Human Pluripotent Stem Cell-Derived Cardiac Stromal Cells and Their Applications in Regenerative Medicine

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Abstract:

Coronary heart disease is one of the leading causes of death in the United States. Recent advances in stem cell biology have led to the development and engineering of human pluripotent stem cell (hPSC)-derived cardiac cells and tissues for application in cellular therapy and cardiotoxicity studies. Initial studies in this area have largely focused on improving differentiation efficiency and maturation states of cardiomyocytes. However, other cell types in the heart, including endothelial and stromal cells, play crucial roles in cardiac development, injury response, and cardiomyocyte function. This review discusses recent advances in differentiation of hPSCs to cardiac stromal cells, identification and classification of cardiac stromal cell types, and application of hPSC-derived cardiac stromal cells and tissues containing these cells in regenerative and drug development applications.

Keywords: cardiac, stromal cells, human pluripotent stem cells, cardiomyocytes

Abbreviations: FGF2, basic fibroblast growth factor, **β-MHC,** beta-myosin heavy chain, **BMP4,** bone morphogenic protein 4, **T,** brachyury, **CAPN9,** calpain 9, **CNN1,** calponin 1, **cTnT,** cardiac troponin T, **CM**, cardiomyocyte, **COL3A1,** collagen type III alpha 1 chain**, Cx40,** connexin 40, **Cx43,** connexin 43, **DDR2,** discoidin domain receptor tyrosine kinase 2, **EGM2,** endothelium growth medium-2, **ELISA,** enzyme-linked immunosorbent assay, **EGF,** epidermal growth factor, **ECM,** extracellular matrix, **FBN1,** fibrillin 1, **FB,** fibroblast, **FACS**, fluorescence activated cell sorting, **Gata4,** GATA binding protein 4, **Gli1,** GLI family zing finger 1, **HAND2,** heart- and neural crest derivatives-expressed protein 2, **HOXA2,** homeobox protein Hox-A2, **hPSC,** human pluripotent stem cell, **Kir2.1,** inward rectifier potassium channel 2, **KDR,** kinase

insert domain receptor, **MACS,** magnetic activated cell sorting, **MSC,** mesenchymal stromal cell, **MEK,** mitogen-activated protein kinase kinase, **MIXL1,** mix paired-like homeobox, **MSX2,** Msh homeobox 2, **MYOCD,** myocardin, **Mef2c,** myocyte enhance factor 2c, **NGFR,** nerve growth factor receptor, **NCAM,** neural cell adhesion molecule, **NG2,** neuron-glial antigen 2, **OCT4,** octamer-binding transcription factor 4, **PITX2,** paired-like homeodomain transcription factor 2, **PPRX1,** paired related homeobox 1, **PTHrP,** parathyroid hormone-related protein, **PC,** pericyte, **POSTN,** periostin, **PDGFBB,** platelet-derived growth factor, **PDGFRα,** platelet derived growth factor receptor alpha, **PDGFRβ,** platelet derived growth factor receptor beta, **PGA,** polyglycolic acid, **SMA and ACTA2,** smooth muscle actin, **SMC,** smooth muscle cell, **SMMHC and MYH11,** smooth muscle myosin heavy chain, **SM22,** smooth muscle protein 22-alpha, **SMTN,** smoothelin, **Nav1.5,** sodium voltage-gated channel alpha subunit 5, **TBX1,** T-box 1, **TBX5,** T-box 5, **TBX18,** T-box 18, **TFAP2A,** transcription factor AP-2 alpha, **TCF21,** transcription factor 21, **TAGLN,** transgelin**, TGFβ**, transforming growth factor beta, **HUVEC,** human umbilical vein endothelial cell, **VCAM1,** vascular cell adhesion protein 1, **VEGF,** vascular endothelial growth factor, **VIM,** vimentin, **WT1,** Wilms tumor protein

1.1 Introduction

Since the discovery of human embryonic stem cells and induced pluripotent stem cells, collectively known as human pluripotent stem cells (hPSCs) (Takahashi and Yamanaka, 2006; Thomson et al., 1998; Yu et al., 2007), researchers have worked to develop simple, rapid, and robust processes to direct these hPSCs to terminally differentiated cell types for *in vitro* and *in vivo* applications. One of the cell types of interest are hPSC-derived cardiac cells, because they can be used in cell-based therapies to improve heart function in patients suffering from heart disease, the leading cause of death worldwide (CDC, 2018). In 2017, 8% of patients awaiting heart transplant died and approximately 50% of patients were still awaiting treatment after 6 months, so the urgent need for cardiac cellular therapies continues to drive stem cell research in this area (Colvin et al., 2019). Furthermore, 13% of all drugs removed from the market from 1950 to 2017 due to safety concerns were the result of cardiac toxicity (Kocadal et al., 2019), indicating the need for a more predictive *in vitro* model of human cardiotoxicity.

The heart is one of the first organs formed during development and is required for the embryo to grow beyond the size at which nutrients can be delivered by diffusion (Vincent and Buckingham, 2010). Structurally, the heart wall is comprised of three layers – the endocardium, myocardium, and epicardium (Figure 1). The myocardium is the thickest of these layers and contains cardiomyocytes (CMs), cardiac stromal cells, and blood vessels. Smooth muscle cells (SMCs) and pericytes (PCs) are perivascularly located and regulate vascular structure and blood flow. Fibroblasts (FBs), mesenchymal stromal cells (MSCs), and PCs can be found in the myocardial tissue and are thought to support heart tissue by providing extracellular matrix (ECM) and electrical coupling amongst other paracrine signaling induced effects.

To date, most efforts have focused on derivation and maturation of hPSC-derived CMs, because as the contractile cells of the heart they hold promise to restore heart function and are thought to be the most susceptible to drug-induced cardiotoxicity (Laflamme et al., 2007; Lian et al., 2015). However, CMs are estimated to comprise only 30-40% of the cells in the human adult heart (Zhou and Pu, 2016). Interactions with other cardiac cell types are crucial for proper CM and cardiac tissue development and changes in interactions contribute to disease phenotypes (Anbara et al., 2019; Bruns et al., 2019; Cartledge et al., 2015; Colliva et al., 2019; Dunn and Palecek, 2018; Furtado et al., 2016; Hastie, 2017; Kapustin et al., 2017; Kivelä et al., 2019; Liu et al., 2019a; Mayourian et al., 2018; Moyes and Hobbs, 2019; Nagaraju et al., 2019; Peters et al., 2019; Ranchoux et al., 2019; Roostalu et al., 2018; Tong et al., 2019; Wang et al., 2018; Zhang et al., 2017; Zouein et al., 2019). Researchers have included these non-myocyte cardiac cell types in 2D cocultures and 3D engineered tissues to better mimic the native heart niche and investigate the role of other cardiac cell types on CMs (Dunn and Palecek, 2018; Kapuria et al., 2018; Morishige et al., 2019; Varzideh et al., 2019; Zhang et al., 2019c).

Cardiac stromal cells, defined as the connective tissue cells of the heart, include FBs, PCs, MSCs, and SMCs that have been shown to contribute to heart function through contraction and ECM deposition amongst other paracrine signaling induced effects (Avolio et al., 2017; Cartledge et al., 2015; Dueñas et al., 2017; Kohl et al., 2005; Mayourian et al., 2018; Ongstad and Kohl, 2016; Plotnikov et al., 2008; Ravenscroft et al., 2016). Further analysis of these populations via lineage tracing studies have demonstrated that cardiac stromal cells comprise a heterogeneous population, which can be correlated to functional differences (Alanazi et al., 2019; Bargehr et al., 2016; Dias Moura Prazeres et al., 2017; Ivey and Tallquist, 2016; Kanisicak et al., 2016; Liu et al., 2018a; Moore-Morris et al., 2014; Pfaltzgraff and Bader, 2015; Sheriff et al., 2018; Subramani et

al., 2016). Transcriptomics, especially single cell sequencing, has further emphasized similarities and differences between the multitude of cell subtypes in the heart, although more research is needed to better understand the diversity and complexity of cardiac stromal cells (Cao et al., 2016; Farbehi et al., 2019; Jonsson et al., 2016; Liu et al., 2019b; Sacchetti et al., 2016; Skelly et al., 2018). In this review, we discuss current protocols for differentiation from hPSCs to cardiac stromal cells, identification and classification of stromal cells, and applications of hPSC-derived cardiac stromal cells.

3.1 Key Identifiers of Cardiac Stromal Cells

Identifying and classifying cardiac stromal cells is a key step in developing processes to differentiate hPSCs to these cells. However, similarities in marker expression, structure, and function of different cardiac stromal cell types complicates their classification. Furthermore, researchers have used different markers and functional assays in cardiac stromal cell characterization, making comparisons between differentiation protocols difficult. We suggest that researchers ensure that the differentiation protocol they chose generates cells with the desired phenotype for their particular application. We discuss common protein markers and functional assays below.

