Adv Exp Med Biol - Cell Biology and Translational Medicine (2021) 11: 51–73
https://doi.org/10.1007/5584_2020_594

Published online: 7 November 2020

Cardiac Progenitor Cells

Shaimaa Shouman, Amr Zaher, Alaa Abdelhameed, Sara Elshaboury, Samar Sakr, Bahaa Eldin Fouda, Haya Mohamed, and Nagwa El-Badri

Abstract

Cardiovascular diseases top the list of fatal illnesses worldwide. Cardiac tissues is known to be one of te least proliferative in the human body, with very limited regenraive capacity. Stem cell therapy has shown great potential for treatment of cardiovascular diseases in the experimental setting, but success in human trials has been limited. Applications of stem cell therapy for cardiovascular regeneration necessitate understamding of the complex and unique structure of the heart unit, and the embryologic development of the heart muscles and vessels. This chapter aims to provide an insight into cardiac progenitor cells and their potential applications in regenerative medicine. It also provides an overview of the embryological development of cardiac tissue, and the major findings on the development of cardiac stem cells, their characterization, and differentiation, and their regenerative potential. It concludes with clinical applications in treating cardiac disease using different approaches, and concludes with areas for future research.

Keywords

Cardiac progenitor cell · Heart embryology · CVD · Stem cell application

Shaimaa Shouman and Amr Zaher contributed equally with all other contributors.

S. Shouman, A. Abdelhameed, S. Elshaboury, and N. El-Badri (✉)
Center of Excellence for Stem Cells and Regenerative Medicine (CESC), Zewail City of Science and Technology, 6th of October City, Egypt
e-mail: sshouman@zewailcity.edu.eg; nelbadri@zewailcity.edu.eg

A. Zaher
Center of Excellence for Stem Cells and Regenerative Medicine (CESC), Zewail City of Science and Technology, 6th of October City, Egypt

National Heart Institute, Giza, Egypt

S. Sakr
Department of Biochemistry, Faculty of Science, Mansoura University, Mansoura, Egypt

B. E. Fouda
National Heart Institute, Giza, Egypt

H. Mohamed
Faculty of Pharmacy, Tanta University, Tanta, Egypt

1 Introduction

The heart is the ultimate blood-pumping organ in the body. Anatomically, it consists of four main chambers connected to the rest of the body by a set of vessels, namely, veins and arteries, and wrapped inside the chest by a pericardial sac that provides protection and support (Fig. 1a). The heart consists of two receiving chambers (atria) and two pumping chambers (ventricles). Physiologically, the heart is divided into a right side t and a left side heart. The right heart consists of the right atrium, which receives blood from all

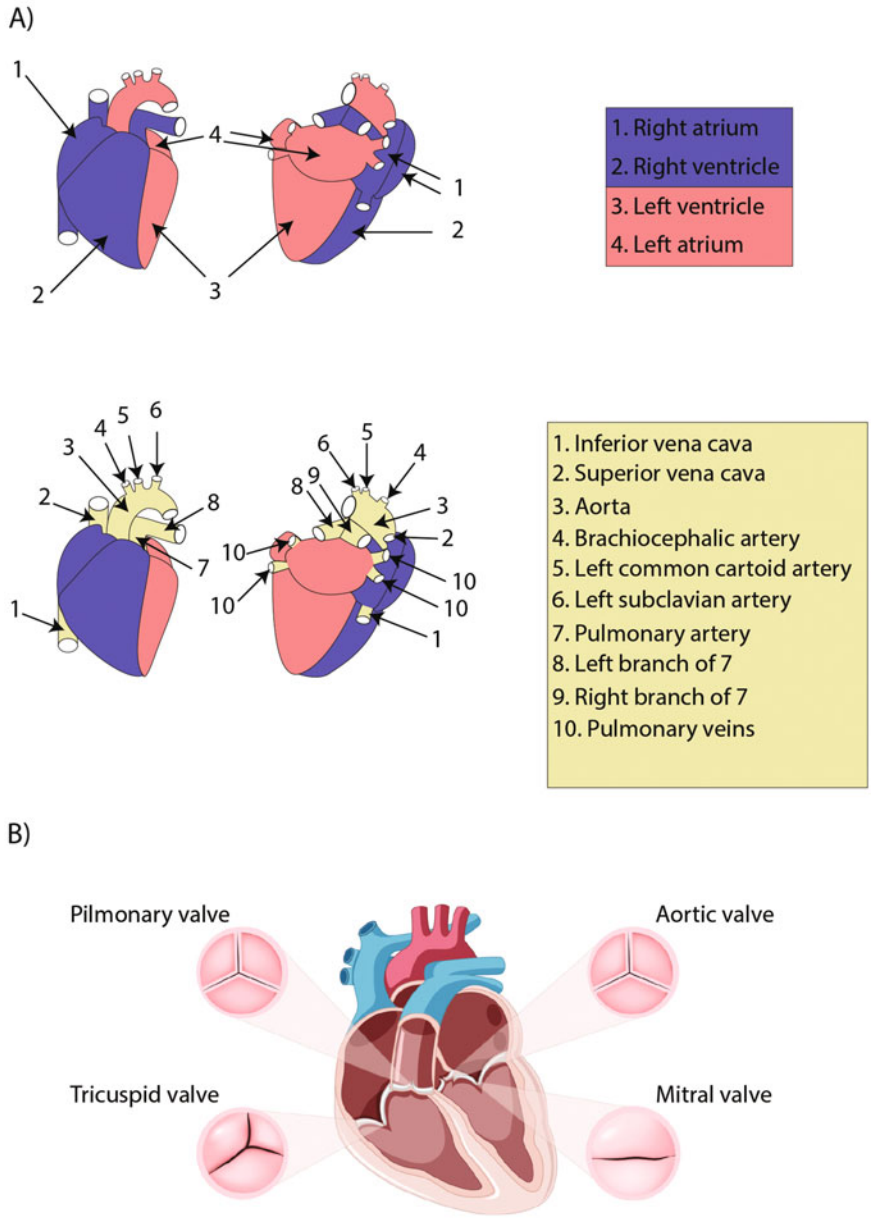


Fig. 1 Heart structure. (**a**) Demonstration of the structure of the heart. (**b**) Heart chambers and inter-chamber valves

over the body via two large veins, the superior vena cava, and the inferior vena cava. Normally, the blood follows a route to the right ventricle where it is pumped to the lungs via the pulmonary artery, where a series of events lead to more oxygenation and less carbonation of the blood. The blood then enters the left heart via four pulmonary veins, where it receives oxygenated blood from the lungs. Oxygenated blood flows to the left ventricle, where it is pumped to all of the organs and tissues of the body via the aorta. This cycle repeats itself with every heartbeat. The cardiovascular circuit is a closed circuit between the two right and left halves of the circulation, which function to perfuse the body with nutrients and carry out metabolic activities in different

organs. Two types of one-way valve between the various chambers maintain the continuity of this circuit and vessels; the atrioventricular (A-V) valves connect the atria with the ventricles, whereas the semilunar valves connect the heart with the aorta and the pulmonary trunk. The mitral valve is bicuspid with two leaflets and connects the left atrium to the left ventricle. The tricuspid valve has three leaflets and connects the right atrium to the right ventricle. The aortic valve connects the left ventricle with the aorta, whereas the pulmonary valve connects the right ventricle with the pulmonary trunk (Fig. 1b). The heart acts as a single functional unit (syncytium), and any defect in this unit will lead to diseases ranging from mild to severe and even fatal conditions. Cardiomyocytes are known to be unable to regenerate after an injury (Laflamme and Murry 2011). To obtain a comprehensive understanding of cardiac regeneration and the role of stem cell therapy in heart repair, it is essential to understand the structure and development of the heart and the role of cardiac progenitors in its regeneration.

