Review Article

The heart of the matter: cardiac stem cells

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Abstract: Cardiovascular diseases are the primary cause of death in the world. Pharmacological and surgical approaches are the main treatment options for heart disease; however, heart transplantation may be the only option for advanced heart failure patients despite its limited nature. Recent advances enabled stem cell-based therapies to become a promising treatment approach for injured or weakened cardiac tissue. With the identification of resident and heart-specific stem cells in early 2000, new avenues of research have been opened to understand heart development, disease, and regeneration. In this review article, different cardiac stem cell subpopulations are classified and defined based on the expression of various characteristic surface or intracellular proteins, including, but not limited to, C-kit, Sca-1, Isl-1, Nkx2.5, HCN4, SIRPA, Flt-1, and KDR. Understanding cardiac stem cell biology, self-renewal, and differentiation mechanisms holds great promise for directing these processes and utilizing these cells to repair or even build new hearts.

Key words: Stem cell, cardiac stem cells, heart disease, cardiac development

1. Introduction

Cardiovascular disease and resulting heart failure remain the leading causes of death and disability in the world. Pharmacological (beta-blockers, ACE inhibitors), electrophysiological (implantable cardioverter defibrillators, pace-makers), and surgical (coronary artery bypass grafts, left ventricular assist devices) interventions and devices are the main therapy options. However, for end-stage heart failure, the only therapeutic option is heart transplantation. Due to the limited number of organ donors and the associated complications of the surgery, alternative pharmacological or cell-based therapies have been continued to be investigated. From the current search for new therapy options, stem cell-based approaches have gained momentum over the last decade, especially after the identification of resident cardiac stem cells (CSC). CSCs are tissue-specific progenitors that contribute to cardiac tissue maintenance and repair throughout adult life.

After a cardiac injury as often seen after a myocardial infarct (MI), due to the ischemia in the myocardium, over a billion cardiomyocytes could be lost (Olivetti, 1997). The heart can regenerate itself with the help of the resident CSCs or with the contribution of recruited mesenchymal stem cells (MSCs) to a small extent. However, given the magnitude of myocardial damage and the constant demand for ventricular contraction to deliver blood to the body, a timely recovery is mostly inadequate. Thus, delivery of CSCs or activation of CSCs within the heart by small molecules of growth factors could represent one of the alternative sources for cell therapies because of their capacity to generate various cell types forming the heart.

Resident CSCs or cardiac progenitor cells arise from a certain population of mesodermal precursors during embryogenesis (Martin-Puig et al., 2008). Various populations of CSCs have been identified that possess the fundamental properties of stem cells, such as multipotency, clonogenicity, and self-renewal capacity (Beltrami et al., 2001). An adult heart is mainly composed of cardiomyocytes, endothelial cells, smooth muscle cells, cardiac fibroblasts, and pericytes. Many CSC populations have the capacity to differentiate into cardiomyocytes, and some populations of so-called cardiovascular progenitors can give rise to smooth muscle cells (SMCs) and endothelial cells (ECs), as well (Bearzi et al., 2007). Beltrami et al. (2001) detected a Ki-67-positive cell population by immunolabeling in 4% of myocyte nuclei, suggesting proliferative cells after myocardial infarction. This observation implicates a stem cell population that could be activated or recruited to the infarct zone and proliferate to repair the tissue. Although still controversial, other reports have also proposed that

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some cardiomyocytes can renew themselves, albeit at low levels. In support of this idea, Bergmann et al. (2009) utilized ^{14}C, which integrates into DNA to establish the age of cardiomyocytes in humans, and showed that less than 50% of cardiomyocytes are exchanged during a normal lifespan (Bergmann et al., 2009).

Although heart size, beating rate, and cardiac physiology differ significantly among different species, common protein families lead heart development during embryogenesis and adulthood in mammals. Model organisms and small and large mammals are widely used to explore CSC biology. In combination with elaborate bioinformatics approaches, both nuclear and cell-surface proteins have been identified as markers for CSCs and mature cell types in the heart. Some widely studied markers of CSC include, but are not limited to, C-kit, Sca-1, SIRPA, KDR, GATA4, HCN4, Nkx2.5, and Isl-1.

In this review, we aim to summarize mainstream studies on various CSC populations that are verified by independent reports, briefly explain their characterization based on specific surface antigens or specific transcription factors that they express (Figure; Table), and briefly explain basic clinical applications. The classifications of cardiac stem cells based on metabolic activity, dye exclusion, and "or else" criteria are excluded due to space limitations and are reviewed in other reports.

2. Cardiac stem cells

2.1. C-kit+ cells
C-kit, defined as a mast/stem cell factor protein receptor (CD117), is one of the most widely studied cell-surface

markers that define a subpopulation of CSCs (Orlic et al., 2001; Beltrami et al., 2003; Messina et al., 2004; Castaldo et al., 2008). C-kit-expressing stem cells have been reported to originate from and reside in the bone marrow niche and are recruited to the heart after a MI (Orlic et al., 2001). Beltrami et al. (2003) reported that C-kit+ cells were a subpopulation CSCs and those cells were negative for expression for hematopoietic lineage markers (Lin). Freshly isolated C-kit+/Lin- cells from adult rat myocardium tissue can self-renew and differentiate into cardiomyocyte, SMC, and EC lineages but failed to spontaneously contract in vitro. It was also shown that isolated C-kit+ cells expressed cardiac-specific transcription factors including *NKX2.5*, *GATA-4*, and *MEF-2* in the early myocyte lineage (Henning, 2011). C-kit+ cells were then injected into an ischemic heart, and cardiac-derived C-kit+/Lin- cells proliferated and regenerated myocytes, SMCs, and ECs in vivo (Beltrami et al., 2003).