3.1.1 Protein markers

PCs typically are identified by expression of neuron-glial antigen 2 (NG2), platelet derived growth factor receptor beta (PDGFRβ), and smooth muscle protein 22-alpha (SM22) (Kumar et al., 2017; Orlova et al., 2014a; Stebbins et al., 2019). SMCs also express SM22 as well as smooth muscle actin (SMA), smooth muscle myosin heavy chain (SMMHC), and calponin (Cheung et al.,

2012; Wanjare et al., 2013; Yang et al., 2016). Numerous FB markers have been proposed, however many of these were also found to be expressed in other cardiac cell types. For example, CD90 is also expressed in MSCs and endothelial cells (Furtado et al., 2014; Rege and Hagood, 2006). Within the $CD90^+$ and $CD90^-$ populations of primary cardiac FBs, there was varying expression of CD29, CD44, CD49e, CD51, CD140a, and SCA1, markers commonly used to stain MSCs, suggesting that cardiac FB populations are heterogeneous (Furtado et al., 2014). Currently, FBs are identified by expression of TE7 fibroblast marker, periostin (POSTN), vimentin (VIM) and TCF21 (Furtado et al., 2014; Ivey and Tallquist, 2016; Zhang et al., 2019c). However, TE7 binds to an unknown antigen, VIM is a general marker of mesenchymal fate, TCF21 is an epicardial-derived transcription factor and does not identify all FBs, and POSTN is an ECM protein. MSCs are express many cell surface markers including CD44, CD73, and CD105, but definitive markers of MSCs have been highly debated (Chen et al., 2012; Chijimatsu et al., 2017; Tran et al., 2012).

Unique markers that identify cardiac stromal cell populations have not been conclusively established. The majority of proposed markers are expressed in multiple cardiac stromal cells at various levels. Secreted factors such as collagen, elastin, and fibronectin are not cell specific and cannot be used for antibody-based single cell identification. Similarly, gap junction proteins such as connexin 40 (Cx40) and connexin 43 (Cx43) are commonly expressed by many cell types and are not amenable for antibody-based sorting. Negative markers, such as CD31 to eliminate endothelial cells and CD45 for leukocytes, have also been suggested in combination with positive markers. However, without a single definitive marker, lineage tracing studies and other assays in which cell types are isolated are susceptible to misinterpretation. Comparison of expression between differentiation protocols is difficult since not all authors have used the same markers. We

have summarized markers that have been used in published studies to classify cardiac stromal cells in Table 1.

3.1.2 Functional assays

Since there are no definitive protein markers for most types of cardiac stromal cells, functional assays can be used to compare hPSC-derived stromal cells to their putative *in vivo* corollary. A major challenge is designing assays that will distinguish various stromal cells since they have similar functions (Christy et al., 2019; Hookway et al., 2019). Assays that have commonly been used to characterize cardiac stromal cells are summarized in Table 2.

Motility of stromal cells is important for recruitment of PCs, SMCs, FBs, and MSCs during development and post-injury. Migration is not specific to cardiac stromal cells but is an important characteristic (Costa et al., 2017; Yang et al., 2016; Zhang et al., 2019a). The scratch assay, or wound healing assay, has also been widely used to study endothelial and epithelial cell migration (Iwanabe et al., 2016; Jonkman et al., 2014; Liang et al., 2007; Rieger et al., 2018; Wu et al., 2017). In this assay, cells are plated and a pipette tip or other physical tool is used to scratch an area, effectively removing cells. (Cappiello et al., 2018; De Ieso and Pei, 2018; Jonkman et al., 2014). Then, migration into the gap is typically measured over 12-30 hours using live-cell time lapse microscopy. Cardiac stromal cell migration can be accelerated with the addition of platelet-derived growth factor (PDGFBB) or Angiotensin-II receptor agonists (Zhang et al., 2019a; Zhao et al., 2019; Zhou et al., 2016). Although the scratch assay is relatively inexpensive and easy to perform, rate of migration varies widely amongst trials and between researchers, and may not be relevant to many heart injuries which do not involve wound closure, including myocardial infarction.

Cardiac stromal cells are situated near CMs, endothelial cells, and epicardial cells and interactions between the various cell types are critical during development, response to injury and homeostasis (Anbara et al., 2019; Bruns et al., 2019; Cartledge et al., 2015; Colliva et al., 2019; Dunn and Palecek, 2018; Furtado et al., 2016; Hastie, 2017; Kapustin et al., 2017; Kivelä et al., 2019; Liu et al., 2019a; Mayourian et al., 2018; Nagaraju et al., 2019; Peters et al., 2019; Ranchoux et al., 2019; Roostalu et al., 2018; Zhang et al., 2017; Zouein et al., 2019). Stromal cells can be cocultured with endothelial cells to evaluate their ability to mediate tubule formation and localization; a key characteristic for PCs, MSCs, and SMCs (Bargehr et al., 2016; Chen et al., 2015; Wanjare et al., 2013). Electrical coupling and current propagation in cocultures with CMs is of particular interest for FBs but has been studied in MSCs as well (Cartledge et al., 2015; Hookway et al., 2019; Kohl et al., 2005; Nakanishi et al., 2019; Zhang et al., 2019a, 2019c). For example, optical mapping of cardiomyocyte monolayers stained with a membrane potential sensitive dye has been used to show that CMs cocultured with dermal FBs exhibit slower electrical propagation compared to CMs cocultured with cardiac FBs (Zhang et al., 2019c). Although both tubule formation and electrical conduction are difficult functional assays to perform with low throughput, they likely provide valuable insight into vascularization and electrical properties of hPSC-derived cardiac tissues.

PCs and SMCs located near and around blood vessels are known to regulate vessel constriction (Brozovich et al., 2016; Yamazaki and Mukouyama, 2018). Activated FBs, or myofibroblasts, that arise under disease conditions have phenotypes more similar to SMCs, including contractile properties. *In vitro*, cell contraction is analyzed using live-cell time lapse imaging after treatment with drugs increase intracellular Ca^{2+} levels. Treatments typically take between 5-30 minutes and drugs include: potassium chloride, Endothelin-1, U46619, and

carbachol (Bajpai et al., 2012; Bargehr et al., 2016; Costa et al., 2017; Iyer et al., 2015; Kakkar et al., 2019; Kumar et al., 2017; Lis et al.; Zhou et al., 2016). Alternatively, researchers have quantified contraction in a gel lattice (Lin et al., 2012, 2019; Yang et al., 2016). Cells can be suspended in a number of different gels including fibrinogen/thrombin, collagen, or Matrigel and contraction is measured as a function of change in gel surface area, thickness, and stiffness (Ehrlich and Moyer, 2013; Han et al., 2018; Jin et al., 2015; Tarpila et al., 1998; Yang et al., 2016). Since altering contractile properties in stromal cells is one way to clinically control blood pressure, these assays are highly relevant.

The role, function, and nomenclature of MSCs from various origins has recently been debated as heterogeneity within the MSC population is becoming better understood (Bargehr et al., 2016; Caplan, 2019; Marklein et al., 2018; Phinney, 2012; Phinney and Sensebé, 2013; Tan et al., 2019; Wilson et al., 2019). Initially, the tri-lineage differentiation potential of MSCs to osteoblasts, adipocytes, and chondrocytes was used as a defining criterion (Kobolak et al., 2016). After treatment with various inductive media, differentiated cells are typically stained for Alizarin Red S, Oil-Red O, and Toluidine Blue (Dominici et al., 2006; Kobolak et al., 2016; Wang et al., 2019). Now, MSCs are often defined by their ability to modulate immune response, since MSCs have primarily been used for immunomodulatory clinical applications (Caplan, 2019; Dominici et al., 2006; Galipeau et al., 2016; Kennedy-Lydon, 2017; Kobolak et al., 2016; Wilson et al., 2019; de Wolf et al., 2017; Zarychta-Wiśniewska et al., 2019). For example, MSCs treated with INF-γ, IL-4, or IL-13, respond by becoming elongated, which can be measured by live-cell microscopy (Marklein et al., 2018; de Wolf et al., 2017, 2017). More rigorously, MSC regulation of T-cell inflammatory secretory profiles can be analyzed using an enzyme-linked immunosorbent assay (ELISA) on cocultured systems (Wang et al., 2019; de Wolf et al., 2017, 2017). Although these

assays may be useful, it is yet to be determined if they form the basis for sufficient release criteria for a successful MSC-based immunotherapy.

