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REVIEW

Developmental origin and lineage plasticity of endogenous cardiac stem cells

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ABSTRACT

Over the past two decades, several populations of cardiac stem cells have been described in the adult mammalian heart. For the most part, however, their lineage origins and *in vivo* functions remain largely unexplored. This Review summarizes what is known about different populations of embryonic and adult cardiac stem cells, including KIT$^+$, PDGFRα^+, ISL1$^+$ and SCA1$^+$ cells, side population cells, cardiospheres and epicardial cells. We discuss their developmental origins and defining characteristics, and consider their possible contribution to heart organogenesis and regeneration. We also summarize the origin and plasticity of cardiac fibroblasts and circulating endothelial progenitor cells, and consider what role these cells have in contributing to cardiac repair.

KEY WORDS: Adult mammalian heart, Cardiac stem cells, Cardiovascular cells, Embryonic precursor cells, Heart organogenesis, Regeneration

Introduction

In mammals and birds, the heart is a four-chambered muscular pump dedicated to delivering deoxygenated blood to the lungs and oxygenated blood to the rest of the body (Fig. 1). Although fetal and neonatal hearts show a strong capacity for regeneration (Drenckhahn et al., 2008; Porrello et al., 2011), pathologies of the adult heart, including myocardial infarction and dilated cardiomyopathy, which are accompanied by severe loss of cardiomyocytes and functional output, are repaired through a process related to wound healing, leading to fibrosis. Here, tissue damage resulting from ischemia and inflammation overwhelms any endogenous regenerative response, with the outcome being scar formation, loss of tissue integrity and reduced cardiac contractile function. However, the discovery of endogenous cardiac stem and progenitor cells has begun to erode the long-held view that the mammalian heart cannot regenerate. This idea has been further strengthened by recent findings that some cardiomyocytes may proliferate during physiological aging (Bergmann et al., 2015; Kimura et al., 2015) and in response to certain pathologies (Anversa et al., 2006; Beltrami et al., 2001) or biochemical pathway stimulation (D'Uva et al., 2015; Lin et al., 2014; Naqvi et al., 2014). These findings have promoted extensive investigations and clinical trials of novel therapies aimed at replacing lost, damaged and fibrotic cardiac tissue. In this Review, we summarize what is known regarding the origin and lineage plasticity of the numerous different cardiac stem and progenitor cell populations that have been described to date, as well as cardiac fibroblasts and circulating endothelial progenitor cells. We briefly describe cardiac development and its constituent lineages to provide a context for this Review, and consider the potential of each population for cell therapies or as targets in novel therapeutic interventions for heart disease.

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The origin of cardiac lineages in development

The heart comprises three principal layers: the endocardium, which forms the innermost layer, the myocardium and the epicardium, which forms the outermost layer. Fate-mapping studies in chicken and mouse demonstrate that cardiac tissues derive mostly from the mesodermal layer produced at gastrulation, with additional contributions from anterior neural crest (Vincent and Buckingham, 2010). Gastrulation is the principal morphogenetic event that leads to the formation and distribution of the three canonical germ layers: endoderm, ectoderm and mesoderm. During gastrulation, the primitive epiblast layer undergoes epithelial-to-mesenchymal transition (EMT) at a midline furrow termed the primitive streak. Sheets of endoderm and subjacent mesoderm emerge from the primitive streak and migrate bilaterally. Pre-cardiac mesodermal territories then migrate towards the future anterior part of the embryo to form a distinct crescent-shaped epithelium called the cardiac crescent (Fig. 2A,B) (Buckingham et al., 2005).