Several studies have shown that, when injected into an ischemic rat heart, C-kit+ cells contributed to the functional myocardium by being detected in approximately 70% of the ventricle (Sullivan et al., 2015). Another study demonstrated that human heart explant cultures contained C-kit+ CSCs at a rate of about 4% and were capable of both endocardial and cardiomyogenic differentiation (Sandstedt et al., 2014). Moreover, infusion of C-kit+ resident CSCs reduced the rate of apoptosis and oxidative stress in cardiomyocytes and noncardiomyocyte cells (Kazakov et al., 2015). The molecular mechanisms behind C-kit+ CSCs' survival/growth and migration were linked to the activation of PI3K and MAPK pathways (Vajravelu et al., 2015).
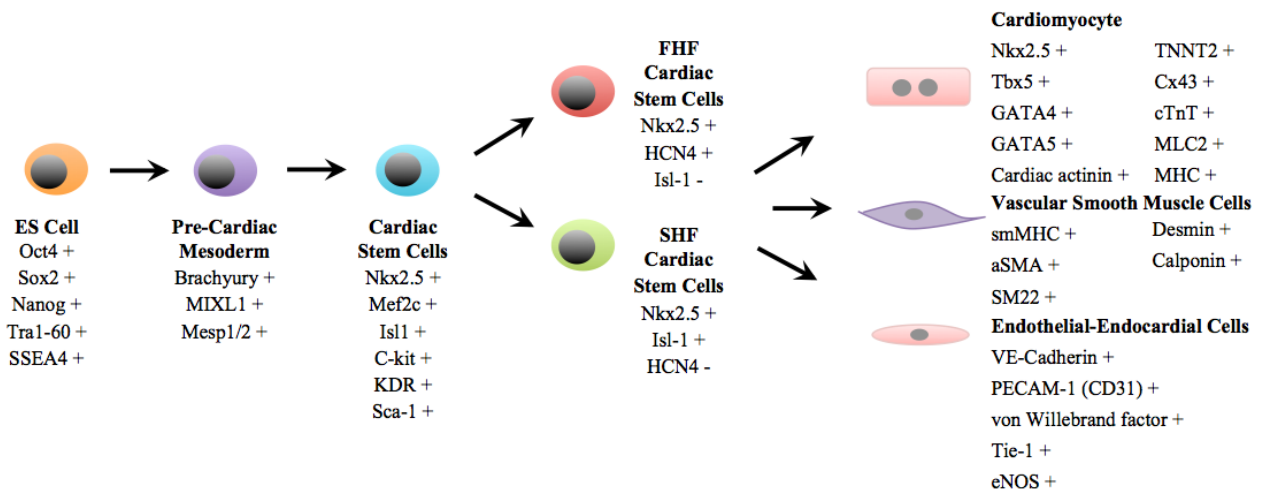

Figure. Schematic presentation of lineage commitment of stem cells towards mature cardiac cells. Characteristic marker gene expression has been depicted under each population. aSMA: alpha-Smooth muscle actinin, cTnT: cardiac troponin T, Cx43: connexin 43, eNOS: endothelial nitric oxide synthase, FHF: first heart field, MIXL1: mix paired-like homeobox, MLC2: myosin light chain 2, PECAM-1: platelet endothelial cell adhesion molecule-1, SHF: second heart field, smMHC: smooth muscle myosin heavy chain, SSEA: stage-specific embryonic antigen, Tie1: tyrosine kinase with immunoglobulin-like and EGF-like domains, TNNT2: troponin T-Type 2, VE-Cadherin: vascular endothelial cadherin.

Table. Name, tissue origin, cardiac differentiation potential, and characteristic cell surface or nuclear protein marker expression of distinct CSC subpopulations.

Name of population	Origin of cells	Differentiation potential	Markers	References
C-kit$^+$/Lin$^-$ cells	Bone marrow-derived mesenchymal stem cells; can be detected in heart tissue after injury	Cardiomyocytes, smooth muscle cells, and endothelial cells	C-kit$^+$ Lin$^-$ Gata4$^+$ CD31$^-$ CD45$^-$ Isl-1$^-$	Beltrami et al., 2003; Di Felice et al., 2009
Sca-1$^+$ cells	Hematopoietic stem cells; can be detected in heart tissue after injury	Cardiomyocytes (after treatment with 5'-azacytidine)	Sca-1$^+$ Mef2c$^+$ Gata4$^+$ CD31$^+$ CD105$^+$	Freire et al., 2014; Valente et al., 2014
KDR$^+$ cells	Mesodermal precursors, ESC and iPSC-derived cells	Cardiomyocytes, smooth muscle cells, and endothelial cells	KDR$^+$ C-kit$^+$ Sca-1$^+$ MLC2v$^+$ cTn-I$^+$	Kattman et al., 2006; Yang et al, 2008
Flt-1$^+$/Flt-4$^+$ cells	ESC and iPSC-derived cells	Cardiomyocytes, smooth muscle cells, and endothelial cells	Flt-1$^+$ Flt-4$^+$ Isl-1$^+$ Nkx2.5$^+$ KDR$^+$ C-kit$^+$	Lui et al., 2013; Nsair et al., 2012
Isl-1$^+$ cells	Second heart field progenitors, embryonic and adult heart	Cardiomyocytes, smooth muscle cells, and endothelial cells	Isl-1$^+$ Nkx2.5$^-$ Gata4$^+$ C-kit$^-$ Sca-1$^-$	Cohen et al., 2007, Qyang et al., 2007; Cagavi et al., 2014
Nkx2.5$^+$ cells	First heart field progenitors, embryonic and adult heart	Cardiomyocytes, smooth muscle cells, and endothelial cells	Nkx2.5$^+$ Gata4$^+$ Tbx5$^+$ Tbx18$^+$	Stennard et al., 2003; Zhang et al., 2014; Ellesøe et al., 2015
Mesp1$^+$/Mesp2$^+$ cells	Early marker for the common hematopoietic and cardiac lineages	Cardiomyocytes, smooth muscle cells, and endothelial cells	Tbx5$^+$ Nkx2.5$^+$	Kitajima et al., 2000; Lindsley et al., 2008
Wt1$^+$/Tbx18$^+$ cells	Cardiac endothelial progenitors, embryonic heart	Endocardial/endothelial cells	Wt1$^+$ Tbx18$^+$ Nkx2.5$^+$ Isl1$^+$	Zhou et al., 2008; Takeichi et al., 2013
SIRPA$^+$/VCAM1$^+$ cells	ESC and iPSC-derived cells, embryonic heart	Cardiovascular cell lineage differentiation	Nkx2.5$^+$ SIRPA$^+$ VCAM1$^+$ CD34$^+$	Elliot et al., 2011; Den Hartogh et al., 2015
HCN4$^+$ cells	First heart field progenitors and sinoatrial node	Cardiomyocytes and heart conduction system	Gata4$^+$ HCN4$^+$ Isl-1$^-$ Nkx2.5$^+$ Tbx3$^+$	Grant, 2009; Später et al., 2013

