platform for genetic reprogramming into a pluripotent state using a doxycycline conditional expression system that avoids re-expression of oncogenic/pluripotent factors. Human EPC-derived iPSC can be differentiated into functional CM that expresses characteristic markers of atrial identity. This work provided the platform for our study to develop an efficient protocol that enables the in vivo culture human atrial myocytes from clinical blood samples. These tissue specific models of disease will help to better understand patient specific mutations at a fundamental level and will open the gateway for truly personalized medicine through individualized therapies.

18. REFERENCES

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