Cells that contribute to the primary heart tube (Fig. 2D) arise from a unique set of progenitors termed the first heart field (FHF), which occupy a distinct anterior-lateral territory within the cardiac crescent (Fig. 2C) (Buckingham et al., 2005; Lescroart et al., 2014), distinguished by expression of the gene for hyperpolarization-activated cyclic nucleotide-gated channel 4 (*Hcn4*) (Liang et al., 2013). The primary heart tube acts as a type of scaffold for heart tube elongation through the addition of new myocardial, endocardial and smooth muscle cell progenitors to its inflow and outflow poles (Fig. 2D,E, arrows). These new progenitors have their origins in a distinct heart progenitor field termed the second heart field (SHF), which probably exits the primitive streak somewhat later than FHF progenitors (Lescroart et al., 2014). SHF cells are therefore delayed in their differentiation. They come to occupy a position medial to FHF progenitors in the crescent, and during heart tube formation constitute a reservoir of undifferentiated cells within the pharyngeal mesoderm (Fig. 2A-E). SHF cells are marked by the persistent expression of the LIM-homeodomain transcription factor islet 1 (ISL1) (Cai et al., 2003). Lineage analysis has shown that FHF cells contribute almost exclusively to the left ventricle, as well as to the atrioventricular canal and parts of the atria. In addition, it has been shown that cells forming the nodal elements of the proximal cardiac conduction system originate from the FHF and continue to express HCN4 (Liang et al., 2013). By contrast, SHF cells contribute almost exclusively to the right ventricle and outflow tract, with contributions also to the atria and inflow vessels (Buckingham et al., 2005; Meilhac et al.,

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

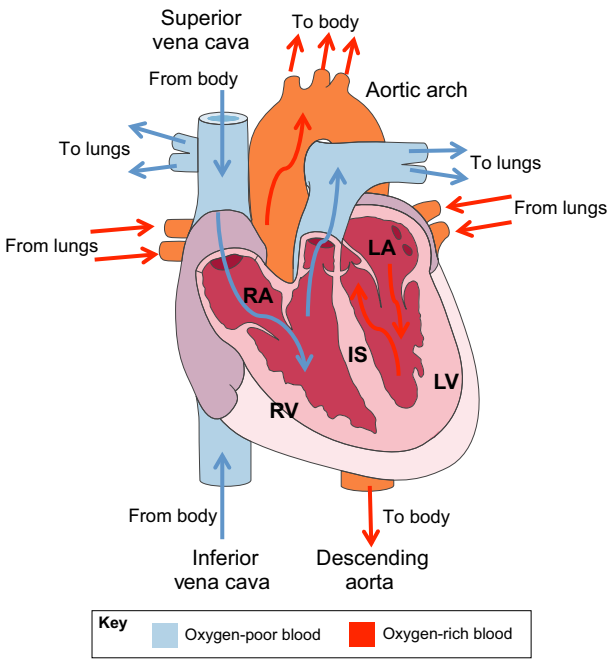

Fig. 1. The adult mammalian heart. The adult mammalian heart is made up of four chambers: the right and left ventricles (RV and LV) and right and left atria (RA and LA). The ventricles are separated by the interventricular septum (IS). The vena cava and the aorta carry the flow of blood to and from the heart, respectively. Blood low in oxygen (blue arrows) from the different tissues is collected into the right atrium via the superior and inferior vena cava and flows to the lungs through the right ventricle. Oxygenated blood (red arrows) from the lungs flows into the left atrium and is pumped into the aorta by the left ventricle. This system allows oxygenated and non-oxygenated blood to be completely separate. Figure created using Servier medical art.

2004) (Fig. 2E-G). Later phases of heart growth are driven by the proliferation of the myocardium and endocardium, and hypertrophic growth of cardiomyocytes.