Table. (Continued).

Cardiac side population	Side population cells in the heart that exclude Hoechst 33342 dye	Cardiomyocytes (when cocultured with neonatal rat ventricular cardiomyocytes)	Abcg2+ Nkx2.5+ C-kit- Sca-1-	Oyama et al., 2007; Nagai et al., 2013
Cardiospheres	Atrial and ventricular heart tissue biopsies	Cardiomyocytes	C-kit+ Sca-1+ KDR+ CD34+ Nkx2.5+ Gata4+	Messina et al., 2004
Cardiac atrial appendage cells	Human atrial appendage	Cardiomyocytes	ALDH+ C-kit+ CD45- CD34+ KDR+	He et al., 2011; Koninckx et al., 2013

Another study demonstrated that C-kit+ cells were localized to the subepicardium by expression of laminin-1 and integrin alpha-6 subunits, whose coexpression may facilitate the activation of regeneration through the epithelial–mesenchymal transition in adult hearts (Castaldo et al., 2008). Recently, using rodent models, Ellison et al. (2013) showed that endogenous C-kit+ CSCs regenerated lost cardiomyocytes after myocardial damage and argued that these cells are necessary and sufficient for cardiac regeneration. In order to specifically show the contribution of C-kit+ CSCs in repair, they selectively directed apoptosis of exogenous CSCs and showed a loss of regeneration following selective death (Ellison et al., 2013).

Contrary to the previous reports, Sultana et al. demonstrated that C-kit+ cells predominantly labeled a cardiac endothelial cell population in developing and adult murine hearts and that resident C-kit+ population were not capable of generating cardiomyocytes (Sultana et al., 2015). After acute cardiac injury, they showed that C-kit+ cells retained their endothelial cell identity and did not become myogenic progenitors or cardiomyocytes (Di Felice et al., 2009; Sultana et al., 2015). Collectively, the true origin and the capacity of C-kit+ cardiac cells have been questioned vigorously. Whether C-kit+ cells can engraft to the myocardium and directly contribute to repair by differentiating into cardiomyocytes or assist healing of damaged cardiac tissue by paracrine signaling and indirectly contribute to repair still remain controversial.

2.2. Sca-1+ cells
Stem cell antigen-1 (Sca-1)-expressing cells have hematopoietic stem cell origin and can be readily obtained from bone marrow (Van De Run et al., 1989). Sca-1+ cardiac progenitors are nontumorigenic in nature and have been shown to propagate long-term in culture, express stemness and cardiac-specific markers, and differentiate into cardiac cell lineages, specifically beating cardiomyocytes (Oh et al., 2003; Matsuura et al., 2004; Wang et al., 2006, 2014).

Sca-1+/C-kit- cells from rodent hearts were shown to induce differentiation into the cardiomyogenic lineages in response to 5-azacytidine, a DNA methyltransferase inhibitor, in vitro (Oh et al., 2003). Transplantation of Sca-1+/CD31- cells into acute infarcted mouse heart ameliorated the functional decline and improved myocardial neovascularization with modest cardiomyocyte regeneration (Wang et al., 2006). Another study reported that delivery of insulin-like growth factor-1 and hepatocyte growth factor-1 combined with Sca-1+/CD31- cell populations gave positive results after transplantation therapy in an altered hostile microenvironment following MI (Wang et al., 2014). These data indicate that accurate factors combined with CSCs could serve as a good candidate approach for myocardial repair.