FBs are known to change during disease states to a filamentous myofibroblast, or activated cell type (Baum and Duffy, 2011; Ivey and Tallquist, 2016; Shinde et al., 2017; Zhou et al., 2017). Treatment with transforming growth factor beta (TGFβ-1) or Angiotensin-II increases ECM production similar to scar formation (Humeres and Frangogiannis, 2019; Ivey and Tallquist, 2016; Xu et al., 2018; Zhang et al., 2019a). FB activation to myofibroblasts is also typically signified by increased SMA expression, measured by immunocytochemistry or flow cytometry (Humeres and Frangogiannis, 2019; Ivey and Tallquist, 2016; Shinde et al., 2017; Xu et al., 2018; Zhang et al., 2019a, 2019c; Zhou et al., 2017). FBs that can be activated into this stress phenotype may more closely resemble primary adult FBs and could potentially be used to screen and analyze fibrotic and anti-fibrotic drugs.

4.1 Specification, Differentiation and Maturation of Cardiac Stromal Cells

hPSC differentiation protocols typically strive to guide cells through developmental pathways to generate somatic cell types of interest, so we will first briefly discuss cardiac development followed by differentiation protocols for each cell type and will direct readers to more in depth developmental reviews for further detail.

Early in human development the three germ layers, endoderm, ectoderm, and mesoderm, are specified. The majority of cardiac cells arise from the BRY^+ $EOMES^+$ mesoderm, become $MESP1^+$ cardiac mesoderm, and divide into two heart fields comprising the cardiac crescent (Brade et al., 2013; Kelly et al., 2014). These heart fields form tubes which loop to form the four chambers of the heart – the left ventricle, right ventricle, left atrium, and right atrium – which has

been reviewed in detail (Brade et al., 2013; Vincent and Buckingham, 2010). A fully developed heart wall has three distinct layers: the endocardium which is primarily comprised of specialized endothelial cells, the myocardium which includes CMs, and the epicardium made up of epicardial cells.

Cardiac stromal cells arise from three lineages: the endocardium, cardiac neural crest, and the proepicardium. The endocardium contributes to the cardiac cushion, valves, trabecular endocardial cells, and mural cells of the heart through endothelial to mesenchymal transition (EndoMT) (Zhang Hui et al., 2018). The cardiac neural crest is a subpopulation of the ectoderm-derived cranial neural crest cells specified by expression of SOX9 (Prasad et al., 2019). Cardiac neural crest cells migrate to form the aorta and pulmonary trunk (Brade et al., 2013; Moore-Morris et al., 2014; Prasad et al., 2019). The proepicardium is a transiently distinct structure which forms a single epicardial cell layer that encases the myocardium (Niderla-BieliŃska et al., 2018; Santini et al., 2016; Zhou et al., 2008). Some of the epicardial cells migrate into the myocardium and undergo epithelial to mesenchymal transition (EMT) (Katz et al., 2012; Moore-Morris et al., 2016).

During differentiation hPSCs do not always follow the same developmental path or timing as cells do *in vivo*. A review of cardiac stromal cell differentiation protocols has been summarized in Figure 2 and Table 3. Markers to indicate the germ layers and cardiac progenitors transited during cardiac stromal cell differentiation have been included in Table 4. hPSC-derived PCs, MSCs, and some FBs can proliferate and maintain stromal identity *in vitro* for 5-10 passages, similar to primary cells, based on division rates and expression of cell specific markers (Chen et al., 2012; Chijimatsu et al., 2017; Kumar et al., 2017; Laco et al., 2018; Stebbins et al., 2019; Zhang et al., 2019c). However, whether these hPSC-derived stromal cells can maintain

functionality such as interactions with hPSC-derived CMs or regenerative capabilities over multiple passages has yet to be determined.

4.1.1 Cardiac MSCs

Early protocols to differentiate hPSCs to MSCs using serum or mouse embryonic fibroblast conditioned media supplemented with basic fibroblast growth factor (FGF2) did not demonstrate germ layer progression (Barberi et al., 2005; Trivedi and Hematti, 2008). Without generation of a specific lineage, there was some concern in the field about the similarity to *in vivo* cell types. Since then, a monolayer protocol was developed to differentiate hPSCs to MSCs through mesoderm (homeobox protein Hox-A2 (HOXA2), neural cell adhesion molecule (NCAM), Msh homeobox 2 (MSX2)) with addition of TGFβ inhibitor SB431542 for 10 days (Chen et al., 2012). Then, cells were cultured in serum-containing medium for 2 days and resulting MSCs expressed CD29, CD44, CD73, CD90, CD105, and CD146, had tri-lineage differentiation potential, and exhibited transcriptomic profiles similar to primary MSCs. Another group generated MSCs by treating hPSCs with Activin A and bone morphogenetic protein 4 (BMP4) signaling for 3 days to generate mesoderm, as shown by upregulation of brachyury (T) and mix paired-like homeobox (MIXL1) proteins and downregulation of octamer-binding transcription factor 4 (OCT4) protein (Tran et al., 2012). Following this, they treated the mesoderm cells with FGF2 and epidermal growth factor (EGF) in serum replacement supplemented medium for 10 days followed by magnetic activated cell sorting (MACS) of CD105+ cells. The resulting population expressed CD29, CD44, CD73, and CD90 and had tri-lineage differentiation potential. MSCs have also been differentiated through neural crest cells marked by transcription factor AP-2 alpha (TFAP2A), SOX10, and nerve growth factor receptor (NGFR) and decreased transcriptome level expression of *Oct3/4* and *Nanog*

(Chijimatsu et al., 2017). After treatment with MSC induction medium containing FGF2 and serum, the resulting population expressed CD44, CD73, and CD105 at similar levels to primary bone marrow-derived MSCs and could be further differentiated into osteogenic and chondrogenic lineages. In another protocol, PCs, MSCs, and SMCs were differentiated through mesoderm and mesenchymangioblast colonies expressing PDGFRB, CD271, and paired related homeobox 1 (PPRX1) (Kumar et al., 2017; Vodyanik et al., 2010). MSCs were differentiated by culture in mesenchymal serum-free expansion medium supplemented with FGF, expressed CD73, CD140a, CD140b, CD146, and CD166, had tri-lineage differentiation potential, were contractile, and associated with human umbilical vein cell (HUVEC) networks in coculture. Very little is known about cardiac-specific MSC-like cells, so it is difficult to say if any of these hPSC-MSCs have similar phenotypes to an *in vivo* counterpart.

4.1.2 Cardiac PCs

PCs arise from the endocardium, and upregulation of Notch3 in PCs gives rise to coronary artery SMCs (Chen et al., 2016; Volz et al., 2015). PCs have also been shown to originate from the epicardium via lineage tracing studies of Wilms tumor protein (WT1), T-box 18 (TBX18), and transcription factor 21 (TCF21) (Cao and Poss, 2018; Rudat and Kispert, 2012; Yamaguchi et al., 2015; Yamazaki and Mukouyama, 2018). In other organs, PCs have been shown derive from neural crest, so it is also likely that some PCs in the heart may arise from cardiac neural crest (Prasad et al., 2019; Yamazaki and Mukouyama, 2018).

PCs have been differentiated through mesoderm-derived vascular progenitors in two separate protocols. In the first, mesoderm was induced with Activin A, BMP4, vascular endothelial growth factor (VEGF), and CHIR99021 for three days followed by vascular specification medium

containing VEGF and TGFβ inhibitor SB431542 with transcript level upregulation of *T*, *EVT2*, and kinase insert domain receptor (*KDR)* during this time period (Orlova et al., 2014a, 2014b). At day 10, the $CD31^-$ population was generated with addition of TGFβ and PDGFBB in serum-containing medium for 3 days. The resulting population expressed many protein markers including SM22, PDGRβ, NG2, CD73, CD44, and CD105 and upon coculture with hPSC-derived endothelial cells formed vascular-like structures. Alternatively, mesenchymangioblasts treated with PDFBB and SB421542 led to PCs expressing NG2, CD274, and vascular cell adhesion protein 1 (VCAM1) which were contractile and formed networks in coculture with HUVECs (Kumar et al., 2017; Vodyanik et al., 2010). PCs have also been differentiated from hPSCs via addition of CHIR99021, SB431642, dorsomorphin and FGF2 through ectoderm-derived NGFR+HNK1+ neural crest cells which were purified via MACs (Stebbins et al., 2019). After nine days in serum containing medium, PCs arose which expressed NG2, PDGFRB, SM22, and calponin and formed vascular structures in HUVEC cocultures. These PC were suggested to be brain PC-like cells due to their capability to induce blood-brain barrier properties in cocultured brain microvascular endothelial cells. Each of these three protocols offers a general method to produce PCs, but little is currently known about tissue-specific PC development. We expect that as our understanding of PC development advances, researchers will develop cardiac-specific hPSC-PCs.