2 Development of Cardiac Progenitor Cells

2.1 Embryology of the Heart

Heart development begins soon after gastrulation. The heart develops from the splanchnic mesoderm on both sides of the primitive foregut, forming a heart tube that undergoes a sequence of looping, septation, realignment, and remodeling (Fig. 2). It was recently hypothesized that the heart develops via a sequence of events involving the interaction of different cell groups (Aguilar-Sanchez et al. 2018). In early development, bilateral groups of cells from the splanchnic mesoderm migrate and distribute along the ventral midline in the cardiac crescent (Fig. 2a); this is called the primary or first heart field (Wu et al. 2008). This is followed by another wave of migration from some progenitor cells from the underlying pharyngeal mesoderm, forming the second heart field (Rochais et al. 2009). The cells of the first heart field are positioned more laterally, while those of the second heart field are located more medially and caudally. The cells of the first heart field will form the initial tubular heart. However, the extension of the tubular heart at the venous and arterial pole is assigned to the second heart field. This process gives rise to the right ventricle and outflow tract. The second heart field contributes to the outflow tract, the majority of the right ventricle, and parts of the atria, while the first heart field cells were found to contribute to the entire left ventricle, the majority of both atria, and parts of the right ventricle (Fig. 2b). The merging of the cardiac crescent in the midline forms the primitive heart tube, which is composed of beating cardiomyocytes and is subsequently lined by the endothelium of the endocardium. All parts are surrounded by an extracellular matrix called the cardiac jelly. The endocardial cells appear to be committed even before migration to the heart field.(Ishii et al. 2009) With the first heartbeat, the primitive cardiac tube undergoes canalization and then further elongation, looping, and chambering (Forouhar et al. 2006) (Fig. 2c). This stage is followed by the third wave of cell migration, referred to as the third heart field (Christoffels et al. 2006). However, this time, the neural crest cells come from outside the cardiac mesoderm and are thought to be derived from the dorsal neural tube. They are responsible for completing the separation of the outflow tracts. The third field cells are arranged anteriorly in the early heart tissue forming proepicardium, which is believed to be responsible for forming the membrane covering the entire heart later on, as well as coronary vasculature (Männer et al. 2001). The three waves of cell migration denoted as the first, second, and third heart fields act synchronously to form the ultimate structure of the heart

2.2 Role of Cardiac Progenitor Cells in Heart Development

Cardiac progenitors are precursor multipotent cells that can differentiate into different types of myocytes and non-myocyte cells in the heart (Brade et al. 2013). Identifying the origin of

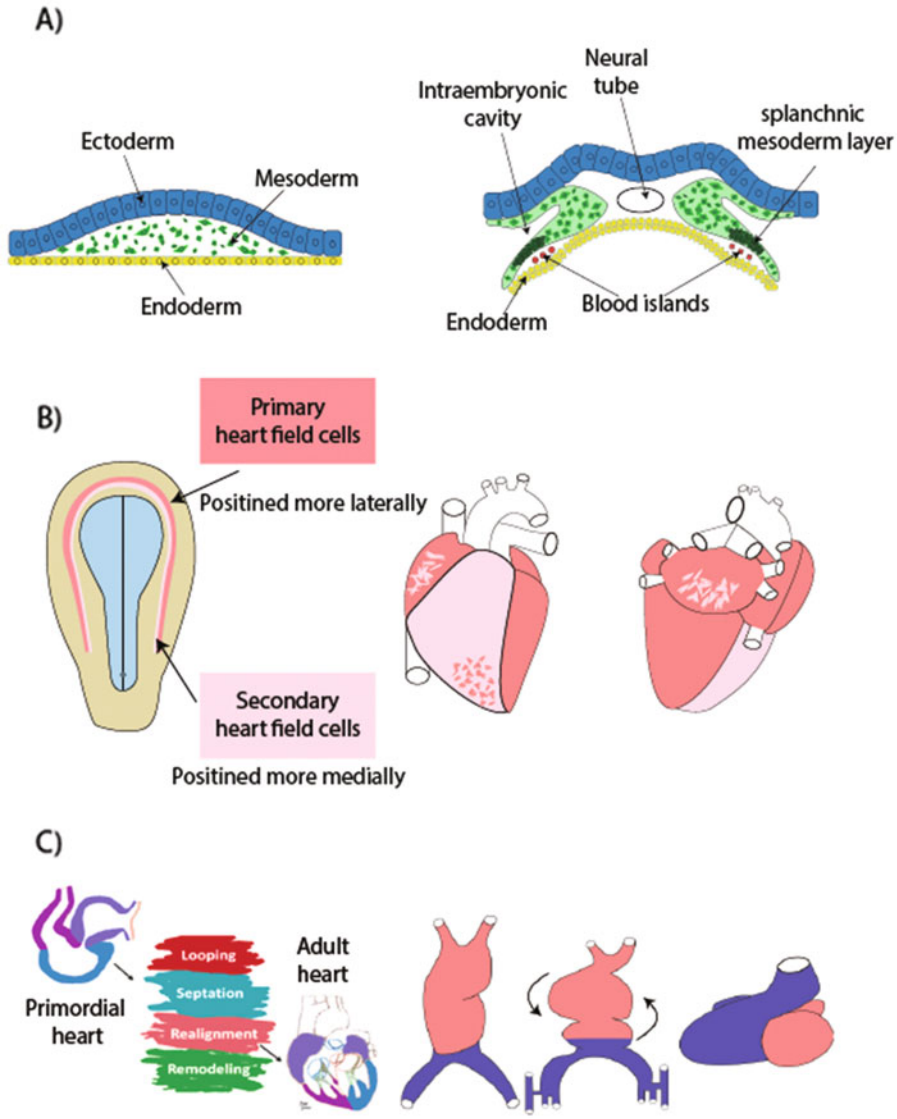


Fig. 2 Heart development. (**a**) Early stage of heart development at embryonic gastrulation**.** (**b**) Development of cardiogenic fields. (**c**) Heart tube formation undergoes a sequence of looping, septation, realignment, and remodeling

these cells and how they differentiate into different cell populations forming a whole functional heart has been the target of extensive research. Determination of the molecular mechanisms that regulate the heart lineage specification provides insight into the potential to reactivate these pathways as a means to treat the loss or damage of adult cardiomyocytes (Xin et al. 2013). During human embryonic development, after the third week of gastrulation, the heart begins to form from the mesodermal germ layer. At this stage, the embryo is converted from being bilaminar (i.e., consisting of two layers of epiblast and hypoblast tissues) into a trilaminar embryonic disk. This process is called gastrulation, where the three germ layers are formed (ectoderm, mesoderm, and endoderm). Initially, a primitive streak (PS) with a node is formed in the bilaminar

embryonic oval disk toward its caudal end. This streak allows the cardiac mesoderm cells (CMCs) to migrate inwardly through PS during gastrulation and to localize in the region anterior of the streak, called the splanchnic mesoderm. Several regulatory pathways from the adjacent endoderm and ectoderm germ layers regulate the induction of the cardiac mesoderm. These include bone morphogenetic protein (BMP), nodal pathway, fibroblast growth factors (FGF), and Wnt signaling pathway, as well as the morphogen gradient (Noseda et al. 2011).