On the contrary, numerous recent reports challenged whether Sca-1+ cells have authentic cardiac or MSC origin. Houlihan et al. argued that Sca-1+ cells are a subpopulation of MSCs based on the detected expression of PDGFRα (Houlihan et al., 2012). Moreover, the Sca-1+/Lin- heart subset was found to be heterogeneous and displayed a mesenchymal profile characterized by a restricted ability to generate cardiomyocytes in vitro and in vivo, even after injury (Valente et al., 2014). Furthermore, reporter animal lines and lineage-tracing experiments examining cells with different myocardial Sca-1 expression levels demonstrated

that Sca-1 expression was low but continuous in the myocardium during adulthood and after injury (Freire et al., 2014). Sca-1^{+} cells were claimed as a ready-to-use CSC population for cardiac repair; however, whether the positive effects of Sca-1^{+} cells on cardiac recovery are due to incorporation and cardiac differentiation or simply inducing neighboring cells indirectly by paracrine signaling still remains to be shown. Nevertheless, due to their easily accessible nature and established culture conditions, Sca-1^{+} cells have been widely used in clinical trials and transplantation experiments to investigate engraftment, survival, adhesion, and migration capacity in the cardiac tissue (Jha et al., 2015).

2.3. KDR[+] (Flk-1^{+}) cells
Kinase insert domain receptor (KDR) is a type III receptor tyrosine kinase, which is also known as vascular endothelial growth factor receptor 2 (VEGFR-2) and fetal liver kinase-1 (Flk-1). KDR was first identified as a protein that is specific for early embryonic hematopoietic precursors (Kabrun et al., 1997). Later reports suggested that Flk-1^{+}/CXCR4^{+}/vascular endothelial cadherin^{-} cells could be cardiac-specific progenitors based on the observation of these cells in early stages of rodent embryos and in ES-cell differentiation cultures (Yamashita et al., 2005). Flk-1^{+} cells were shown to be panmesodermal populations as they gave rise to CSCs and mature cardiac lineages in both the first heart field (FHF) and second heart field (SHF) during embryonic development. To test the cardiomyogenic potential of these cells, Brachyury[+]/Flk-1^{-} population lineages were traced and shown to generate a Flk-1^{+} population that displayed robust cardiac potential in vivo (Kattman et al., 2006).

In support of the observation that KDR-expressing cells are CSCs, Yang et al. demonstrated that KDR^{+} cells isolated from human embryonic stem cell (ESC) cultures were capable of differentiating into cardiomyocytes, SMCs, and ECs in vitro (Yang et al., 2008). In fact, KDR expressions were shown to increase between days 4 and 6 of human ESC differentiation, which mimics the developmental stage after the establishment of the primitive-streak-like population in the embryo. In the same report, gene expression analysis showed that cardiac-specific genes including *NKX2.5*, *ISL1*, and *TBX5* were expressed at the highest levels in the KDR[low]/C-kit[-] cell population (Yang et al., 2008). These findings suggest KDR[+] CSCs as a representative of one of the earliest stages in mesoderm specification to the cardiovascular lineages that needs to be further verified in human embryonic development.

2.4. Flt-1^{+}/Flt-4^{+} cells
Vascular endothelial growth factor receptors (VEGFR) VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), and VEGFR-3 (Flt-4) play important roles in angiogenesis, vascular remodeling, and cellular hypoxic response. Human and mouse Flt-

1[+] and Flt-4^{+} coexpressing cells have been shown to differentiate into all three cell types of the cardiovascular lineage in vitro (Nsair et al., 2012). In vivo evaluation of Flt-1^{+}/Flt-4^{+} CSCs transplanted into the left ventricle showed successful engraftment and differentiation into mature beating cardiomyocytes (Nsair et al., 2012). Gene expression analysis of endogenous mouse Flt-1^{+}/Flt-4^{+} cells found that they were able to expand in vitro and they were demonstrated to express high levels of progenitor cell markers such as Isl-1, Nkx2.5, Flk-1, Flt-1, Flt-4, and C-kit, but not genes associated with differentiated cardiovascular cells (Nsair et al., 2012). It needs to be further investigated whether these Flt-1^{+}/Flt-4^{+} cells are true CSCs or a subpopulation of Isl-1 or Nkx2.5 CSC populations.

2.5. Isl-1^{+} cells
A novel population of cardiovascular progenitors was identified based on the expression of the LIM-homeodomain transcription factor Islet-1 (Isl-1). Isl-1^{+} CSCs belong to the SHF progenitors during embryogenesis and contribute to the majority of the heart including the right atrium, right ventricle, left atria, and outflow tract, and are also present in the adult human heart (Cai et al., 2003; Laugwitz et al., 2005; Bu et al., 2009). Lineage tracing experiments implied that Isl-1^{+} CSCs do not contribute to the left ventricle, suggesting the contribution of other CSC populations during heart development and possible compartmentalization and clonality based on CSC subpopulations (Cai et al., 2003). Both in vitro and in vivo characterization studies revealed that Isl-1^{+} CSCs are able to differentiate into the three cardiovascular lineages: cardiomyocytes, SMCs, and ECs. Isl-1^{+} CSCs were also found positive for some VEGFRs, Flt-1, and KDR (Lui et al., 2013).

In vivo activation of β-catenin has been shown to promote the expansion of Isl-1^{+} CSCs through the regulation of fibroblast growth factor (FGF) signaling, suggesting that the Wnt/beta-catenin signaling pathway regulates renewal of Isl-1^{+} progenitors (Cohen et al., 2007; Qyang et al., 2007). Functional cardiomyocytes can be differentiated from Isl-1^{+} CSC population through bone morphogenic protein 4 (BMP4) signal activation (Cagavi et al., 2014). Another study showed that Notch1 signaling also regulates self-renewal, maintenance of cardiac progenitors, and the requirement for Notch1 activity in cardiac differentiation (Kwon et al., 2009). Moreover, a first clinical case study on severe heart failure conducted with cardiac progenitors recently reported that ESC-derived Isl-1^{+} CSCs in clinical trials for MI repair in heart failure patients had positive outcomes in the short term (Menasché et al., 2015).