4.1.3 Cardiac FBs

Cardiac fibroblasts have been shown to arise from various lineages. Using Tie-2 lineage tracing in a mouse model, endocardial-derived FBs were found to contribute primarily to the ventricles and septum (Ali et al., 2014). Other lineage tracing studies have shown that a population

of FBs arise from epicardial cells (Acharya et al., 2012; Ali et al., 2014; Cai et al., 2008; Cao and Poss, 2018; Gittenberger-de Groot et al., 1998; Rudat and Kispert, 2012). Lastly, FBs in the coronary trunk and aorta are primarily come from cardiac neural crest cells (Prasad et al., 2019).

Cardiac FBs have recently been differentiated from hPSCs through T^+ mesoderm using the GiFGF protocol (Zhang et al., 2019c). In this protocol, hPSCs were treated with CHIR99021 followed by FGF2 to generate double positive T-box1 (TBX1) and heart- and neural crest derivatives-expressed protein 2 (HAND2) second heart field progenitors. Further treatment with FGF2 resulted in $TE7^+POSTN^+$ FBs that secreted similar levels of ECM to primary cardiac FBs, have increased expression of SMA after treatment with TGFβ1 or Angiotensin-II, and electrically coupled with CMs. Cardiac FBs have also been generated by treating hPSC-derived epicardial cells with FGF2 (Bao et al., 2016; Iyer et al., 2015; Witty et al., 2014; Zhang et al., 2019a; Zhao et al., 2017). Addition of the TGFβ inhibitor SB431542 enhanced the cardiac FB yield by inhibiting differentiation to PC and SMC lineages (Zhang et al., 2019a). Resulting FBs expressed discoidin domain receptor tyrosine kinase 2 (DDR2), POSTN, and TCF21, had increased migration in a scratch assay with the addition of PDGFBB, and could be activated with TGFβ1, causing increased SMA expression. From single cell sequencing datasets of the developing human heart, four distinct FB-like populations have been identified which can largely be separated by developmental origin and location within the heart (Asp et al., 2019; Cui et al., 2019). However, little is known about functional differences between these FB populations. It is likely that these two hPSC-cardiac FB differentiation protocols produce different FB populations recapitulating aspects of cardiac FB developmental specification. However, there is still the need for further investigation into the heterogeneity of cardiac FBs and the development of hPSC differentiation protocols to generate cells that mimic *in vivo* cardiac FB populations.

4.1.4 Cardiac SMCs

Coronary artery SMCs are thought to arise from endoderm-derived PCs via upregulation of Notch3 (Chen et al., 2016; Volz et al., 2015). Lineage tracing studies via Wilms tumor protein (WT1), T-box 18 (TBX18), and transcription factor 21 (TCF21) have shown that SMCs also originate from epicardial cells (Acharya et al., 2012; Cai et al., 2008; Cao and Poss, 2018; Grieskamp et al., 2011; Rudat and Kispert, 2012).

Treatment of epicardial cells with TGFβ1 produced SMCs that expressed calponin, SMA, SMMHC, and smoothelin (Bao et al., 2016; Iyer et al., 2015; Witty et al., 2014; Zhang et al., 2019a; Zhao et al., 2017). Another protocol differentiated hPSCs through KDR^{+} lateral plate mesoderm, from which cardiac progenitor cells are derived, via addition of FGF2, LY294002, and BMP4 (Cheung et al., 2012; Tirosh-Finkel et al., 2006). SMCs were induced by the addition of PDGF-BB and TGFβ1, resulting in cells that expressed *smooth muscle myosin heavy chain 11* (*MYH11), transgelin (TAGLN), calponin 1 (CNN1), smoothelin (SMTN)*, and *smooth muscle actin alpha 2 ACTA2*, displayed contractile response to carbachol, and contributed to vessel formation in cocultured HUVECs. SMCs were also differentiated from mesenchyangioblasts by treatment with TGFβ3. The resulting cells expressed calponin and SMMHC, were contractile, and in coculture with HUVECs formed tubular networks (Kumar et al., 2017; Vodyanik et al., 2010). An alternative protocol treated hPSCs with serum for 6 days followed by PDGF-BB and TGFβ1 for 6 more days to achieve SMCs which expressed SMA, SMHHC, SM22, and calponin, contracted in response to carbachol, and migrated to mouse vasculature when transplanted (Wanjare et al., 2013). Finally, a recent protocol was able to achieve specification to a more contractile or synthetic SMC (Yang et al., 2016). Both lineages went through a mesodermal progenitor induced by

CHIR99021, BMP4, and B27 which was followed with SMC induction with VEGF-A, FGFβ, and B27. Finally, contractile SMCs were induced with addition of PDGFβ, TGFβ, and B27 and then 4-6 days of lactate metabolic purification to increase the percentage of SMA^+ cells. Synthetic SMCs were differentiated by treatment with VEGF-A, FGFβ, and B27 followed by treatment with PDGFβ, TGFβ, and B27 then 4-6 days of lactate metabolic purification. Contractile SMCs had a greater response to carbachol treatment and gels containing these cells contracted more compared to synthetic SMCs. They also expressed SMMHC, calponin, and transgelin, which were not detected on a transcript level in synthetic SMCs. Conversely, synthetic SMCs exhibited faster wound closure in a scratch assay and were more proliferative than contractile SMCs. These differentiation protocols collectively allow for differentiation of hPSCs into SMCs that range in contractility and proliferation capabilities. It is likely that these hPSC-SMCs span a similar range of heterogeneity to SMCs *in vivo;* however, cardiac SMC developmental and functional heterogeneity is still being explored.

4.1.5 Co-differentiation of cardiac stromal cells

In hPSC differentiations, it is nearly impossible to achieve an absolutely pure population of the desired cell type. For example, efficiency of hPSC differentiation to endothelial cells efficiency is typically 20-90%, with other populations primarily consisting of PCs and SMCs (Lian et al., 2014; Marchand et al., 2014; Palpant et al., 2017). Endothelial populations are easily positively sorted using fluorescence activated cell sorting (FACS) or MACS on definitive endothelial markers such as CD31 to achieve greater than 90% endothelial populations. Since endothelial and stromal cell types are commonly used together for *in vitro* vascular engineering, some researchers have proposed using the codifferentiated stromal population within engineered

tissues (Gao et al., 2018; Generali et al., 2019; Kong et al., 2019; Moldovan et al., 2017). In cardiac specific tissues, Masumoto et al. codifferentiated CMs, endothelial cells, and vascular mural cells from endothelial cells and directly incorporated these cells into a cardiac patch which restored contraction in a rat myocardial infarct model without the need for a cell purification step (Masumoto et al., 2014; Matsuo et al., 2015). However, the ease of using an unpurified cell population must be balanced against potential variability in cell composition in across batches or cell lines.

4.1.6 Maturation

Cardiac stromal cells are known to mature *in vivo*, and functionally, fetal and adult stromal cells differ in regenerative capability and crosstalk (Jonsson et al., 2016; Li et al., 2017; Liau et al., 2017; Santiago-Torres et al., 2015). For example, implanted fetal MSCs and adult MSCs following induced left ventricular cryoinjury in a rat had differing arteriole formation capabilities (Iop et al., 2008). hPSC-derived cardiac stromal cells differentiated without a maturation phase are more fetal-like than adult-like (Kumar et al., 2017; Zhang et al., 2019c). Some cardiac stromal cell differentiation protocols have included a maturation phase (Kumar et al., 2017; Wanjare et al., 2013). For example, maintaining hPSC-derived SMCs in endothelium growth medium-2 (EGM2) with mitogen-activated protein kinase kinase (MEK) inhibitor PD0325901 for 6 additional days resulted in higher protein expression of SMMHC and decreased expression of myocardin (MYOCD) (Kumar et al., 2017). Going forward, we need to identify if more mature cells improve cardiac cell performance in applications including drug testing and cellular therapies, and if so, we will need to invest in developing effective and rapid maturation strategies.