The heart basically develops from migrating CMCs, which transiently express master regulator mesoderm posterior 1 ($Mesp1^+$) and BHLH transcription factor 1, under control of the T-brachyury transcription factor (Bondue et al. 2008). To maintain the cardiac mesoderm lineage, the Mesp1 transcription factor inhibits the Wnt signaling pathway through the activation of Wnt Dickkopf (WNT signaling pathway inhibitor 1) (Bondue and Blanpain 2010). CMCs express the $Mesp1^+$ marker, which is characterized as the earliest sign of heart development (Bondue et al. 2008; Saga et al. 2000). Those uncommitted CMCs proliferate rapidly and migrate craniolaterally to form the cardiac crescent (at week 2 in the human embryo and day E7.5 in the mouse embryo). Sunbsequently, the sequential commitment of $Mesp1^+$ cells is controlled by spatiotemporal signals to give rise to different cardiac progenitor cells (CPCs), expressing specific markers (Saga et al. 2000). Three CPCs have been identified in the embryonic heart: cardiogenic mesoderm cells (CMCs), cardiac neural crest cells (CNCCs), and the proepicardium (PE) (Brade et al. 2013). Cardiogenic mesoderm cells form two fields, the first heart field (FHF) forms the left ventricle and atria, while the second heart field (SHF) forms the right ventricle and outflow tract (OFT) (Buckingham et al. 2005). Cardiac precursors in the FHF and SHF express specific markers such as Gata-4, Nkx2.5, Mef2c, and Islet1 (Laugwitz et al. 2008; Molkentin et al. 1997; Dodou et al. 2004). Cardiac progenitors arising from PE can differentiate into interstitial fibroblasts implanted in the myocardium, smooth muscle, endothelial cells of coronary vessels, and a small number of myocytes located in the atrioventricular A-V septum (Brade et al. 2013). Therefore, the interaction among cardiomyocyte, epicardial, endocardial, and CNCCs results in the formation of the septated four chambers of the fetal heart (Garry and Olson 2006).

3 Types of Cardiac Progenitor Cell

3.1 Cardiac Progenitor Cells in Embryonic Heart

Most of the knowledge obtained about cardiac embryogenesis has been from animal studies, which has resulted in significant gaps of knowledge that limit our understanding of the development of cardiac cells in humans. The anatomical convergence between mice and humans is a key factor in understanding the developmental stages of the heart (Sahara et al. 2019). Animal studies have shown that CPCs play a key role in regulating the sequential assembly of different heart cells during embryogenesis. These progenitors include cardiogenic mesoderm cells (CMCs), CNCCs, and the PE.

3.1.1 Cardiac Mesoderm Cells

In early vertebrate development, CMCs, derived from a common mesodermal lineage, develop into the FHF and SHF. FHF forms on day 7.5 of gestation in mice and from days 16–18 in humans, when the early cardiac progenitor mesoderm forms the cardiac crescent. Markers for FHF cells are the transcription factor NKX2-5 and the cyclic nucleotide-gated ion channel HCN4 (Brade et al. 2013; Wu et al. 2006). In the cardiac crescent stage, FHF progenitor cells are committed to differentiation by the action of BMP (Schultheiss et al. 1997), FGF (Reifers et al. 2000), and Wnt/β-catenin inhibitors (Marvin et al. 2001). In contrast, SHF progenitors remain undifferentiated and in a proliferating state till they enter the heart tube (Brade et al. 2013). SHF is formed from the pharyngeal mesoderm in the medial and anterior regions of the cardiac crescent and is Islet-1

(ISL1)-positive (Cai et al. 2003). The proliferation of uncommitted SHF cells is regulated by FGF, Notch, canonical WNT, and Hedgehog signaling pathways (Dyer and Kirby 2009). By embryonic day 8 in mice, cells from the cardiac crescent migrate to the midline to form a linear heart tube, serving as a scaffold for subsequent heart growth. Further anterior and posterior expansion of the heart results from the migration of cells from the secondary heart field (Garcia-Martinez and Schoenwolf 1993). Many intermediates originate from the first and second heart field-derived CPCs, which subsequently generate all of the major cells in the heart, including cardiomyocytes (CMs), vascular smooth muscle cells (SMCs), arterial and venous endothelial cells (ECs), fibroblasts, and cells of the cardiac conduction system. Moreover, epigenetic regulation mediated by miRNA and lncRNA is also significant for the progression of CPCs to terminally differentiated muscle and non-muscle cardiac lineages (Liu and Olson 2010).

3.1.2 Cardiac Neural Crest Progenitors

The third multipotent distinct embryonic cardiac progenitors are CNCCs, characterized as non-cardiac cell types. CNCCs arise from the ectoderm, from the dorsal neural tube between mid-otic placode and caudal boundary of somit 3 (Achilleos and Trainor 2012). They undergo epithelial–mesenchymal transition (EMT) and migrate toward the heart at pharyngeal arches 3, 4, and 6. CNCCs contribute to the development of the aorticopulmonary septum, conotruncal cushions (i.e., atrioventricular cushions), and smooth muscle and appropriate patterning of the large arteries (Keyte and Hutson 2012; Bergwerff et al. 1998). In addition, the cardiac neural crest generates cardiac parasympathetic innervation and connective tissue insulation of His-Purkinje fibers (Kirby et al. 1983; Gurjarpadhye et al. 2007). Several signaling pathways, transcription factors, and secreted molecules have been shown to interact to instruct CNCCs during their induction, migration, and differentiation. The key players for neural crest induction and specification are BMP/TGF-β growth factors, FGF, the Wnt/β-catenin signaling pathway, as well as retinoic acid (RA) (Aybar and Mayor 2002; Sauka-Spengler and Bronner-Fraser 2008). The migration of CNCCs to a specific site on the heart outflow tract is guided by chemical attractants, such as semaphorin 3C and connexin 43, alongside FGF signaling molecule (Toyofuku et al. 2008; Xu et al. 2006; Sato et al. 2011). The myocardium underlying the outflow tract expresses semaphorin, which binds to its receptor on CNCCs, leading to cytoskeletal rearrangement and cell migration (Toyofuku et al. 2008). The final process of patterning the aortic arch artery is controlled by TGF-β and PDGF signaling pathways. Mutations arising in the genes encoding these signaling pathways or molecules result in various congenital heart diseases (Keyte and Hutson 2012). Unfortunately, no unique molecular marker that allows the identification and tracking of CNCCs is currently available. Instead, molecular lineage labeling and chick–quail chimeras allow the indirect tracking of CNCCs (Phillips et al. 1987). This chick–quail chimera technique allows the transplantation of quail tissues into chick embryo or vice versa, in order to follow the fate of specific regions during embryonic development (Phillips et al. 1987). However, a study reported a multipotent CNCC population in neonatal and adult mouse hearts, precisely within the cardiac side population (Golebiewska et al. 2011). Side population (SP) cells are dormant tissue-resident progenitors that were first identified by their distinctive ability to efflux Hoechst-33342 dyes through ATP-binding cassette (ABC) transporters (Golebiewska et al. 2011). SP cells were isolated and formed a cardiosphere upon culture, similar to the case for neurosphere formation. This cardiosphere was shown to express Nestin and Musashi-1 markers and, upon dissociation, differentiated into neurons, glia, melanocytes, chondrocytes, and myofibroblasts. Once the labeled cardiosphere cells were transplanted into chick embryo, they migrated to the heart, similar to endogenous CNCCs, and contributed to contraction of the cushion and outflow tract (Youn et al. 2003).

3.1.3 Proepicardium (PE)

The outermost layer of the heart enveloping both the endocardium and the myocardium is called the epicardium. PE cells are embryonic progenitor cells that give rise to epicardial cells . During the looping stages of the heart, proepicardial cells migrate to cover the heart surface with an epicardial sheet. Some epicardial cells detach and undergo EMT, invade the myocardial walls, and give rise to the epicardial-derived cells (EPDCs) (Perez-Pomares et al. 2002; Gittenberger-de Groot et al. 1998). Invasive EPDCs differentiate into coronary vascular SMCs, ECs, and subepicardial and intramyocardial fibroblasts (Dettman et al. 1998; Smart et al. 2007). The induction and maintenance of PE are regulated by the opposite interaction between FGF and BMP signaling pathways. FGF signaling induces a proepicardial fate from the splanchnic mesoderm, while BMP signaling induces myocardial differentiation (Kruithof et al. 2006). Important signaling molecules driving EPDC differentiation into primary coronary blood vessels are the TGF-β superfamily, FGFs, retinoic acid, as well as Hedgehog and VEGF (Lavine and Ornitz 2008; Perez-Pomares and de la Pompa 2011).