2.6. Nkx2.5^{+} cells
The *NKX2.5* gene encodes a homeobox-containing transcription factor that functions in heart development

in vertebrates and invertebrates (Turbay et al., 1996; Durocher et al., 1997; Tanaka et al., 1999; Stennard et al., 2003; Akazawa and Komuro, 2005). Nkx2.5 is a FHF marker; however, its contribution to both FHF and SHF ventricular identity and cardiac function into adulthood has been demonstrated by several reports (Akazawa and Komuro, 2005; Zhang et al., 2014; George et al., 2015). Nkx2.5[+] CSCs were also shown to contribute to endothelial differentiation of the embryogenic heart (Paffett-Lugassy et al., 2013). C-kit[+] and Sca-1^{+} progenitor cells were found to express Nkx2.5, implying their commitment to the cardiac lineage (Beltrami et al., 2003; Matsuura et al., 2004).

The cardiac transcription factors *NKX2.5* and *GATA4* are known to cooperate and play important roles in vertebrate heart development (Durocher et al., 1997). *NKX2.5* has been shown to interact with *GATA4* and cardiac T-box factor (*TBX-5*). Moreover, *TBX-20* also directly interacts with *NKX2.5*, *GATA4*, and *GATA5* during heart development (Stennard et al., 2003).

Global knockout of *Nkx2.5* in mice results in severe growth retardation, perturbed cardiac morphogenesis, and lethality at approximately embryonic day 9.5 (Tanaka et al., 1999). Mutations in the *Nkx2.5* gene are known to cause congenital heart diseases such as familial atrial septal defect and sudden cardiac death (Ellesøe et al., 2015). Overexpression of *Nkx2.5* can induce proper function of the adult heart (George et al., 2015). All these findings underline the necessary role of Nkx2.5[+] CSCs in both embryonic heart development and adult human heart maintenance.

2.7. Mesp1^{+} and Mesp2^{+} cells

Mesoderm posterior 1 (Mesp1) is one of the earliest known markers for common progenitors of hematopoietic and cardiac lineages. In 1996, Saga et al. showed Mesp1 gene expression at embryonic day 6.5 in the early cardiac mesodermal population that contributes to FHF and SHF (Saga et al., 1996). Later studies showed *Mesp* genes as essential transcription factors for precardiac mesoderm formation in mice and humans (Lindsley et al., 2008). Double knockout studies for *Mesp1* and *Mesp2* resulted in loss of mesodermal structures (Kitajima et al., 2000). In fate mapping analysis, the *Mesp1* gene was found to contribute almost all cardiac cell layers, including the epicardium, endocardium, and myocardium (Kitajima et al., 2006). In a single-cell analysis, the nascent mesodermal gene *Mesp1* was found to gradually terminate as the expression of the cardiac genes *TBX-5* and *Nkx2.5* increased, suggesting a role for *Mesp1* at earlier stages than the CSC population during development (Kokkinopoulos et al., 2015).

2.8. Wt1^{+} and Tbx18^{+} cells

An epicardial progenitor population in the heart was first described by the expression of transcription factors *Tbx18* and Wilms tumor 1 homolog (*Wt1*) (Cai et al., 2008; Zhou et al., 2008). These *Tbx18*- and *Wt1*-expressing progenitors are located in the epicardium and can differentiate into fully functional cardiomyocytes (Zeng et al., 2011). Cai et al. (2008) also reported that Tbx18^{+} migrated onto the outer surface of the heart, forming the epicardium and giving rise to myocytes in the ventricular septum and atrial and ventricular walls. Tbx18-expressing cardiac progenitors are also shown to contribute to differentiation into cardiac fibroblasts and coronary smooth muscle cells. Zhou et al. (2008) stated that Wt1^{+} proepicardial cells arose from progenitors that expressed both Nkx2.5 and Isl-1 strongly, suggesting that these progenitors have the capability to differentiate (Zeng et al., 2011). It is noteworthy that Tbx18 and Wt1 are shown to bidirectionally control the epicardial to mesenchymal transition in the heart (Takeichi et al., 2013).

2.9. SIRPA[+]/VCAM1^{+} cells

Signal-regulatory protein-alpha (SIRPA, SHPS-1, or CD172a) and vascular cell adhesion protein-1 (VCAM-1, CD106) are cell surface proteins and their coexpression was shown to be enriched in human cardiac lineages (Elliot et al., 2011). By using a transgenic human ESC cell line in which GFP expression was reported with *NKX-2.5* gene expression (NKX2.5-eGFP) and bioinformatic analysis, Elliot et al. identified the coexistence of SIRPA and VCAM1 on purified NKX2.5-eGFP CSCs and their progeny (Elliot et al., 2011). Further characterization of SIRPA-VCAM1 cell populations showed cardiac troponin T (cTnT) expressions in differentiated cardiomyocytes that were derived from NKX2.5-eGFP cells isolated from human ESCs (Den Hartogh et al., 2015). SIRPA was coexpressed with cTnT, indicating that it was specifically expressed on cardiomyocyte lineages in differentiated populations. These studies suggested SIRPA and VCAM1 as convenient and candidate cell-surface markers for purification of live CPCs from tissue or cultured cells. One important consideration is to pay attention to coexpression of SIRPA-VCAM1 and the embryonic timing, as these markers could be expressed in differentiated cardiac cells as well as CSCs.