The molecular principles that pattern the heart into chambers and other structures are poorly understood. It seems likely that pattern and regional function are partly enshrined in the identity of FHF and SHF cells as they exit the primitive streak. Because SHF cells share clonal relationships with head muscles (Lescroart et al., 2010), the craniopharyngeal mesoderm is proposed to have played a major role in evolution of the vertebrate heart, head and neck (Diogo et al., 2015). It is generally thought that cardiac precursor cells represent multipotent lineage progenitors for cardiomyocytes, the endocardium and smooth muscle cells, a view that has been much influenced by studies of differentiating embryonic stem cells (ESCs) (summarized in Box 1). However, recent lineage-tracing studies have shown that although FHF and SHF cells arise from a common progenitor, probably in the pre-gastrulation embryo, their territories become geographically separate (Ali et al., 2014; Buckingham et al., 2005; Meilhac et al., 2004). FHF progenitors that form the primary heart tube and future left ventricle (Buckingham et al., 2005; Lescroart et al., 2014) are unipotent for cardiomyocytes or endothelial cell fates (Cohen-Gould and Mikawa, 1996; Lescroart et al., 2014). By contrast, SHF cells exiting the primitive streak later and contributing to other regions of the heart are either unipotent or bipotent for cardiomyocyte and endothelial cells, or cardiomyocytes and smooth muscle cells. Signaling gradients across the pre-gastrula and transcription factors expressed in broad mesodermal domains during gastrulation, such as eomesodermin and MESP1/2, have profound influences in establishing cardiogenesis (Bondue et al., 2008; Costello et al., 2011). Adding further complexity to heart patterning, the endocardium and other heart lineages have both cardiac field and extra-cardiac origins (Cohen-Gould and Mikawa, 1996; Harris and Black, 2010; Nakano et al., 2013). How these early events ultimately shape the embryonic heart and whether they also determine adult cardiac stem cell identity remain unclear.

By embryonic day (E) 8.5 in the mouse, the heart tube has undergone rightward looping and begun to beat, and distinct cardiac chambers are clearly visible (Fig. 2E,F). From E9.5, a mesothelial cell population envelopes the surface of the myocardium to create the epicardial layer. These cells emanate from a transitory progenitor structure appended to the primary heart tube called the proepicardium. Both the proepicardium and epicardium are marked by expression of the transcription factor Wilms tumor 1 (WT1). The proepicardium has its origins within the cardiac progenitor fields expressing transcription factors ISL1 and NKX2-5, although these factors are not expressed in the proepicardium itself (Zhou et al., 2008b). During migration and spreading of the epicardium, a subset of cells undergo EMT in response to myocardial signals and penetrate the matrix-rich subepicardium and myocardial interstitium. These cells, termed epicardium-derived cells (EPDCs), differentiate into interstitial and valvular fibroblasts, and coronary vascular smooth muscle cells and endothelial cells (Gittenberger-de Groot et al., 1998; Katz et al., 2012; Perez-Pomares et al., 2002; Viragh and Challice, 1981). A fraction of cardiomyocytes has also been suggested to derive from the epicardium (Cai et al., 2008; Zhou et al., 2008a), although this remains contentious (Christoffels et al., 2009; Kikuchi et al., 2011; Rudat and Kispert, 2012). The epicardium is heterogeneous in both its cellular composition and its origin, with a population of bone marrow-derived $CD45^+$ (CD45 is also known as PTPRC) cells taking up residence in the embryonic epicardium as early as E12.5 (Balmer et al., 2014; Tallini et al., 2009). These are distinct from the $WT1^+$ proepicardium-derived cells. Postnatally, $CD45^+$ cells form clusters within a matrix-rich niche in the proximity of the coronary vessels (Balmer et al., 2014). Lineage tracing has shown that $CD45^+$ epicardial cells can differentiate into pericytes, although their broader functions and lineage descendants are unknown. Hemopoietic cells also contribute to cardiac valvular interstitial cells (Hajdu et al., 2011).

Vessels provide a niche for many adult stem cell populations. The coronary vascular tree emerges as endothelial cell and perivascular cell precursors located within the sub-epicardium and myocardial interstitium condense at around E11.5-E12.5 (Fig. 3). Although the perivascular compartment of coronary vessels appears to derive from the epicardium (including resident $CD45^+$ cells), recent lineage-tracing studies show that coronary endothelial cells have heterogeneous origins. The details are still being debated, but it is clear that distinct populations of endothelial cells arise from the sinus venosus and the endocardium, with a minor population deriving directly from the epicardium (Chen et al., 2014; Del Monte and Harvey, 2012; Katz et al., 2012; Tian et al., 2014; Wu et al., 2012). These populations deploy angioblasts with distinct kinetics and spatial signatures (Chen et al., 2014), with the endocardium also contributing to the coronary vascular tree postnatally during a process called trabecular compaction (Tian et al., 2014). Cardiac lymphatics also have a dual origin from the endothelial cells of the cardinal veins, as well as yolk sac endothelial or hemogenic cells (Klotz et al., 2015).