5.1 Effects of cardiac stromal cells on differentiation and maturation of hPSC-derived cardiomyocytes

5.1.1 Differentiation

hPSC-derived CMs are commonly derived using a monolayer differentiation protocol where stage-specific small molecule modulation of Wnt/β-catenin signaling is used to achieve ~70-90% cardiac troponin T (cTnT) positive beating cells within two weeks (Burridge et al., 2014; Lian et al., 2015). This protocol has been adapted to suspension culture allowing for large-scale bioreactor production (Fonoudi et al., 2016; Halloin et al., 2019). Compared to *in vivo* human adult CMs, hPSC-derived CMs cultured *in vitro* are metabolically, structurally, and electromechanically immature and more closely resemble fetal CMs (Dunn and Palecek, 2018; Jiang et al., 2018; Lundy et al., 2013; Marchianò et al., 2019; Ronaldson-Bouchard et al., 2018). They can be matured by a number of methods including, but not limited to, extended time in culture, coculture, hormone treatment, metabolite availability, electrical stimulation, surface chemistry modifications, and mechanical stimuli (Hu et al., 2018; Jiang et al., 2018; Lundy et al., 2013; Parikh et al., 2017; Ronaldson-Bouchard et al., 2018; Shiekh et al., 2018; Yoshida et al., 2018).

The non-myocyte population generated during cardiomyocyte differentiation has been shown to include stromal cells and endothelial cells via qPCR and single cell RNA sequencing (Dubois et al., 2011; Friedman et al., 2018). Single cell transcriptomics showed that at day 30 of differentiation, the stromal population expressed CD90 and is similar to *in vivo* outflow tract derivatives based on expression levels of a number of cardiac transcription factors including paired-like homeodomain transcription factor 2 (PITX2) and correlation to *in vivo* single cell samples (Friedman et al., 2018). The stromal population in the differentiation may impact on the differentiation efficiency or functionality of the resulting CMs since cardiac stromal cells have

been shown to interact with CMs in coculture studies (Baum et al., 2012; Hookway et al., 2019; Kohl et al., 2005; Lee et al., 2015; Miklíková et al., 2018; Suhaeri et al., 2017; Varzideh et al., 2019; Ye et al., 2018; Yoshida et al., 2018; Zhang et al., 2019c). The impact of this stromal population during differentiation has not been thoroughly explored yet. For regenerative strategies, Iseoka et al. built upon work by Thavandiran et al. and incorporated different ratios of CMs to non-CMs in scaffold free cardiac patches, using two populations generated by sorting on CD172a (Iseoka et al., 2018; Thavandiran et al., 2013). They found that 50-70% CMs was optimal for cardiac regeneration in a rat myocardial infarction model as measured by increased ejection fraction, increased capillary density, and decreased fibrotic area. In a study by Biendarra-Tiegs et al., day 10 non-CMs and CMs were sorted and replated at ratios of 7:3, 1:1, 3:7, and 1:9. After one month, cocultures with 70-90% CMs exhibited increased action potential amplitude and upstroke velocity measured by optically by ArcLight imaging (Biendarra-Tiegs et al., 2019). Furthermore, the authors observed that negative effects of the non-CMs on CM electrophysiology could be reduced by short hairpin RNA depletion of Cx43 in the non-CM population, suggesting that Cx43-mediated cell-cell communication has an impact on CM maturation. If these strategies to incorporate co-differentiated cardiac stromal populations in cardiac regenerative therapies are to be pursued, analysis of variability within the non-myocyte population between differentiations and their effect on CM phenotype is needed.

One major concern with any cellular therapy is the possibility of teratoma or aberrant tissue formation which could be caused by undifferentiated hPSCs or cells that do not properly engraft (Masuda et al., 2015; Nussbaum et al., 2007; Park and Yoon, 2018). Additionally, since FBs have been implicated many of the negative phenotypes associated with heart failure, there is concern that addition of FBs will only amplify fibrosis and detrimentally alter electrical propagation,

undermining any beneficial effects from the cell therapy (Baudino et al., 2006; Pellman et al., 2016). Finally, as a process development consideration, there is a need for a consistent cell source and characterization of the cell preparation so that release criteria can be set to provide a safe and effective therapy (Abraham et al., 2016; Lin-Gibson et al., 2017). Thus, if non-purified CMs are to be used in cell therapies, this population must be more closely characterized on a molecular and functional level to more thoroughly determine their potential in a cellular therapy.

5.1.2 CM maturation by stromal cells

Immaturity of hPSC-derived CMs limits their potential for coupling with and improving injured hearts in cell therapies. Efforts to improve hPSC-derived CM maturation have focused on mimicking the native heart environment and have included coculture with other cardiac cells (Jiang et al., 2018). In this section, we will discuss interactions between CMs and cardiac stromal cells.

MSCs have been tested in clinical trials for their interactions with immune cells and other cell types, primarily through secreted factors (Li et al., 2019). In a transwell study, MSC-secreted factors improved hPSC-derived CM maturation as measured by increased cell size, increased filament length, increased contraction and relaxation velocity, and enhanced metabolic energetics (Yoshida et al., 2018). Bone marrow-derived mouse MSCs directly cocultured *in vitro* with neonatal mouse ventricular CMs have also been shown to enhance CM proliferation as a result of activation of CM Notch-1 receptors by the secreted ligand Jagged-1 (Sassoli et al., 2011). In addition to MSC capability to increase proliferation of CMs, they have also been used in engineered tissues. For example, CMs cocultured with endothelial cells and MSCs in cardiac tissues formed lumen-like structures with upregulated sarcomeric transcripts and more mature beating patterns compared to monocultured tissues (Varzideh et al., 2019).

The differences in fetal and adult FB crosstalk with CMs has been studied by Li et al (Li et al., 2017). Using a 3D engineered heart tissue, they showed that adult, but not fetal, mouse cardiac FBs deteriorated electromechanical function of rat CMs by altering action potential propagation and tissue stiffness. Another group also showed that rat neonatal cardiac FBs improved Cx43 gap junction formation and stabilized the contraction rhythm in rat neonatal CMs in direct coculture (Hachiro et al., 2007). In a 3D *in vitro* hydrogel, FBs were implicated as important for human heart muscle viscoelastic stiffening as measured by engineered tissue compaction and changes in force of contraction (Schlick et al., 2019). In a different study using a patterned hydrogel microtissue, coculture of neonatal rat CMs and FBs led to increased expression of Cx43 and β1-integrin and structural organization of F-actin and α-actinin (Saini et al., 2015). Furthermore, culture on a scaffold coated with ECM secreted by mouse FB cells led to increased Cx43 expression, α-actinin expression, and decreased circularity in neonatal rat CMs (Suhaeri et al., 2017).

FB secrete paracrine signaling factors which interact with CMs. In conditioned media studies, differing results on cardiac maturation have been observed. Similar to effects of fibrosis, cardiac FB conditioned media slowed neonatal rat CM conduction velocity, increased action potential duration, and increased membrane depolarization in a dose dependent manner while increasing CM cell size and beta-myosin heavy chain (β-MHC) expression (Pedrotty et al., 2009). These effects were not observed by treatment with shared media CM and FB cocultures, suggesting a protective effect by CM. On the other hand, a study using mouse embryonic stem cell-derived CMs directly cocultured with fetal mouse cardiac FBs showed increased expression of inward rectifier potassium channel 2 (Kir2.1), sodium voltage-gated channel alpha subunit 5 (Nav1.5), Cx43, and α-actinin in the cocultured CMs compared to CMs in monoculture (Liau et al., 2017).

They found that cardiac FB conditioned medium alone enhanced CM spreading and contractile activity mediated by MEK/ERK signaling. Hence, FB direct coculture or conditioned media alter CM maturation and electrical conduction in a FB age-dependent manner.

Interactions between SMCs and CMs have been investigated in disease-like conditions, since transplantation of SMCs after myocardial infarction inhibited undesired cardiac remodeling potentially through angiogenic factors such as FGF2 (Wang et al., 2015). Rat SMCs were shown to secrete FGF2 which directly increased viability of cocultured CMs, as measured by Annexin V and propidium iodide staining, via the PI3K/Akt pathway (Ye et al., 2018). Another study showed that parathyroid hormone-related protein (PTHrP) synthesized by SMCs preserved cardiac function post-myocardial infarction in a mouse model and prolonged life-span (Zhang et al., 2019b). Together, these results suggest that cardiac stromal cells may be able to impart maturation phenotypes hPSC-derived CMs, but additional studies are needed to determine optimal coculture compositions, length of coculture, and best time in CM differentiation for initiation of coculture.

6.1 Applications of cardiac stromal cells in regenerative medicine

6.1.1 Tissue construction and cellular therapy

Studies discussed above illustrate the potential advantages of including cardiac stromal cells in engineered heart tissues. Stromal cells have also been explored as therapies to treat heart damage in preclinical models and clinical trials. For example, in a mouse myocardial infarction model, the transplantation of PC progenitors reduced scar size, reduced fibrosis, and improved ejection fraction via paracrine signaling (Katare et al., 2011). hPSC-derived smooth muscle sheets implanted into a rat myocardial infarction model reduced cardiac remodeling by decreasing scar expansion and improving cardiac function as measured by ejection fraction (Harada et al., 2016).