2.10. HCN4^{+} cells

Until 2011, the isolation and purification of CSCs was primarily based on two cell surface antigens: C-kit[+] (Beltrami et al., 2003) and Sca-1^{+} (Matsuura et al., 2004). Recently, a distinct CSC population was identified by expression of hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4), a voltage-gated ion channel. HCN4 is expressed specifically in the heart, known to be a cardiac conduction system marker, and it was recently shown to be expressed in the early cardiac mesoderm and determined to be a FHF marker in mice and humans (Später et al., 2013). This channel is mostly used as a marker for the sinoatrial node and is

correlated with the funny current, which is responsible for pacemaking and heart rate control. HCN4 mutations in humans result in severe bradycardia, QT prolongation, and torsade de pointes (Grant, 2009). HCN4 is strongly expressed in human ESCs and cardiac progenitors at the embryonic stage; however, HCN4 is prominently expressed in the pacemaker region in the adult heart. This suggests that HCN4 could be a good surface marker for isolation of a subpopulation of embryonic CSCs; however, HCN4 would not be good option to separate CSCs in the adult heart.

2.11. Cardiac side population cells
Side population cells, which can be identified by their ability to exclude Hoechst 33342 dye, can generate cardiomyocytes in vitro and in vivo and part of this population coexpresses C-kit and/or Sca-1 (Oyama et al., 2007). Side population cells were first described in the bone marrow, but they are also found in other organs, including the heart. Thus, side population cells are not specific to the heart (Segers and Lee, 2008), but cardiac side population cells have the potential to migrate and home into the injured heart, and these cells can differentiate into cardiomyocytes, ECs, or SMCs in vivo (Oyama et al., 2007; Nagai et al., 2013). It remains unclear whether cardiac side population cells and Sca-1$^+$ cells emerge from the same population or have distinct characters.

2.12. Cardiospheres
Cardiospheres were first isolated from postnatal atrial or ventricular human biopsies and from mouse hearts and are described as self-adherent clusters of undifferentiated cells expressing endothelial progenitor markers (Messina et al., 2004). In later studies, cardiospheres were shown to contain both primitive cells and progenitors for three cardiac lineages; that is, they can differentiate into cardiomyocytes, ECs, and SMCs (Barile et al., 2013).

Cardiospheres were found to express C-kit, Sca-1, and KDR proteins and were reported to self-renew and differentiate into cardiomyocytes through coculture with postnatal rat cardiomyocytes (Messina et al., 2004). Smith et al. (2007) showed that human cardiospheres expressed C-Kit and CD105, which was the regulatory component of the transforming growth factor-β receptor complex important in angiogenesis and hematopoiesis. Cardiospheres were proliferative, identified by Ki-67 expression (Smith et al., 2007). Cardiospheres contain primitive and differentiating cells that express cardiomyocyte proteins (Nkx2.5, GATA4 cTnI, α-sarcomeric actinin) and connexin-43 (Barile et al., 2013).

Although obtaining human heart tissues may be difficult and contains risks, the main advantage of cardiospheres is that CSCs can be isolated from very small tissue samples of human myocardium and can be expanded in vitro without losing their differentiation capacity. Moreover, autologous transplantation using cardiosphere cultures from the same patient is an alternative cell source for cell therapy.

2.13. Cardiac atrial appendage cells
Cardiac atrial appendage stem cells (CASCs) were isolated from the human left atrial appendage. CASCs were first identified based on the high expression of aldehyde dehydrogenase (ALDH) as well as the presence of CD34 and absence of CD45 surface marker expression (Koninckx et al., 2013). In vitro analysis demonstrated that CASCs possess cardiomyogenic differentiation capacity (Koninckx et al., 2013). Importantly, following transplantation of CASCs to the MI zone, CASCs were shown to differentiate into cardiomyocytes in a minipig model. When compared with the sham control, 98% of engrafted CASCs had differentiated into cardiomyocytes (Fanton et al., 2015). Cells obtained from samples freshly isolated from human atrial appendages were discovered to contain a small population of C-kit$^+$/Lin$^-$ cells, and also were positive for expression of KDR and CD31 proteins. Cells were able to differentiate into different cardiac cell lineages under directed cardiac differentiation protocols (He et al., 2011). Although CASCs have CSC-like properties and allow autologous cell transplantation benefits, the requirement of an invasive approach to acquire CASCs remains an important consideration and limitation.

2.14. Cardiac stem cell applications
2.14.1. Clinical trials
Embryonic or adult stem cells are important cell sources for basic research and clinical research. Over the last decade, a large number of clinical trials have been started in order to evaluate the therapeutic role of MSC, bone marrow-derived stem cells, and CSCs for cardiac repair in humans. Over 650 studies for CSCs and 3661 studies for MI were conducted up to 2016 based on the data supplied by the official website of the National Institute of Health (https://clinicaltrials.gov/ct2/results/details?term=cardiac+stem+cell). Here we discussed several representative trials that are either ongoing or completed.

Several CSC populations provide an option to carry out autologous cell therapy. From those, cardiosphere-derived cells were examined and shown to provide regenerative effects in the CArdiosphere-Derived aUtologous stem "CEIls to reverse ventricUlar dySfunction" (CADUCEUS) clinical trial. The CADUCEUS trial was conducted on acute MI patients to investigate the effectiveness of cardiosphere-derived stem cells that were obtained from right ventricular endomyocardial biopsies and transplanted to the same patient. After 36 days of expansion in culture, 12.5–25 million autologous cardiosphere-derived cells were administered via intracoronary infusion between 6 and 12 weeks post-MI of 25 patients (Malliaras et al., 2014). At the end of the this trial, there were no functional

improvements observed in the left ventricular ejection fraction (LVEF) of patients; however, the scar tissue was reduced significantly compared to control patients and regional wall thickness was improved (Malliaras et al., 2014).