Neural crest cells also contribute to the embryonic heart after their delamination from the neural plate. Cardiac neural crest

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

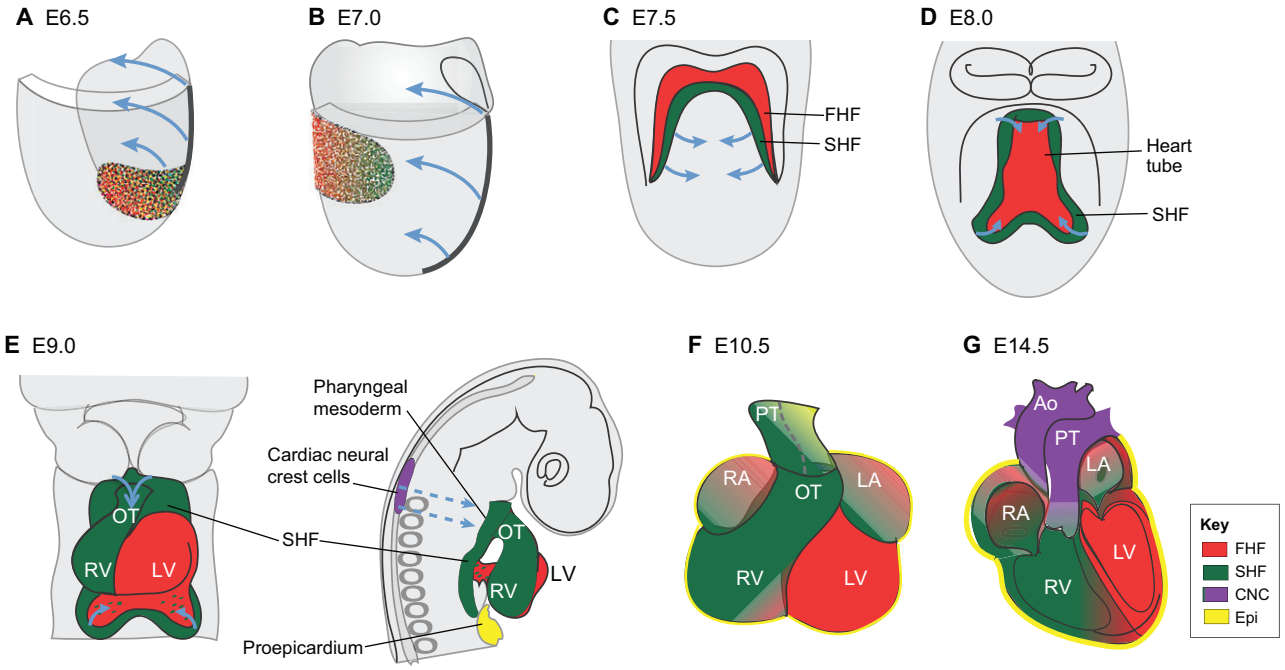

Fig. 2. Lineage contributions to the forming heart. (A-D) During gastrulation at E6.5-E7.0 (A,B), mesodermal and endodermal cells corresponding to distinct organ territories, including heart progenitors from the first heart field (FHF, red) and second heart field (SHF, green), undergo lateral migration (arrows) away from the primitive streak (thick black line). At the cardiac crescent stage at E7.5 (C) and during the formation of the early heart tube at E8.0 (D), FHF and SHF descendants are shown in red and green, respectively. Solid arrows indicate ongoing rearrangement of FHF and SHF cells from the cardiac crescent as FHF cells form the primary heart tube, with SHF cells being added to the poles of the primary heart tube (D). (E) Frontal and lateral views of the heart region at E9.0 showing looped heart tube. Dashed arrows indicate migration of cardiac neural crest cells to the heart. (F,G) The chambered heart at E10.5 (F) and E14.5 (G), with lineage contributions from the FHF, SHF, cardiac neural crest (CNC) and epicardium (Epi). Ao, aorta; RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; OT, outflow tract; PT, pulmonary trunk.