For a long time, it was thought that the PE is an extracardiac population of cells; however, recent molecular analysis and lineage tracing studies found that CPCs expressed Nkx2-5- and Isl1 markers as SHF progenitors, contributing to the formation of proepicardial cells. In addition, these proepicardial progenitor cells expressed Wt1 and Tbx18 markers and could differentiate into cardiomyocytes, ECs, and SMCs (Zhou et al. 2008a). This supports the assertion that SHF progenitor (Nkx2-5- and Isl1-positive) cells contribute to the formation of PE during cardiac development (Zhou et al. 2008a).

3.2 Cardiac Progenitor Cells in Adult Heart

Cardiac cells were long believed to lack the capacity to self-renew (post-mitotic organ) and thus to have limited potential to regenerate after injury (Laflamme and Murry 2011). The regenerative potential of administering stem cells directly into the heart is still impeded by many challenges, such as limited yield and differentiation potential (Madonna et al. 2016). The benefits of stem cell therapy in cardiac patients have been proposed to be caused by a paracrine action, such as the angiogenesis mediated by the CPC-driven chemokine CXCL6 (Torán et al. 2017; Ptaszek et al. 2012; Mercola et al. 2011; Sebastião et al. 2019). During normal physiological aging, cardiomyocyte genesis is a caused by the slow division of pre-existing cardiomyocytes (Senyo et al. 2013). However, recent studies have shown that the generation of new cardiomyocytes occurs in the adult heart, generating renewed interest in cardiac regeneration (Kuhn and Wu 2010). The discovery of CPCs in embryos encouraged a further search for such progenitors in the adult heart (Kuhn and Wu 2010). An endogenous heterogeneous population of cells that is widely distributed throughout the adult heart, in the atria, ventricles, and other parts, was found to play a role in myocardial regeneration (Anversa and Nadal-Ginard 2002; Bergmann et al. 2009). These CPCs are quiescent cells that make a minimal contribution to repair damage to myocardial cells under normal physiological conditions (Hsieh et al. 2007). The specific biological role of CPCs in maintaining homeostasis or their reparative function in the injured heart is still unclear . Adult CPCs are subclassified into different types, according to their expression of specific cellular markers such as c-kit (CD117), Isl-1 (insulin gene enhancer protein), and Sca-l (stem *cell* antigen-1(Galvez et al. 2008; Oh et al. 2003). However, those markers are not specific and overlap with other tissue markers. The main characteristics of those cells are their self-renewal and clonogenic properties, in addition to their multipotent potential to differentiate into cells of cardiac lineages, such as myocytes, SMCs, and ECs (Beltrami et al. 2003a). The *in vitro* propagation of these cells in culture is characterized by being adherent or spheroid (the adherent cells grow in monolayers, while in the spheroid model the cells grow as 3D aggregates), termed cardiospheres (Messina et al. 2004; Shenje et al. 2008). Spheroids are non-adherent, multicellular

floating clusters of cells that were first defined in neural stem cells (Reynolds and Weiss 1992). Human cardiospheres (CS) were reported from the culture of patients' atrial appendage specimens on non-adhesive substrates supplemented with cardiosphere-forming medium. This medium contains epidermal growth factor, basic fibroblast growth factor, thrombin, cardiotrophin-1, and B27. CS showed heterogeneous populations of both primitive and committed progenitors expressing mesenchymal stem cell markers, such as CD105, CD13, CD73, and CD166, as well as early and late cardiac markers (Nkx2.5, GATA4, and connexin 43) (Barile et al. 2013). CS exhibited the multipotent ability to differentiate into cardiomyocytes, SMCs, and ECs, serving as a promising cell source for treating myocardial infarction in phase 1 clinical trials (Makkar et al. 2012). Additionally, CPCs of proepicardial origin, expressing platelet-derived growth factor receptor-alpha ($PDGFR\alpha^+$) and c-kit, have been found in the adult human heart (Chong et al. 2011). $PDGFR\alpha^+$ cells could differentiate into SMCs and ECs, providing a source of vascular and interstitial tissues of the injured heart . A recent study demonstrated that some types of CPC, such as $Bmi1^+$ cells, contribute to the regeneration of cardiomyocytes after injury, serving as a source of progenitors in cardiac repair (Valiente-Alandi et al. 2016).

3.3 Cardiac Progenitor Cells Derived from Human Pluripotent Stem Cells

Developmental cardiac progenitor cells are the *in vitro* version of CPCs that can be generated from either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). The main characteristics of these cells are their self-renewal and clonogenic properties, in addition to their multipotent differentiation potential, from which different cardiac lineages such as CMs, SMCs, and ECs arise (Sanganalmath and Bolli 2013; Mauretti et al. 2017; Beltrami et al. 2003b). Generating cardiovascular cells from ESCs has been shown to have many advantages. For example, ESCs are natural pluripotent cells, and can be scaled up and genetically tagged for cell selection or tracing. CPCs were shown to be derived from ESCs by in vitro manipulation of the essential signaling pathways involved in embryonic carcinogenesis, as described by Pucéat's protocol (Jebeniani et al. 1994). ESCs were cultured in serum-free mesogenic induction medium supplemented with small-molecule that inhibit FGF and Wnt signaling pathways in the presence of the cardiogenic morphogen BMP2. CPCs were isolated by the expressions of SEA-1 and MESP1 markers and showed a commitment to three lineages: cardiomyocytes, SMCs, and ECs (Blin et al. 2010a). A phase 1 clinical trial using hESC-derived cardiac progenitors was conducted for patients with severe heart failure (see ClinicalTrials.gov Identifier: NCT02057900).

The discovery of iPSCs and gene editing have promised an attractive approach for generating cardiac cells with specific mutations. This technology allowed the mimicking of inherited cardiac diseases and elucidation of their developmental mechanism. Congenital long QT syndrome is caused by mutation of the KCNH2 gene encoding potassium ion channels that regulate the cardiac action potential (Bohnen et al. 2017). CRISPR/Cas9 editing was thus used to introduce specific mutation into the KCNH2 gene (potassium voltage-gated channel, subfamily H and member 2) of healthy hiPSC lines. These iPSC-derived cardiomyocytes (iPSC-CMs) allowed study of the mechanism underlying inherited cardiac channelopathy (Chai et al. 2018). The third approach for direct cellular reprogramming involves a cocktail of transcription factors (Gata4, Mef2c, and Tbx5) essential for early carcinogenesis being directly injected into the cardiac fibroblasts of elderly mice. The transfected cells were directly reprogrammed into adult cardiomyocyte-like cells. These cells that beat upon cardiac stimulation decreased the infarct size and raised hopes for the achievement of *in vivo* cardiac regeneration (Qian et al. 2012).