As another autologous intervention, in the "Stem Cell Infusion in Patients with Ischemic cardiOmyopathy" (SCIPIO) trial, right atrial appendages of 23 patients were obtained during coronary artery bypass grafting surgery (Yacoub and Terrovitis, 2013). After 4 weeks of the surgery, 1×10^6 C-kit+/Lin- CSCs were delivered through the coronary artery. Four months after cell transplantation, the majority of patients showed reduced infarct size and an improvement in LVEF when compared to the control group (Yacoub and Terrovitis, 2013). From 2007 to 2014, in which years the trial was conducted, no long-term amelioration of cardiac function was reported.

The "AutoLogous human CArdiac-Derived stem cell to Treat Ischemic cARdiomyopathy" (ALCADIA) project is another completed trial that was conducted between 2009 and 2015. Different from previous trials, autologous CSCs were delivered in combination with bioengineered gelatin-hydrogel scaffolds that release basic FGF. Briefly, CSCs were collected from ischemic cardiomyopathy patients and cultured for 4 weeks before intramyocardial injection. Six months after transplantation, cardiac imaging indicated a significant increase in ejection fraction and regional wall motion as well as a reduction in infarct size (Yacoub and Terrovitis, 2013).

A large number of clinical trials have evaluated the safety and efficacy of stem cells that have hematopoietic or bone marrow origin for MI or heart failure patients. Among those, "Transplantation Of Progenitor Cells And Regeneration Enhancement in Acute Myocardial Infarction" (TOPCARE-AMI) investigated the effects of intracoronary infusion of circulating stem cells and BM-MSCs in patients who had acute MI. After cell transplantation, 53% of the patients exhibited an improvement of LVEF and a reduction in infarct size (Leistner et al., 2011). In another study, the C-CURE "Cardiopoietic stem Cell therapy in heart failURE" trial used BM-MSCs that were exposed to a cardiogenic cocktail containing BMP-4, bFGF, and cardiotrophin (Bartunek et al., 2013). The so called "cardiopoietic" cells were delivered by endomyocardial injections to patients with ischemia-originated heart failure. The evaluation of patients who received cardiopoietic stem cells showed an increase in LVEF (Bartunek et al., 2013).

From 2001 to 2009, the "Tight Glycemic Control Increases Cardiac Stem Cells During Acute Myocardial Infarction" phase IV trial was conducted to monitor the regenerative myocardial effects of tight glycemic control of patients with acute MI. Three days before coronary bypass surgery, selected patients with various glycemic levels were administered insulin. During surgery, myocyte precursor cells (C-kit+/MEF2C+/GATA4+ cells) were isolated from biopsy specimens taken from the periinfarcted area and analyzed for oxidative stress and apoptosis parameters. Biopsy specimens from patients in the tight glycemic control group compared to the conventional glycemic group demonstrated an increase in regenerative capacity of the ischemic myocardium that was associated with reduced senescence of myocyte precursor cells (Marfella et al., 2012).

There are several ongoing clinical trials about CSCs in order to evaluate their therapeutic biological effects on heart disease patients. One example is a phase II clinical trial testing C-kit+ CSCs alone or in combination with MSCs named "Combination of Mesenchymal and C-kit+ Cardiac Stem Cells as Regenerative Therapy for Heart Failure" (CONCERT-HF), which was started in 2015. Ischemic cardiomyopathy patients are being recruited to this trial and 15 transendocardial injections of C-kit+ CSCs alone or together with MSCs are planned to be administered to the left ventricle of accepted participants.

Another stem cell source for cardiac repair is human cord blood-derived stromal MSCs. Due to their low immunogenicity and availability as an allogeneic transplantation option, the Human Umbilical Cord Stroma MSC in Myocardial Infarction (HUC-HEART) trial was initiated and has started to enroll participants to evaluate the efficacy of this cell type for cardiac repair and remodeling in humans (Can et al., 2015).

2.14.2. Cardiac tissue and organ engineering

Tissue engineering aims to regenerate damaged cardiac tissues by combining CSCs or mature cardiac cells together with biomaterials and/or nanoparticles loaded with growth factors or small molecules. Cardiac issue engineering studies have been widely conducted with stem cells (autologous or not) and combine them with natural or synthetic polymers, which forms a scaffold to facilitate cell adherence and engraftment to the cardiac tissue. Biological polymers such as collagen, gelatin, Matrigel, and alginate are mostly utilized for seeding cardiomyocytes and they possess several advantages including promoting cell adhesion, survival, and differentiation (Alcon et al., 2012; Huyer et al., 2015). Synthetic biomaterials such as polyglycolic acid (PGA), polylactic acid (PLA), and poly ε-caprolactone (PCL) scaffolds are mostly preferred as they provide appropriate dispersion, spontaneous contractility, and suitable habitats for cardiac cells to adhere to, and they have low toxicity (Alcon et al., 2012; Huyer et al., 2015). On the other hand, synthetic polymers have some disadvantages such as poor cell survival, difficulty in manipulation, toxicity, and invoking immune reactions. As an alternative, engineered intact cardiac cell-sheets have

been developed in order to achieve less immunogenicity and better biocompatibility (Haraguchi et al., 2014).