migrates to the cardiogenic region and contributes to smooth muscle cells of the aorta and branchial arch arteries, valves and conduction tissue, and to the parasympathetic innervation of the heart (Creazzo et al., 1998; Engleka et al., 2012; Nakamura et al., 2006) (Fig. 2B,C). Transient paracrine signaling roles for cardiac neural crest in the SHF, outflow tract and valve development have been reported (Creazzo et al., 1998; Engleka et al., 2012; Waldo et al., 1999). Neural crest cells persist in the adult heart within valves and proximal conduction tissue, with some cells expressing melanocytic, neurogenic and gliogenic markers (Engleka et al., 2012). Rare neural crest-derived multipotent progenitor cells might also exist in the developing and adult heart (Engleka et al., 2012; Hatzistergos et al., 2015) (see below). The overall picture of cardiac lineage development is one of complexity and heterogeneity. Linking adult cardiac stem cells to their cell of origin in the embryonic heart is an exacting but important task if a working understanding of cardiac stem cells and their clinical potential is to be achieved.

Box 1. Insights into cardiac development from embryonic stem cells

Several groups have isolated primitive cardiac precursor cells from embryonic stem cells (ESCs) that give rise to cardiomyocytes, smooth muscle cells and endothelial cells. ESCs generate aggregates called embryoid bodies in which lineage derivatives of all three germ layers form, mimicking embryonic development. Several studies showed that murine myocardium and endocardium originate from a common population expressing vascular endothelial growth factor receptor 2 (VEGFR2; also known as FLK1 and KDR) (Ema et al., 2006). ESCs expressing GFP under the control of a brachyury early mesodermal promoter/enhancer (referred to as *Bry*) revealed that VEGFR2^{+} cells arising from the *Bry*-GFP^{+} mesodermal population contained blast colony-forming cells, which generated vascular endothelial cells and blood islands (Kattman et al., 2006). The VEGFR2^{+}/*Bry*-GFP^{+} population also gave rise to cardiovascular colony-forming cells that yielded cardiomyocytes, endothelial cells and smooth muscle cells *in vitro*. Treatment of human ESC-derived embryoid bodies with BMP4, FGF2, VEGFA and dickkopf homolog 1 (DKK1) generated a VEGFR2^{low}/KIT^{-} progenitor population with cardiomyocyte, endothelial cell and smooth muscle cell potential *in vitro* and after transplantation (Yang et al., 2008). Taken together, these data suggest a common cardiovascular lineage progenitor, although how these ESC findings relate to *in vivo* development requires further study.

Endogenous adult cardiac stem cells

Numerous cells with the properties of stem and progenitor cells have been detected in the adult heart using different experimental approaches (Fig. 4; Table 1) (Chong et al., 2014a). These cells are rare *in vivo*, comprising just 0.005-2% of all adult cardiac cells, which presents a technical challenge for studying their roles *in vivo*. In the following sections, we describe what it is known about the different putative adult cardiac stem/progenitor-like populations that reside around vessels and among cardiac muscle fibers in the adult heart. A recurring theme among these studies is that although *in vitro* evidence might suggest a degree of lineage plasticity, *in vivo* functions are less clear. Furthermore, in many cell transplantation studies, the observed positive effects on cardiac repair might be due to paracrine pro-survival and angiogenic functions of injected or infused cells, and not from stem cell expansion and differentiation (Gnecchi et al., 2008; Keith and Bolli, 2015). Such studies hint at the largely uncharted non-lineage functions of cardiac stem, progenitor and stromal cells (also known generically as cardiac fibroblasts) *in vivo* (Liang et al., 2014).