4 In Vitro Characterization of Cardiac Progenitor Cells in Embryonic and Adult Heart

Cardiac progenitors express many specific proteins. Their markers have yet to be fully elucidated. Adult CPCs are categorized into seven main types expressing overlapping markers: c-kit, Sca1, islet-, SP cells, epicardium-derived cells, cardiac colony forming unit fibroblasts (c-CFU-Fs), and cardiosphere-derived cells (Le and Chong 2016a). They commonly share the expression of c-kit, but at different levels. Most adult CPC populations express surface markers such as Sca-1, Abcg-2, Flk-1, CD34, CD90, and CD10, and the transcription factors Isl-1, NK2 homeobox 5 (Nkx2.5), GATA binding protein 4 (GATA4), and myocyte enhancer factor 2 (MEF2), which are expressed continuously in both adult and embryonic CPCs. Embryonic CPCs express Oct3/4, Bmi-1, and Nanog, supporting their regenerative potential through enhancing the self-renewal and multiple propagation abilities (Van Berlo et al. 2014; Chong et al. 2013), Surface markers can be identified using flow cytometry or immunohistochemistry (Mishra et al. 2011). Table 1 illustrates the cell surface markers for each population (Takamiya et al. 2011). CPCs can also be characterized by their ability to form cardiospheres (Blue Box 1), SP cells to pump out the DNA binding dye (efflux), and colony formation by c-CFU-F cells (Mishra et al. 2011; Unno et al. 2012; Belostotskaya et al. 2018).

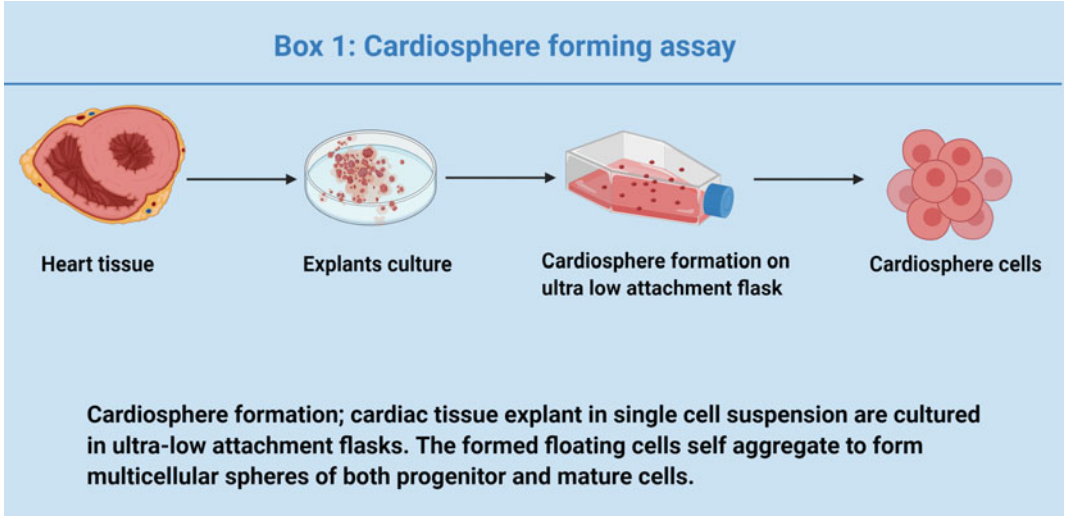
5 Concepts in Cardiac Differentiation

Signaling pathways play an important role in cardiac differentiation (Devalla and Passier 2018). Early-activated signaling pathways are inhibited during the later stages of cardiac differentiation to allow complete differentiation. Wnt proteins encompass a major family of lipid-modified glycoproteins acting as signaling molecules to facilitate cellular communication. They maintain the equilibrium of growth, function, differentiation, and cell death (Willert and Nusse 2012). The activation of Wnt proteins is essential during the generation of the mesoderm, and their inactivation is essential during the differentiation of cardiac progenitors. During early gastrulation, the E-cadherin signaling pathway dominates and the epiblast cells are tightly packed, resulting in an increase in membrane-bound β-catenin and Wnt signaling. The epiblast corresponds to this increase by releasing β-catenin from the membrane into the cytoplasm, leading to its accumulation (Naito et al. 2006; Ueno et al. 2007). Wnt proteins inhibit the phosphorylation of β-catenin in order to prevent its degradation by the proteasome (Pahnke et al. 2016). Subsequently, hypo-phosphorylated β-catenin moves to the nucleus and enhances the transcription of Wnt-induced genes. These genes include Wnt inhibitors that promote cardiac differentiation (Ueno et al. 2007; Lindsley et al. 2008). *In vitro* cardiac differentiation protocols initially used 5-azacitidine, a demethylating agent that alters gene expression and increases Wnt/β signaling, in order to enhance early mesodermal commitment. However, the exact mechanism by which 5-azacitidine acts has not been well characterized. Some protocols combine the usage of 5-azacitidine and TGF-β1 to increase vascularization and certain cardiac markers such as α-smooth muscle actin and vascular endothelial growth factor (VEGF) (Sebastião et al. 2019). Angiotensin II (Ang II) in combination with 5-azacitidine and TGF-β1 at low concentration (Xing et al. 2012) is used to enhance cardiac differentiation. Ang II was shown to stimulate the expression of TGF-β1 in different cell types such as SMCs, cardiac fibroblasts, and myofibroblasts (Williams 2001). To overcome the cytotoxicity of 5-azacytidine, small bioactive lipids were applied to induce Wnt/β-catenin signaling without notable cell damage. Sphingosine-1-phosphate (S-1-P) in combination with lysophosphatidic acid (LPA) was found to activate Wnt/β-catenin signaling, which results in the accumulation of nuclear β-catenin; this in turn facilitates mesodermal induction and subsequent cardiac differentiation. Other differentiation

Table 1 Cardiac progenitor cells types in adult and embryonic heart

Type	Surface marker	Transcription factor	Possible origin	Potency	Differentiates into	Pathway involved in differentiation	Ref.
Cardiac progenitor types (adult)							
Epicardium-derived cells	$CD34^+$	MRTF-A	Proepicardial organ/ epicardium	Multipotent	Smooth muscle myofibroblasts Endothelial cells	TGF-β	Smart et al. (2007), Trembley et al. (2015), Winter et al. (2007), Zhou et al. (2008b)
	c-Kit+	MRTF-B					
	$CD105^+$						
	$CD90^+$						
	$CD44^+$						
	$CD46^+$						
Side population cells	$CD34^+$	Nkx2.5	Bone marrow derived	Multipotent	Cardiomyocytes	c-Jun N -terminal kinase (JNK)	Pfister et al. (2005), Oyama et al. (2007), Liao et al. (2007), Chen et al. (2014)
	$CD45^+$	GATA4	Neural Crest (still heterogenous)				
	c-Kit+						
	Abcg2+						
	Sca-1^+						
Cardio sphere-derived cells	$CD34^+$	MEF2C	Cardiac origin	Multipotent	Cardiomyocytes	Notch 1/J kappa-recombining binding protein (RBPJ) signaling	Walravens et al. (2018), Chimenti et al. (2010), Malliaras et al. (2012), Davis et al. (2009), Chen et al. (2012)
	$CD45^+$	GATA4			Smooth muscle		
	c-$Kit^{(low)}$				Endothelial cells		
	Abcg+						
	$CD31^+$						
	$CD105^+$						
	Sca-1+						
c-Kit+ CPCs	CD34-	Nkx2.5	Cardiogenic mesoderm	Multipotent	Cardiomyocytes	PI3K	Beltrami et al. (2003a), Rota et al. (2007), Fathi et al. (2020)
	CD45-	MEF2C			Smooth muscle	MAPK	
	c-Kit+	GATA4	Bone marrow		Endothelial cells		
	$CD105^+$						
	CD166+						
	Abcg2+						
Cardiac colony forming unit fibroblasts (cCFU-F)	PDGFR	N/A	Proepicardial	Multipotent	Smooth muscle	TGFβ	Chong et al. (2011), Doyle et al. (2015)
	α+				Endothelial cells	BMP	
	CD90+				Cardiomyocytes		
	CD105+						
	CD44+						
	CD29+						