CSCs are invaluable cell sources for engineering whole hearts. This on its own is a special topic that is reviewed elsewhere (reviewed by Murphy and Atala, 2012). An interesting report suggested engineering of a bioartificial heart by decellularizing rat heart by certain chemicals and detergents. Reseeding of the decellularized heart matrix with CSCs and ECs resulted in the recellularization of the heart that was contracting and drug-responsive (Ott et al., 2008). These and other reports are preliminary studies to build an intact heart for its possible use in the clinic for patients who are desperately seeking heart transplantation.

3. Discussion and conclusion

The studies on CSCs aim to understand the mechanisms behind their self-renewal and differentiation patterns. This way, we can direct these cells in their nascent environment or use them for cell-based therapies. The heart being a complex organ, there is no single CSC population; rather, various CSC subpopulations have been identified that contribute to heart development or repair at different stages of an organism (Figure; Table). Interestingly, not all CSCs have the same capacity to generate the same regions in the heart; instead, different CSC subpopulations give rise to different and/or overlapping regions in the heart, as summarized in the Table. Moreover, the in vitro and in vivo potential of different CSCs may vary.

Until recently, isolation of CSCs was based on transgenic models or the expression of CSC-associated surface antigens, such as C-kit and Sca-1. Though these proteins are characteristic markers for hematopoietic lineage, recently numerous studies revealed novel markers for CSCs such as HCN4, SIRPA, VCAM1, and Flt family proteins, giving researchers a wide range of selection. An important consideration is that researchers need to choose the best CSC subpopulations for the desired study, as differences in expression in embryonic and adult hearts (as in HCN4^{+} cells) as well as differences in species-specific expression ranges (Isl-1^{+} CSCs) of these factors have been reported.

Studies on CSCs and other pluripotent or multipotent stem cells have been conducted to compare their regenerative potential in the heart. In one such study, Oskouei et al. determined higher potency of CSCs compared with BM-MSCs in cardiac repair in the ischemic heart failure model in mice (Oskouei et al., 2012). Furthermore, transplantation of human CSCs together with BM-MSCs was found to reduce infarct size and recover cardiac function after MI (Williams et al., 2013). Interestingly, adult-derived C-kit[+] CSCs were less effective than fetal CSCs, but more potent than high-dose MSCs as determined by engraftment and cardiac differentiation

potential, suggestive of functional differences between adult and embryonic CSCs (Oskouei et al., 2012). Collectively, these and other reports underline a stronger developmental potential for embryonic CSCs compared to adult CSCs and the possibility of better restoration capacity of CSCs when administered in combination with MSCs for heart tissue repair in vivo.

In recent years, researchers have identified a novel population of stromal cells, called cardiac telocytes, which contribute to renewal, regeneration, and repair of tissues (Albulescu et al., 2015). In the failing human heart, a decreased number of telocytes was detected due to apoptosis and extracellular matrix deformities in the damaged area (Richter and Kostin, 2015). Interestingly, C-kit[+] cardiac telocytes were also shown to coexpress CD34/PDGFR-α, suggesting the importance of telocytes in vascularization of heart tissue (Li et al., 2015; Zhou et al., 2015). Moreover, the hypoxic environment created in the epicardial and subepicardial regions may serve as a way to regulate CSCs via Hif-1α signaling (Kocabas et al., 2012). To this end, after knockdown of Hif-1α, there was an increase in cardiomyocyte and endothelial lineage differentiation suggestive of the utilization of glycolytic metabolism in some CSC populations (Kocabas et al., 2012).

Recently, the B lymphoma Mo-MLV insertion region 1 (Bmi1^{+}) population was suggested to be a novel cardiac progenitor with substantial self-maintenance potential. The Bmi1^{+} CSC population was seen to contribute to the generation of endothelial and smooth muscle cell lineages in vivo (Valiente-Alandi et al., 2015). This cell population needs to be further investigated to understand its potential as CSCs.

Isolation of CSCs from neonatal or adult hearts is an important approach to gain access to these cells; however, CSCs are present in minute amounts in the heart. A solution to this problem comes from the generation of ESC-derived or induced pluripotent stem cell (iPSC)-derived CSCs that can be obtained in unlimited amounts. Efficient differentiation protocols for ESCs are being established to direct lineage commitment of progenitors and be able to study different stages of cardiac development and disease. Unless using a transgenic animal model, the requirement for efficient cell surface markers for CSCs is a prerequisite for isolation and purification of any CSC population, whether from intact heart tissue or from differentiation cultures of ESCs or iPSCs. Thus, discovery of surface markers for CSCs (SIRPA, VCAM1, Flt-1, Flt-4, HCN4) paved the way to purify and characterize CSC subpopulations. Importantly, these factors can be expressed in other adult stem cell types or mature cardiac cells. This should alert researchers to be careful about the

stage of cells and expression of surface markers in the right combination when isolating CSCs from heart or differentiation cultures.

Another current research area is the direct differentiation of CSCs from terminally differentiated human tissue. In 2010, fibroblasts were directly converted to cardiomyocyte-like cells using the viral delivery of a combination of three master transcription factors: *GATA4*, *MEF2C*, and *TBX5* (Ieda et al., 2010). One recent study used a protein-based approach in which a combination of QQ-reagent-modified GATA4, Hand2, Mef2c, and Tbx5 together with BMP4, activin A, and bFGF promoted reprogramming of human dermal fibroblasts into cardiac progenitors. When these reprogrammed cardiac cells were

transplanted into rat hearts, functional recovery in the damaged area was observed (Li et al., 2015).

In future studies, the true potential and implications of different CSC populations will continue to be investigated in basic and translational research. Combinatorial therapies of CSCs with other progenitor populations or gene delivery options may be developed and tested for therapy in clinical applications, which may give hope to patients suffering from cardiovascular disorders.

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