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

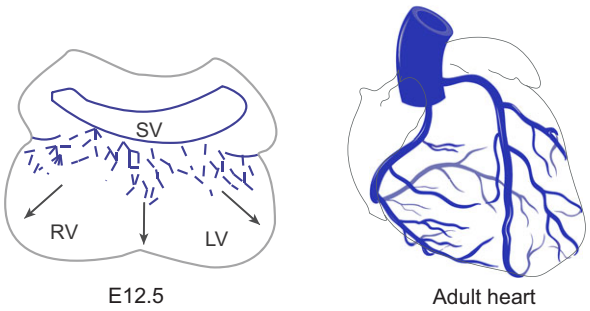

Fig. 3. Formation of the coronary vasculature. At E12.5 (left) the coronary vessels begin to form around the sinus venosus (SV) progressing apically (arrows) across the right ventricle (RV) and left ventricle (LV). The schematic on the right illustrates the adult coronary vascular tree.

KIT$^+$ cardiac stem cells

Identification and developmental origins

KIT (previously known as c-KIT) was first identified in the 1980s as a proto-oncogene in mammalian cells (Yarden et al., 1987). In 2003, a landmark study showed that KIT marks rare cardiac-resident adult stem cells, establishing adult cardiac stem cell biology as a field of investigation (Beltrami et al., 2003) (Fig. 4). Heterogeneous KIT$^+$ cardiac cells, some positive for the cardiomyocyte progenitor markers NKX2-5, GATA4 and MEF2C but negative for hemopoietic lineage markers (LIN$^-$), were clonogenic, self-renewing and able to differentiate into cardiomyocytes, smooth muscle cells and endothelial cells *in vitro* and after transplantation of culture-expanded cells (Beltrami et al., 2003). However, KIT is not a unique marker of this proposed cardiac stem cell population. KIT expression has been reported in postnatal cardiomyocytes (Li et al., 2008), in adult cardiomyocytes induced to dedifferentiate (Jesty et al., 2012; Kubin et al., 2011; Zhang et al., 2010), as well as in coronary endothelial cells and epicardial cells (Castaldo et al., 2008; Limana et al., 2007; Tallini et al., 2009). KIT expression can also be induced from KIT$^-$ cells *in vitro* (Keith and Bolli, 2015). The adult heart also contains resident KIT$^+$ mast cells (Kubo et al., 2008; Pouly et al., 2008) and many cardioprotective KIT$^+$ cells of bone marrow origin enter the heart after injury (Chong et al., 2011; Cimini et al., 2007; Fazel et al., 2006, 2008). In adult *c-kit*$^{W/Wv}$ mutant mice, which have diminished KIT tyrosine kinase activity, cardiac pressure overload induced by aortic constriction triggered postnatal cardiomyocytes to maintain cell cycle activity into adulthood and the expansion of a KIT$^+$/GATA4$^+$ interstitial population. These results suggest that KIT functions to limit cell proliferation in both cardiomyocytes and progenitors (Li et al., 2008), and is therefore a marker of cell state rather than cell lineage.

The developmental origins of the heterogeneous KIT$^+$ cells in the adult heart remain unclear, although some experimental approaches hint at a connection between embryonic and adult populations. Murine ESCs differentiated *in vitro* can give rise to immature mesodermal cells expressing KIT as well as the cardiac transcription factor NKX2-5, which can further differentiate into cardiomyocytes and smooth muscle cells in a clonal fashion (Hatzistergos et al., 2015; Wu et al., 2006). KIT$^+$/NKX2-5$^+$ cells with similar bipotential differentiation capacity *in vitro* have been isolated