	Sca-1+ CD31- CD45- Flk-1^{-} C-kit$^{(low)}$						
Islet-1+ CPCs	C-kit +/- CD31- Sca-1^{-}	Isl-1+ Nkx2.5+ GATA4+	SHF	Multipotent	Smooth muscle Endothelial cells Cardiomyocytes		Laugwitz et al. (2005), Moretti et al. (2006), Genead et al. (2010)
Sca 1+ CPCs	Sca-1+ C-kit +/- CD105^{+} CD34- CD45-		N/A	Cardiomyocytes Chondrocytes Osteocytes	Cardiomyocytes		Oh et al. (2003), Le and Chong (2016b), Wang et al. (2006), Uchida et al. (2013)
Cardiac progenitor types (embryonic)							
Mesoderm CPCs	Islet-1+ C-kit+ FlK1-	NKX2-5 – Mesp-1+	SHF FHF	Multipotent	Cardiomyocytes smooth muscle cells arterial and venous endothelial cells fibroblasts	Notch, canonical WNT Hedgehog (Bergmann et al. 2009) signaling pathways	Bondue and Blanpain (2010), Wu et al. (2006), Dyer and Kirby (2009)
Neural crest CPCs	CD49+ Integrin-alpha-4 beta 1	Mesp-1- Sox10 MafB Krox20 Nestin	Ectoderm Neural tube	Multipotent	Smooth muscle (in vitro) Aortic arch arteries (in vivo)	TGF-β, and PDGF	Keyte and Hutson (2012), Tani-Matsuhana et al. (2018), Tomita et al. (2005)
Proepicardium	–	Nkx2-5+ Isl1+ Tbx18 WT1(Zhou et al. 2008a)	Extra cardiac mesenchyme and SHF	Multipotent	cardiomyocytes, endothelial cell smooth muscle	TGF-β Hedgehog (Keyte and Hutson 2012; Bergmann et al. 2009)	Keyte and Hutson (2012), Zhou et al. (2008a), Manner et al. (2001)



Blue Box 1 Cardiosphere assay protocol

protocols use glucose synthase kinase 3 inhibitors (GSK3) such as CHIR99021 to increase β-catenin expression, which consequently enhances Wnt signaling, allowing differentiation into cardiomyocytes (Sharma et al. 2018). It was also reported that small molecules such as KY02111 were used to block Wnt/β-catenin signaling in late differentiation in order to efficiently enhance cardiac myocyte differentiation (Minami et al. 2012).

6 Clinical Applications of Cardiac Stem Cells

According to the World Health Organization, the leading cause of mortality globally is cardiovascular diseases (CVDs). In 2016 alone, approximately 31% of deaths worldwide were attributed to these diseases. CVDs include congenital heart diseases, coronary heart diseases, cerebrovascular diseases, peripheral arterial diseases, and rheumatic heart diseases. The use of pharmacological agents and mechanical devices has helped to improve heart function, but most available therapies are symptomatic, do not cure the disease, and require lifelong maintenance. Regenerative medicine could potentially replace damaged heart or vessel cells (Fig. 3).

Stem cells used in cardiac regenerative therapies include:

6.1 Pluripotent Stem Cells

6.1.1 Embryonic Stem Cells

At present, the only performed clinical trial using ESC-derived pluripotent cells (ESCORT) was for the treatment for severe heart failure (ClinicalTrials.gov Identifier: NCT02057900); however, no data from this trial are currently available. Genetic modification of ESCs to promote the expression of the cellular repressor of E1A-stimulated genes (CREG), a glycoprotein that enhances cell survival and differentiation, followed by injection of the CREG-ESC cells into the peri-ischemic region in a myocardial infarction model, showed reductions in infarction, and fibrosis. Moreover, the survival rate of CREG-ESCs was high in the treated mice. Additionally, CREG-ESCs induced reductions in inflammatory cytokines including IL-1β, IL-6, and TNF-α and increases in the pro-inflammatory TGF-β, bFGF, and VEGF165. Enhancing the expression of CREG in CREG-ESCs appeared to prevent teratogenicity as the injection of up to 3.0×10^6 CREG-ESCs did not result in the teratoma formation

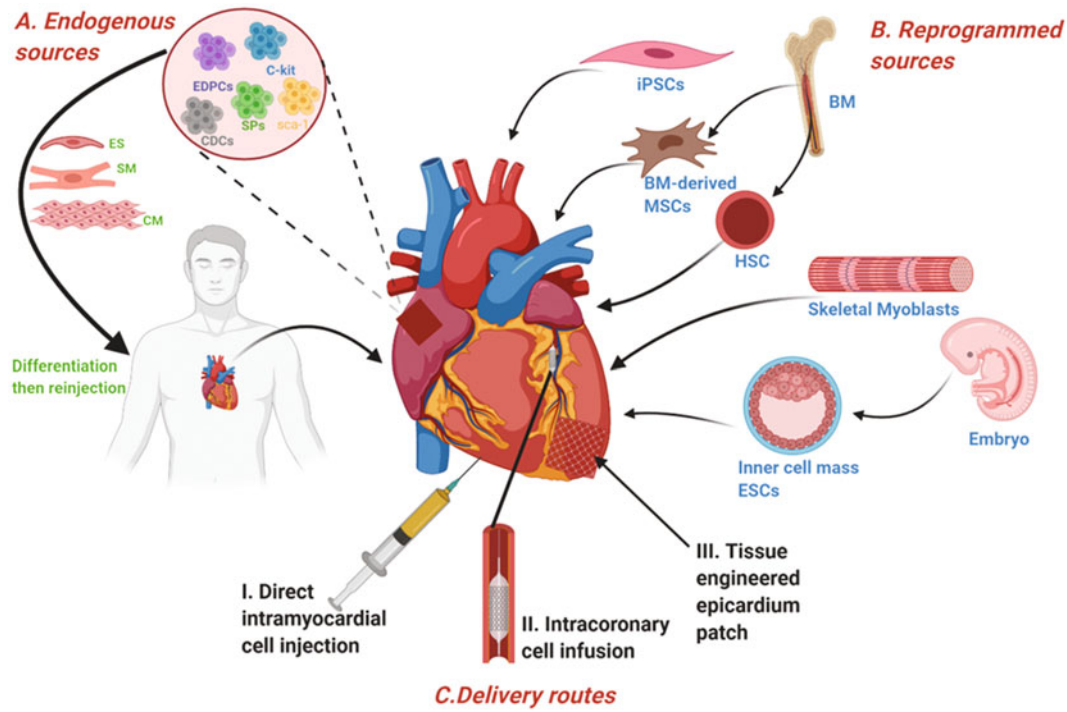


Fig. 3 Summary of different types of stem cell and their roles in heart regeneration

observed with ESCs (Zhang et al. 2018). In addition, Romagnuolo et al. used human ESC-derived cardiomyocytes (hESC-CMs) to treat infarcted heart in a pig model. Although immunosuppressed animals did not reject the grafted cells and no teratoma formation was detected, monomorphic ventricular tachycardia frequently occurred up to 4 weeks after transplantation (Romagnuolo et al. 2019).