MSCs have been in clinical trials as a treatment for cardiomyopathy, and systematic reviews of these trials have shown that MSCs are safe, although use of MSCs alone has not significantly improved mortality rates (Lalu et al., 2018; Li et al., 2019; Yagyu et al., 2019).

hPSC-derived cardiac cells have shown significant improvement of cardiac function in small and large preclinical animal models, and human clinical trials are slated to begin soon (Chong and Murry, 2014; Chong et al., 2014; Gao et al., 2018; Kashiyama et al., 2019; Liu et al., 2018b; Mitamura, 2018). Cardiac stromal cells, especially FBs, have been included with hPSC-derived CMs in regenerative therapies to promote CM maturation and provide support in tissue constructs (Arai et al., 2018; Chikae et al., 2019; Hookway et al., 2019; Noguchi et al., 2016; Norotte et al., 2009; Yeung et al., 2019). One group designed a bioprinted cardiac patch including hPSC-derived CMs, HUVECs, and primary human adult cardiac FBs that reduced scar area, improved the number of vessels in the infarct zone, and improved survival rates in a rat myocardial infarction model (Yeung et al., 2019). FBs have also been used to produce decelluarlized cardiac matrix to create scaffolds for transcatheter vein valves and myocardial patches (Schmuck et al., 2014; Syedain et al., 2019). Bargehr et al. combined hPSC-derived CMs with hPSC-derived epicardial cells (Bargehr et al., 2019). When transplanted into a rat myocardial infarction model, some of the engrafted epicardial cells became FBs, and the epicardial cell-containing grafts were over twofold larger with improved vascularization and led to an improvement in systolic function compared to grafts containing only CMs. These results indicate that researchers should consider incorporating hPSC-derived cardiac stromal cells or their progenitors in their tissue engineering strategies.

It is estimated that on the order of one billion cardiac cells are needed per therapy to treat a myocardial infarction, and vascularization will likely be needed to support cell doses of this size

(Chong and Murry, 2014; Li et al., 2018; Rouwkema and Khademhosseini, 2016). To attempt to induce vascularization in hPSC-derived tissues, one group incorporated hPSC-derived endothelial progenitors and smooth muscle cell progenitors into spheroids and showed the feasibility of printing vascular structures from hPSC-derived cells (Moldovan et al., 2017). Another group cultured hPSC-derived SMCs in polyglycolic acid (PGA) tubes and demonstrated vascular remodeling after two weeks in nude rat aorta grafts (Gui et al., 2016). Vascularization of cardiac tissue constructs was shown in a model including primary cardiac endothelial cells, primary MSCs, and hPSC-derived CMs (Valarmathi et al., 2017). Thus, we anticipate that further advances in cardiac tissue vascularization using hPSC-derived endothelial and vascular stromal cells will accelerate cardiac cell-based regenerative therapies.

6.1.2 Disease modeling

In addition to using hPSC-derived cardiac stromal cells in cell therapies to treat heart failure, they can also be used as an *in vitro* heart disease model serving as a screening platform for regenerative medicine drug and therapeutic discovery. Since hPSC-derived cardiac stromal cell protocols are still early in development, in this section we discuss the role of primary cardiac stromal cells on disease phenotype with an eye toward incorporating hPSC-derived cardiac stromal cells in these disease models.

During a myocardial infarction, CMs die and FBs and immune cells are recruited to the infarct zone (Baudino et al., 2006; Ongstad and Kohl, 2016). These FBs proliferate and become myofibroblasts as well as secrete ECM which forms scars (Baudino et al., 2006; Ongstad and Kohl, 2016). Scarring of the heart leads to reduced cardiac output as a result of mechanical stiffening.

Another aspect of heart failure is cardiac fibrosis, the accumulation of ECM due to overactive fibroblasts (Andrae et al., 2008; Elwakeel et al., 2019; Horowitz and Thannickal, 2019; Humeres and Frangogiannis, 2019; Kumai, 2019; Okamoto et al., 2019; Olson and Soriano, 2009). Mouse models of fibrosis of connective tissues have discovered that increased platelet derived growth factor receptor alpha (PDGFRα) activation can drive fibrosis (Andrae et al., 2008; Olson and Soriano, 2009). In swine models, the transplantation of PCs into an infarct zone showed reduced fibrosis at the infarct zone (Alvino et al., 2018). MSCs that are GLI family zinc finger 1 (Gli1) positive have also been shown to differentiate into myofibroblasts after a cardiac incident, and contribute significantly to fibrosis (Kramann et al., 2015). Ablation of these cells preserved the ejection fraction in a cardiac failure model (Kramann et al., 2015).

In addition to inducing fibrosis, FBs release secreted factors that facilitate communication with CMs during acute responses to cardiac injury (Bode et al., 2019; Bruns et al., 2019). To model the roles of FBs in ischemia/reperfusion injury, hPSC-derived FBs have been combined with hPSC-derived CMs and treated with conditions mimicking nutrient deprivation and oxygen depletion (Bracco Gartner et al., 2019; Chen and Vunjak-Novakovic, 2019). In particular, these models may help identify strategies to reduce fibrosis and improve cardiac excitation-contraction coupling with small molecule treatments (Hou et al., 2013; Mayourian et al., 2018; Ongstad and Kohl, 2016). For example, TGFβ secreted by myofibroblasts was shown to directly cause CM hypertrophy, and conversely CMs release soluble factors which modulate FB proliferation as shown through targeted knockdown of Tgfbr2 signaling in a mouse model and *in vitro* coculture systems (Bhandary et al., 2018; Cartledge et al., 2015; Huang et al., 2019). From this type of mechanistic study, potential therapeutic targets of fibrosis have been identified, including calpain 9 (CAPN9) antagonism (Fix et al., 2019; Kim et al., 2019; Nguyen et al., 2019; Shao et al., 2019).

Complementing *in vivo* models with these *in vitro* models will help to identify viable anti-fibrotic drugs as well as analyze off-target effects in preclinical studies going forward (Fang et al., 2017). Thus, models to analyze heart failure and drug response could be developed using hPSC-derived cardiac stromal cells.

Recently, there has been progress in analyzing effects of genetic diseases in cardiac stromal cells (Peng et al., 2019). For example, researchers studying Marfan syndrome, caused by a mutation in the *fibrillin 1* (*FBN1)* gene, in iPSC-derived SMCs found that this mutation caused reduced fibrillin-1 deposition, vascular stress, and increased SMC apoptosis and decreased proliferation via p38 MAP kinase (Granata et al., 2017). Another study generated an iPSC line from Ehlers-Danlos Syndrome patients with *collagen type III alpha 1 chain (COL3A1)* mutations which the authors hope to use in future studies to generate cardiac tissues representative of patients with weakened heart walls (He et al., 2018). Other mutations in ECM proteins and genes associated with arrhythmias have also been investigated in primary mouse and human FBs and SMCs (Brilla et al., 1995; Cheng et al., 2019; Ji et al., 2017; Sai et al., 2019; Tran et al., 2019). Thus, we expect that iPSC-derived cardiac stromal cells will become more widely used in cardiac *in vitro* disease models as differentiation protocols are refined, interactions with stromal cells are better understood, and reprogramming iPSCs becomes streamlined.

6.1.3 Drug testing

There are number of cardiac-targeted drugs, commonly used after heart failure, used to modulate blood pressure and heart rhythm. Response of cardiac stromal cells to these drugs is important in the resulting heart function. Several studies have proposed the possibility of using *in vitro* systems to analyze SMC response to vasodilators (Bass et al., 2016; Brozovich et al., 2016;

Ding et al., 2018). Another drug-based approach to treat heart failure involves targeting FB ECM production and myofibroblast differentiation to reduce scarring. *In vitro* culture allows for high-throughput methods to analyze ECM deposition and SMA expression (Cai et al., 2013; Du et al., 2019; Fix et al., 2019; Lai et al., 2009; Rehman et al., 2019; Zeng et al., 2019). For example, *in vitro* culture of primary rat ventricular CMs and cardiac FBs with 2,5-dimethylcelecoxib was used to identify how the drug reduced cardiac FB production of fibronectin and suppress isoprenaline-induced CM hypertrophy (Morishige et al., 2019). Another group found that 3D valvular interstitial cell constructs exposed to TGF-β1 had increased SMA expression and scaffold contraction, indicative of myofibroblast differentiation (Gonzalez Rodriguez et al., 2018).

hPSC-derived cardiac cells have also been proposed as an *in vitro* model to complement *in vivo* preclinical toxicology studies. High-throughput, predictive *in vitro* models may help reduce cost of drug development by identifying cardiotoxicity early in the drug discovery pipeline and facilitate identification of mechanisms of cardiotoxicity. Titmarsh et al. recently demonstrated the application of cocultured 3D hPSC-derived vascular constructs for drug applications (Titmarsh et al., 2019). They combined hPSC-derived endothelial cells and SMCs into 3D vascular cocultures and used high throughput screening to test population-based vascular toxin response. Hence, the next step for using this approach in cardiac applications will involve inclusion of combinations of cardiac cells which will better recapitulate the native heart environment and enable insight into how drugs change electrical propagation and cell-cell signaling in addition to leading to cell death (Christoffersson et al., 2019; Hookway et al., 2019).