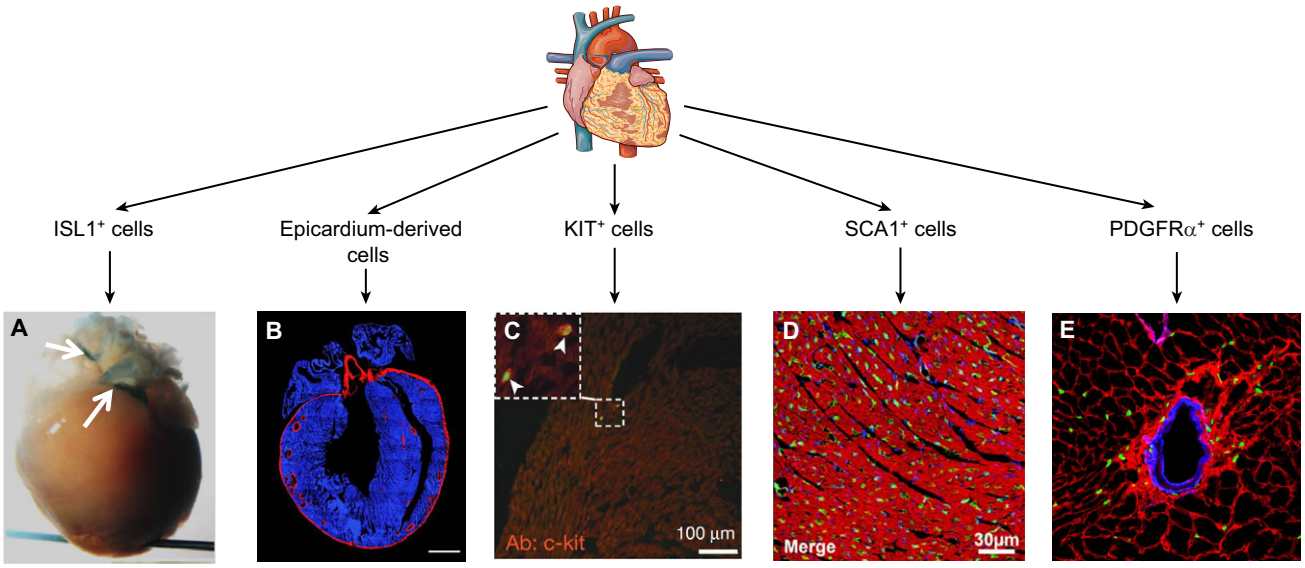

Fig. 4. Stem cell populations in the adult heart. Multiple different stem cell populations have been described in the adult heart, including (from left to right) ISL1$^+$ cells, epicardium-derived cells, KIT$^+$ cells, SCA1$^+$ cells and PDGFRα$^+$ cells as well as cardiospheres and side population cells (not shown). (A) Rare ISL1$^+$ cells are present in the adult heart only in the conduction system including the sinoatrial node (arrows). Cells were marked by β-galactosidase staining (blue) from an *Isl1*-nuclear *lacZ* knock-in allele. Reproduced with permission (Weinberger et al., 2012). (B) The epicardium (shown in red, along with the coronary vessels within the myocardium) gives rise to perivascular cells and stromal fibroblasts in development, and in the adult this potential is retained, which is activated after injury. Other cardiac cells are stained with DAPI (blue). Scale bar: 500 µm. Reproduced with permission from Riley and Smart (2009). (C) KIT$^+$ cells are a minor population in the adult mouse heart shown by expression of a *Kit*-Cre allele (green) and staining for the endogenous KIT protein (red). The inset box shows two mononuclear KIT-expressing cells (arrowheads). Reproduced with permission (van Berlo et al., 2014). (D) Heterogeneous SCA1$^+$ cells are present within the interstitium of the adult heart, shown here with SCA1$^+$ cells marked by GFP (green), phalloidin (red) showing cardiomyocytes, and DAPI (blue). SCA1$^+$ cells include microvascular cells and different stem cell fractions. Reproduced with permission (Uchida et al., 2013). (E) PDGFRα-expressing cells can be detected using a nuclear green fluorescent protein (GFP) expressed from a mouse *Pdgfra* knock-in allele. GFP$^+$ cells are present throughout the cardiomyocyte interstitium and within the adventitial zone of coronary arteries. Red indicates immunofluorescence staining for collagen VI (an adventitial collagen) and blue marks smooth muscle cells positive for α-smooth muscle actin. Original figure reproduced with permission from Vaibhao Janbandhu (Victor Chang Cardiac Research Institute, Australia). Adult heart in figure created using Servier medical art.