6.1.2 Induced Pluripotent Stem Cells (iPSCs)

IPSCs are derived from adult somatic cells (e.g., fibroblasts) that have been reprogrammed into an embryonic-like state, and have the ability to differentiate into different lineages (Takahashi et al. 2007). They display broad differentiation plasticity and are a source of autologous therapy. The in vitro culture of iPSCs treated with BMP2 in the presence of FGF pathway inhibitors was shown to upregulate the expression of connexin-43 and myosin chain complexes in CPCs (Blin et al. 2010b). These progenitors were shown to be multipotent and could generate SMCs, ECs, and cardiomyocytes (Blin et al. 2010b). The cardiomyocyte derivatives of iPSCs (iPSC-CMs) were demonstrated to successfully restore the myocardium after injection into ischemic hearts in animal models (Kawamura et al. 2012; Wang et al. 2013). The administration of CPC-iPSCs was shown to achieve myocardial restoration, increase the formation of new blood vessels, and result in better survival in a hostile ischemic environment compared with terminally committed iPSC-CMs (Mauritz et al. 2011). Carpenter et al. generated CPC-iPSCs that differentiated into smooth muscle and cardiomyocytes and persisted for more than 1 month upon injection into infarcted rat heart (Carpenter et al. 2012). The co-administration of MSC-loaded patch (hMSC-PA) along with hiPSC-CMs in the infarcted heart enhanced their resistance to the hostile ischemic tissue microenvironment and promoted vascular regeneration. This effect was mediated by paracrine factors secreted by hiPSC-CMs. Additionally, the MSC-loaded patch increased the

retention of hiPSC-CMs and prevented their leakage into the epicardial space. Furthermore, the differentiated cells showed striations with Z-bands and more efficient electrical conduction. Dual stem cell therapy led to improved heart function, vascular regeneration, retention, engraftment maturity of hiPSC-CMs, and ameliorated cardiac fibrosis (Park et al. 2019). Ongoing clinical trials [e.g., (HEAL-CHF) ClinicalTrials.gov Identifier: NCT03763136] are limited due to the safety concerns associated with iPSCs.

6.2 Multipotent Stem Cells

6.2.1 Mesenchymal Stem Cells

Human MSCs (hMSCs) are non-hematopoietic, multipotent stem cells capable of differentiating into osteocytes, adipocytes, and chondrocytes, as well as other cell lineages (Ullah et al. 2015). MSCs are extensively used in experimental and clinical studies because of the accessibility of the cell for in vitro modifications, and their immunomodulatory characteristics (Roura et al. 2017; Nauta and Fibbe 2007). HMSCs have been isolated from bone marrow, adipose tissue, umbilical cord, placenta, and amniotic fluid (Nauta and Fibbe 2007). The administration of MSCs into infarcted myocardium resulted in reduction in the size of the infarct and upregulated VEGF secretion, leading to enhanced vascularization and amelioration of the damage to cardiac tissues (Zhao et al. 2014; Rahbarghazi et al. 2014). A paracrine effect mediated by secreted factors and juxtacrine crosstalk between the transplanted MSCs and ECs in the infarcted area were shown to mediate this repair. Soluble factors such as Ang-1, IGF-1, VEGF, SDF-1α, and EGF upregulated the expression of endogenous pro-angiogenic molecules in the infarcted tissue (Rahbarghazi et al. 2014).The proposed mechanisms of repair include the differentiation of administered cells into cardiac cells, release of paracrine signaling factors, and fusion of the administered cells into myocardiac muscle cells (Kajstura et al. 2005; Orlic et al. 2001; Mazhari and Hare 2007). The latter mechanisn was refuted when the injection of Akt-expressing MSCs into infarcted rat heart resulted in transient grafting, infrequent fusion, and very low differentiation (Noiseux et al. 2006). The infarcted microenvironment contributes to the low efficacy of stem cell transplant. Hypoxia and inflammatory cytokines are the main factors that limit the survival of the grafted MSCs in myocardial infarction (Mangi et al. 2003). Transfecting MSCs using genes encoding Akt was shown to enhance their engraftment, differentiation, and ability to repair the damaged heart tissues in a rodent model of MI (Mazhari and Hare 2007; Mangi et al. 2003). In another study, the co-administration of insulin-like growth factor 1 improved the survival of the transplanted MSCs and enhanced their capacity to regenerate the myocardium after MI (Davis et al. 2006). Survival of the MSCs following MI was also enhanced by a hypoxia-regulated heme oxygenase 1-vector modification (Tang et al. 2005). In a phase 1, randomized, double-blind, placebo-controlled clinical trial, allogeneic hMSCs were intravenously injected into patients with a first acute myocardial infarction (Hare et al. 2009). Specific safety monitoring showed that patients who received the cell therapy had better outcomes in terms of cardiac arrhythmias, lung function, left ventricular function, and global symptoms, than those without this therapy (ClinicalTrials.gov Identifier: NCT00114452) (Hare et al. 2009). In addition, autologous BM-MSCs have been injected into 59 patients with ischemic heart failure (ClinicalTrials.gov Identifier: NCT00644410)(Mathiasen et al. 2015). The transplanted patients demonstrated significant improvement in systolic left ventricular (LV) function, left ventricular end-systolic volume, left ventricular ejection fraction (LVEF), systolic volume , and cardiac output, compared with the placebo group. The LV mass and wall thickening were also enhanced(Mathiasen et al. 2015).

Other sources of MSCs include adipose tissues, placenta, cord blood, and Wharton's jelly. Adipose-derived mesenchymal stem cells (AD-MSCs) have also shown promise in MI patients. The cells are usually harvested from subcutaneous lipoaspiration (Davis et al. 2006;

Qayyum et al. 2019). AD-MSCs are easier to obtain and give higher yields of stem cells than bone marrow MSCs. Moreover, a study showed that patients with refractory angina treated with autologous AD-MSCs (ClinicalTrials.gov Identifier: NCT01449032) maintained their exercise abilities, while the exercise performance of patients in a placebo group was significantly decreased. The heart symptoms improved significantly during a 3-year follow-up and the number of weekly angina attacks for patients was significantly reduced (Qayyum et al. 2019)..Umbilical cord MSCs (UC-MSCs), placental MSCs, and those derived from Wharton's jelly present attractive sources for MSCs for cardiac regeneration. UC-MSCs were shown to have a higher capacity for self-renewal than BM-MSCs (Fong et al. 2011). In addition, the intracoronary administration of Wharton's jelly-derived MSCs in patients with ST-elevated MI showed improved myocardial viability and cardiac function, when compared with those transplanted with BMMNC (ClinicalTrials.gov Identifier: NCT01291329) (Gao et al. 2015).

As mentioned above, cardiac repair after MSC therapy is attributed to several mechanisms. The hypoxic conditions of infarcted tissue induce the expression and release of growth factors that promote angiogenesis, the distribution and migration of cardiac progenitors, and the differentiation of MSCs into cardiomyocytes. In addition to the promotion of angiogenesis, factors such as VEGF, hepatocyte increasing factor (HGF), and insulin-like growth factor (IGF) upregulate the expression of cardiac differentiation genes. Additionally, immunomodulatory and trophic factors secreted by MSCs activate resident stem cells to potentiate cardiac repair and enhance vascularization (Madigan and Atoui 2018; Caplan 2017). However, there are still many challenges regarding MSC therapy for cardiac regeneration. Most importantly, poor survival of the transplanted cells after grafting into the host myocardium leads to therapy failure, presumably due to the hostile environment of the ischemic/infarct tissue (Timmers et al. 2011).

6.2.2 Cardiac Progenitor Cells

Upon treatment with 5-azacytidine and TGFβ, CPCs were shown to differentiate into cardiomyocytes that beat spontaneously (Goumans et al. 2008). Additionally, they were differentiated into ECs and SMCs upon treatment with VEGF (Goumans et al. 2008). Upon the transplantation of CPCs into the infarcted zone in mice, cardiac function was improved for 3 months after MI. Over the same period, and despite the remarkable increase in the number of blood vessels, only a small proportion of the infused CPCs survived. The density of the blood vessels increased remarkably when measured only 2 weeks after transplantation, despite of no indication of vascular differentiation. Improved function after MI was not due to their differentiation to replace damaged cardiomyocytes, but due to paracrine mechanisms. The pro-angiogenic potential of extracellular vesicles (EVs) isolated from CPCs was shown to promote revascularization. EVs of CPCs are being introduced as a potential therapy for MI. This is due to the complexity of their content of miRNAs and proteins and their effectiveness as performers of the paracrine therapeutic effect of CPCs (Smits et al. 2009a; Smits et al. 2009b).