7.1 Challenges

Stromal cells are an important component of cardiac tissue and additional study of these cells is crucial to advancing our understanding of cardiac development, designing cardiac drugs, and engineering regenerative cardiac cell therapies. In particular, hPSC-derived stromal cells provide a scalable robust human cell source without the need for donors of cardiac tissue.

In order to do this, first and foremost, the field needs to identify and come to a consensus on a panel of markers and/or functional assays that define specific stromal populations, including FBs, MSCs, PCs, and SMCs so that differentiation protocols can be developed, and researchers can better compare behavior of different cell populations. Second, we need to achieve a clearer mechanistic understanding of interactions between hPSC-derived CMs and other cardiac cell types that influence resulting cardiac tissues. This allows for scalable *in vitro* screening platforms using hPSC-cardiac stromal cells to identify potential regenerative medicine drugs and therapeutics. For a cellular therapy, one should also address potential safety pitfalls of incorporating cardiac stromal cells in tissue constructs, so we can engineer safe and effective cellular therapies. Finally, as clinical trials using hPSC-derived cardiac cell types begin, we should address differentiation consistency in both CMs and stromal cells to expedite FDA approval and to ensure a consistent cell therapy.

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Figures and Tables

Table 1: Comparison of proposed markers of cardiac stromal cells.

Marker	Cell Type				References
	FB	MSC	PC	SMC	
Calponin	-		+/-	+	Bao et al., 2016; Cheung et al., 2012; Iyer et al., 2015; Kumar et al. 2017; Orlova et al., 2014b; Stebbins et al., 2019; Wanjare et al., 2014; Witty et al., 2014; Yang et al. 2016; Zhang et al, 2019a; Zhao et al., 2017
CD13			+	+	Kumar et al. 2017; Stebbins et al., 2019
CD29		+			Tran et al., 2012
CD31		-	-	-	Kumar et al. 2017; Tran et al., 2012
CD34		-	-	-	Chijimatsu et al., 2017; Kumar et al. 2017; Sánchez et al., 2010; Tran et al., 2012
CD44		+	+	+	Chijimatsu et al., 2017; Kumar et al. 2017; Orlova et al., 2014a; Sánchez et al., 2010; Tran et al., 2012
CD45		-	-	-	Chijimatsu et al., 2017; Kumar et al. 2017; Tran et al., 2012
CD73		+	+	+	Chijimatsu et al., 2017; Kumar et al. 2017; Orlova et al., 2014a; Sánchez et al., 2010; Tran et al., 2012
CD90	+/-	+		-	Bao et al., 2016; Chijimatsu et al., 2017; Sánchez et al., 2010; Tran et al., 2012; Witty et al., 2014; Zhang et al., 2019c
CD105		+	+	+/-	Chijimatsu et al., 2017; Kumar et al. 2017; Orlova et al., 2014a; Sánchez et al., 2010; Tran et al., 2012
CD146			+	+	Kumar et al. 2017; Orlova et al., 2014a;
CD271		+			Chijimatsu et al., 2017
COL1A1	+			+	Yang et al. 2016; Zhang et al., 2019a; Zhao et al., 2017
Desmin			+/-		Kumar et al. 2017; Stebbins et al., 2019
DDR2	+				Zhang et al., 2019a
Elastin				+	Wanjare et al., 2013
FSP1	+				Zhao et al., 2017
HLA-ABC		+			Tran et al., 2012
HLA-DR		-			Tran et al., 2012
MCAM	-				Zhang et al., 2019a
NG2	-		+	+	Kumar et al. 2017; Orlova et al., 2014a; Stebbins et al., 2019; Wanjare et al., 2014; Zhang et al., 2019a
PDGFRα	+				Iyer et al., 2015
PDGFRβ	-		+	+	Kumar et al. 2017; Orlova et al., 2014a; Stebbins et al., 2019; Wanjare et al., 2014; Zhang et al., 2019a
POSTN	+				Iyer et al., 2015; Zhang et al., 2019a; Zhao et al., 2017; Zhang et al., 2019c
SMA	-		+/-	+	Cheung et al., 2012; Iyer et al., 2015; Kumar et al. 2017; Orlova et al., 2015; Stebbins et al., 2019; Wanjare et al., 2014; Witty et al., 2014; Yang et al. 2016; Zhang et al., 2019a; Zhao et al., 2017
SM22α			+	+	Orlova et al., 2014b; Stebbins et al., 2019; Wanjare et al., 2013
SMMHC	-		-	+	Bao et al., 2016; Cheung et al., 2012; Iyer et al., 2015; Kumar et al. 2017; Wanjare et al., 2014; Witty et al., 2014; Yang et al. 2016; Zhang et al., 2019a; Zhao et al., 2017
Smoothelin				+	Cheung et al., 2012; Witty et al., 2014
TAGLN	-		+	+	Cheung et al., 2012; Iyer et al., 2016; Stebbins et al., 2019; Witty et al., 2014; Yang et al. 2016; Zhang et al., 2019a; Zhao et al., 2017
TCF21	+				Witty et al., 2014; Zhang et al., 2019a
TE7	+				Zhang et al., 2019c
VE-Cadherin				-	Yang et al. 2016
VIM	+			+	Bao et al., 2016; Iyer et al., 2015; Witty et al., 2014; Yang et al. 2016; Zhang et al, 2019a; Zhang et al., 2019c

Table 2: Functional assays to identify and differentiate cardiac stromal cells.

Assay	Cell Types	Function Analyzed	References
Migration towards PDGFbb or Angiotensin II receptor antagonists	FB, PC, SMC	Migration	Zhao et al., 2019; Zhang et al., 2019; Zhou et al., 2016
Scratch assay	FB, MSC, SMC	Migration	Costa et al., 2017; Yang et al., 2016; Zhang et al., 2019
Mediate tubule formation in coculture with endothelial cells *in vitro*	MSC, PC, SMC	Vascular association	Bargehr et al., 2016; Chen et al., 2015; Wanjare et al., 2013
Cell contraction with addition of drugs (e.g. KCl, Endothelin-1, U46619, or carbachol)	MSC, PC, SMC	Contraction	Bajpai et al., 2012; Bargehr et al., 2016; Costa et al., 2017; Iyer et al., 2015; Kakkar et al., 2019; Kumar et al., 2017; Zhou et al., 2016
Contraction in gel lattice	PC, SMC	Contraction	Lin et al., 2012; Lin et al., 2019; Yang et al., 2016
Contractile changes in coculture with CM	FB, MSC	Electrical coupling	Hookway et al., 2019; Zhang et al., 2019c
Increase in ECM production in response to TGFB1	FB, SMC	ECM Production	Lin et al., 2019; Zhang et al., 2019a
Differentiation potential (osteo, chondro, and adipogenic)	MSC	Differentiation potential	Chen et al., 2016
Myofibroblast activation with TGFB1, Angiotensin II	FB	Differentiation potential	Zhang et al., 2019a; Zhang et al., 2019c
Elongation in response to INF-γ, IL-4, IL-13	MSC	Inflammatory response	Marklein et al., 2018
Coculture with CD3+ T-cells modulates T-cell inflammatory secretions	MSC	Immunomodulatory response	Wang et al., 2019

Table 3: Comparison of cardiac stromal cell differentiation protocols from hPSCs.

Cell Type	Precursors	Signaling factors used to differentiate from progenitor	References
FB	hPSC, Mesoderm, Cardiac progenitor, EpiC	bFGF	Bao et al., 2016; Iyer et al., 2015; Witty et al., 2014; Zhang et al., 2019a, Zhao et al., 2017
FB	hPSC, Mesoderm, Secondary heart field cardiac pr	bFGF	Zhang et al., 2019c
MSC	hPSC	SB-43152	Sánchez et al., 2010
MSC	hPSC, Neural crest	Serum	Chijimatsu et al., 2017
MSC	hPSC, Mesoderm	bFGF, EGF	Tran et al., 2012
PC	hPSC, Neural crest	Serum	Stebbins et al., 2019
PC	hPSC, Hematopoeitc progenitor	SB-43152, VEGF	Kumar et al. 2017
PC	hPSC, Hematopoeitc progenitor	TGFβ, PDGFBB	Orlova et al., 2014a, 2014b
PC	hPSC, Mesoderm, Mesenchymoangioblast	FGF2, PDGFBB	Kumar et al. 2017
SMC	hPSC, Mesoderm, Cardiac progenitor, EpiC	TGFβ1	Bao et al., 2016; Iyer et al., 2015; Witty et al., 2014; Zhang et al., 2019a, Zhao et al., 2017
SMC	hPSC, Mesoderm, Mesenchymoangioblast	SPC, TGFβ3	Kumar et al. 2017
SMC	hPSC, Mesoderm	FBFβ, VEGF, TGFβ1, B27	Yang et al. 2016
SMC	hPSC, Mesoderm	PDGFBB, TGFβ1	Cheung et al., 2012; Wanjare et al. 2013; Wanjare et al., 2014

Table 4: Markers of progenitors during hPSC differentiation to cardiac stromal cells.

Progenitor Cell	Markers of Fate	References
Neural Crest	TFAP2A, SOX10, NGFR	Chijimatsu et al., 2017; Stebbins et al., 2019
Mesoderm	T, MIXL1	Chen et al., 2012; Cheung et al, 2012; Orlova et al., 2014a, 2014b; Tran et al., 2012; Yange et al., 2016; Zhang et al., 2019c
Cardiac Mesoderm	ISL1, NKX2.5, GATA4, MESP1	Bao et al, 2016; Iyer et al., 2015; Witty et al., 2014; Zhang et al., 2019a; Zhang et al., 2019c; Zhao et al, 2017
First heart field cardiac progenitors	TBX5, TBX20, HAND1	Bao et al, 2016; Zhang et al, 2019c
Second heart field cardiac progenitors	TBX1, HAND2	Zhang et al., 2019c
Epicardial Cells	WT1, TBX18, ALDH1A2	Bao et al, 2016; Iyer et al., 2015; Witty et al., 2014; Zhang et al., 2019a; Zhao et al, 2017
Hematopoeitc progenitor	PDGFRβ, NGFR, KDR	Kumar et al, 2017; Vodyanik et al., 2010
Mesenchymoangioblast	MCAM, NGFR, NT5E-	Kumar et al, 2017; Vodyanik et al., 2010

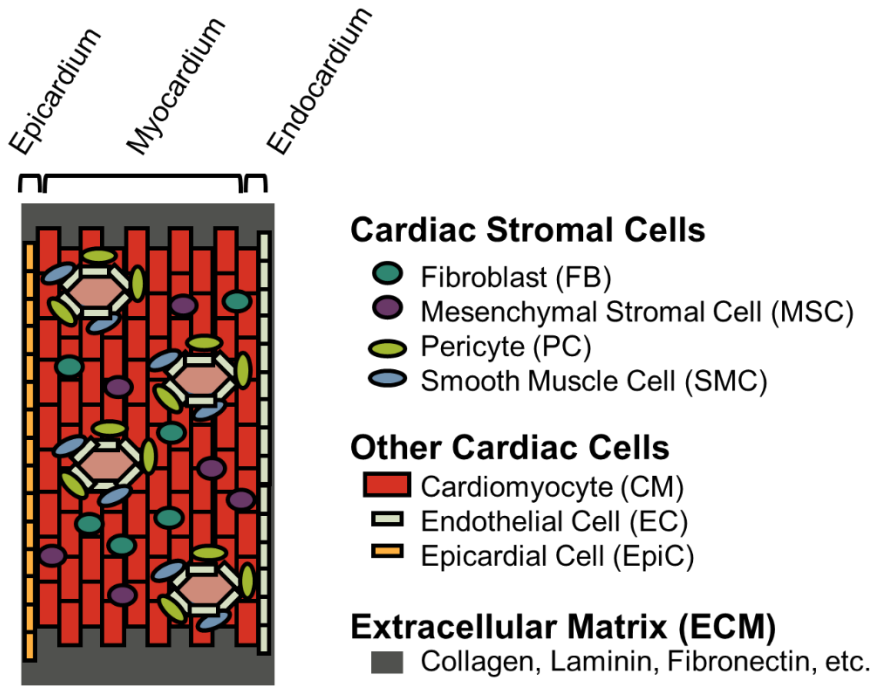

Figure 1: Schematic of cardiac stromal cell composition and localization in cardiac wall.

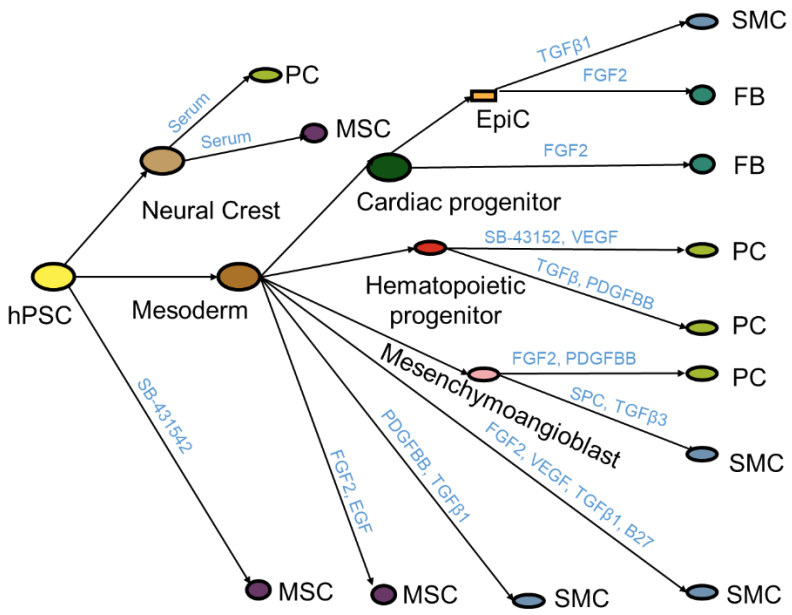

Figure 2: Diagram strategies to induce cardiac stromal cell differentiation from hPSCs where blue text indicates factors and black text indicates cell types.

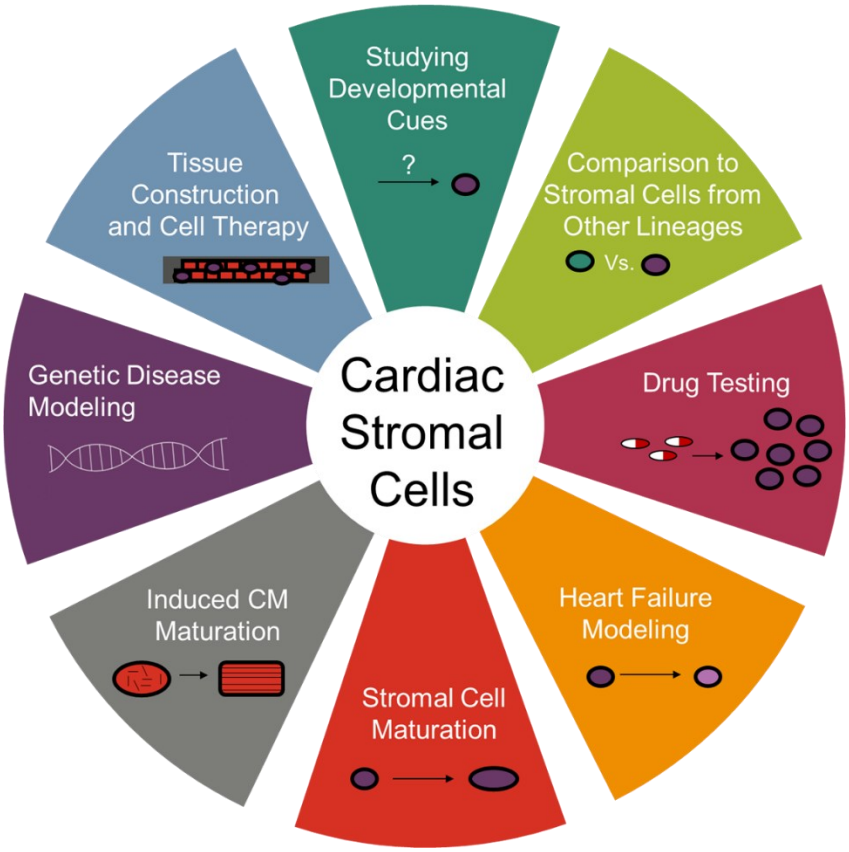

Figure 3: Applications of cardiac stromal cells.