Undifferentiated CPCs were found to secrete a higher level of VEGF. In preclinical studies, they were shown to reduce cardiac damage, enhance proliferation in the left ventricle, lead to the promotion of proliferative markers in the border zone, and stimulate the secretion of pro-angiogenic factors such as endoglin (Goumans et al. 2008; Maring et al. 2019). Andrade et al. reported that the subcutaneous injection of 100 $\mu g.kg^{-1}$ of IGF-1 for up to 7 days enhanced the survival and proliferation of CPCs and ameliorated obesity-induced cardiomyopathy in a rat model (Andrade et al. 2020). Moreover, in a preclinical study, Kannappan et al. investigated the effect of enhanced expression of the p53 tumor suppressor gene on CPC function. A high yield of CPCs was isolated from transgenic mice with an extra allele of the p53 gene. Additionally, those cells showed the ability to withstand oxidative stress upon

injection into a rat model of type I diabetes mellitus. High expression of the p53 gene was also shown to play an important role in protecting CPCs and enhancing their ability to replace damaged cardiomyocytes (Kannappan et al. 2017). The intracoronary injection of 0.3 million/kg autologous CPCs to treat single ventricle physiology (TICAP), also known as single ventricular defect (ClinicalTrials.gov Identifier: NCT01273857), showed no adverse cardiac effects in seven study participants (Tarui et al. 2015).

6.2.3 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are blood-forming multipotent cells that are present in the bone marrow at a low level (about 1 in 10,000 cells). Sources of HSCs include the bone marrow, peripheral blood, and umbilical cord (Fong et al. 2011). The injection of lin-c-kit$^+$ HSCs after coronary ligation in mice was shown to repair 68% of the infarcted heart section, leading to a significant improvement in coronary artery disease (Orlic et al. 2001). The COMPARE-AMI clinical trial (phase II, double-blind, placebo-controlled, randomized study) tested the safety and feasibility of administering CD133$^+$hematopoietic progenitor cells by intracoronary injection in acute myocardial infarction (AMI) patients. No serious adverse events such as arrhythmia, angina, stent thrombosis, heart failure, or death were reported during a 1-year follow-up. This study showed that CD133$^+$ injection was safe and feasible, and effectively improved LV perfusion and function for patients with AMI (Mansour et al. 2011). Another trial (the REGENT trial) aimed to compare intracoronary infusion of bone marrow mononuclear cells and hematopoietic cells (CD34$^+$) in patients with AMI. The study showed no significant difference between selected CD34$^+$and unselected bone marrow mononuclear cells. Both groups showed a 3% increase in LVEF from baseline, in contrast to no significant change in the control group (Tendera et al. 2009).

6.2.4 Skeletal Myoblasts (Satellite Cells)

Skeletal myoblasts are multipotent cells, arise from the muscle stem cells (satellite cells), located beneath the basal lamina of muscle fibers (Yin et al. 2013). Myoblasts express a group of markers, including Pax7, CD34, VCAM 1, MRF4, Desmin, CD56, syndecan3, Pax3, M-cadherin, N-CAM, c-met, Leu-19. When activated, especially after injury, myoblasts firstly express MyoD or/and Myf-5, then differentiation markers myogenin and MRF4 (Durrani et al. 2010) (Fig. 4). Satellite cells were among the first stem cells to be tested in for myocardial regeneration in pre-clinical and clinical studies (Tompkins et al. 2018).

When satellite cells isolated from an adult rat were combined with bromodeoxyuridine and transplanted into syngeneic rat heart, they failed to differentiate into cardiomyocytes after 12 weeks follow up (Reinecke et al. 2002). Co-transplantation of skeletal myoblasts and other stem cells showed to be more effective than using the single types of cells. For example, the combination of mononuclear bone marrow stem cells and skeletal myoblasts were more beneficial for myocardial repair than either cell alone (Ott et al. 2004). However, the major limitation of skeletal myoblast transplantation remains due to poor engraftment. Repeated administration of skeletal myoblasts for infarcted tissue was shown to be required for effective treatment (Gavira et al. 2010). Percutaneous injection of three repeated doses of skeletal myblast in infarcted swine heart showed improved efficacy of the aortic valve ejection fraction (AVEF) and improved cardiac function in general (Gavira et al. 2010).

The first skeletal myoblast transplantation was carried out in 2001 in a patient suffering from severe ischemic heart failure (Menasché et al. 2001; Menasche et al. 2001). Clinical applications of this approach however suffered the limitation of absent control groups, and limited number of participants in general. In a 2015 study, seven patients were transplanted with skeletal myoblasts for treatmet of CHF. After 26 weeks, six of the patient showed an improvement in the left ventricular ejection fraction (Sawa et al. 2015). In and study, thirty patients with class II (mild) and class III (moderate) heart failure were treated by using connexin-43 expressing muscle progenitor cells. When followed up for

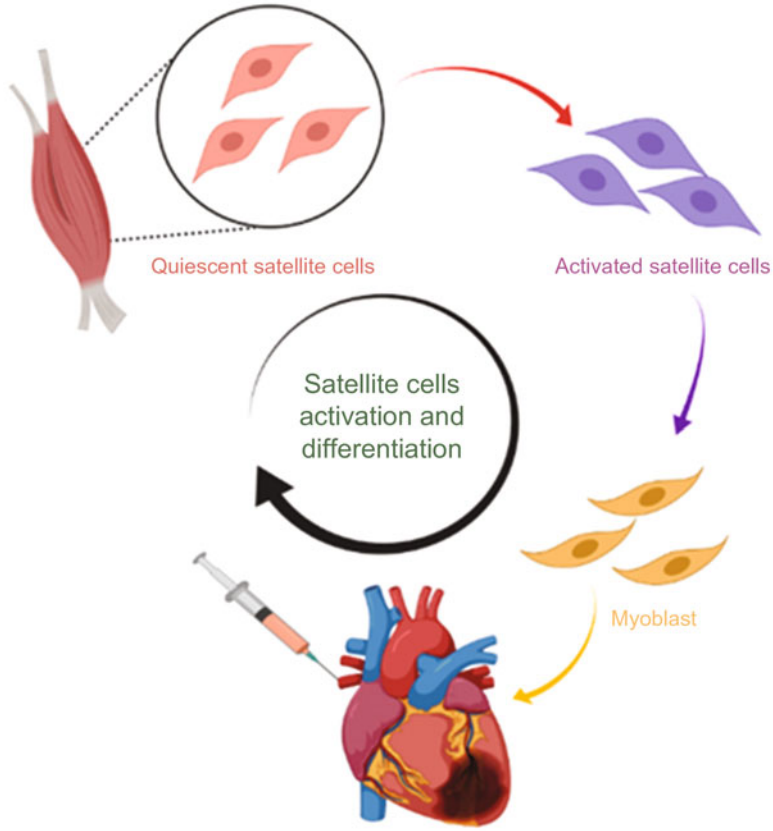


Fig. 4 The pathway cycle for quiescent satellite cell activation and differentiation into myoblasts, followed by transplant into the heart

6 months, the patients showed promising improvement in the targeted tissues especially myocardial viability (Gwizdala et al. 2017). Although, low tumorgenicity encourage the studies at first, but , many side effects were observed ranging from resistant ventricular arrhythmias to ischemic stress (Yin et al. 2013) , in addition to the inability to differentiate into cardiomyocytes in some cases (Cheitlin 2008). Due to these significant risks in patients' lives, the attention on these cells for treatment almost diminished (Müller et al. 2018).

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