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

Table 1. Summary of endogenous adult cardiac stem and progenitor cells

Cell population	Additional markers	Embryonic origin	Adult localization	Differentiation potential		Clinical trials	Additional comments
				In vivo lineage potential*	*In vitro* lineage potential		
KIT$^+$	SCA1, MDR1	Unknown, probably heterogeneous	Epicardium, endocardium, cardiac interstitium	Endothelial cells, smooth muscle cells, cardiomyocytes (rare), fibroblasts (rare)	Culture expanded cells: cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells	SCIPIO (USA, 2009) (Bolli et al., 2011); CONCERT-HF, (study NCT02501811) (USA, 2015)	Controversy over the ability of endogenous adult cardiac KIT$^+$ cells to give rise to cardiomyocytes in meaningful numbers; lineage tracing suggests that KIT$^+$ cells have an endothelial phenotype
cCFU-F	SCA1, PDGFRα	Proepicardial mesoderm	Epicardium, endocardium, cardiac interstitium, coronary vascular adventitia	Unknown, probably give rise to stromal, perivascular and adipogenic cells in homeostasis and myofibroblasts after injury; endogenous cells unlikely to give rise to cardiomyocytes	Culture expanded cells: cardiomyocytes, endothelial cells, smooth muscle cells, adipocytes, chondrocytes, osteocytes	None	Exist within the broader PDGFRα$^+$ cardiac stromal cell fraction
ISL1$^+$	NKX2-5	Unknown (pharyngeal mesoderm proposed)	Rare: sinoatrial node, cardiac ganglia, few cells in the outflow tract	Rare ISL1$^+$ cells in the adult do not appear to give rise to meaningful numbers of cellular progeny	ESC-derived cells: cardiomyocytes, endothelial cells, smooth muscle cells	Embryonic Stem Cell-derived Progenitors in Severe Heart Failure (ESCORT; study NCT02057900) (France, 2013)	This clinical study using human ESC-derived CD15$^+$ ISL1$^+$ cells is ongoing
Epicardial cells	TCF21, TBX18, WT1	Proepicardial mesoderm	Epicardium	Fibroblasts, smooth muscle cells	Explant cultures: endothelial cells, smooth muscle cells	None	TCF21, TBX18 and WT1 are epicardial developmental markers that are reactivated upon injury; the adult epicardium also provides pro-angiogenic and other paracrine signals after injury
SCA1$^+$	PDGFRα, KIT	Unknown, probably heterogeneous	Epicardium, endocardium, cardiac interstitium coronary vascular adventitia	Smooth muscle cells, endothelial cells, cardiomyocytes (rare)	Explanted cells: cardiomyocytes, endothelial cells, smooth muscle cells	None	SCA1 marks a heterogeneous population of cells in the adult, including subpopulations of endothelial, stromal and coronary vascular adventitial cells
Cardiospheres	KIT, SCA1, CD34, markers of MSCs and three main cardiac lineages	Unknown but probably cardiac origin, probably heterogeneous	Unknown, cardiospheres per se do not exist in the adult	n/a	Culture expanded cells: cardiomyocytes, endothelial cells, smooth muscle cells	CADUCEUS (USA, 2009) (Makkar et al., 2012)	Cardiospheres are culture aggregates derived from cardiac tissue outgrowths
Side population	SCA1, GATA4, NKX2-5, MDR1	Unknown, probably heterogeneous	Unknown	n/a	Explanted cells: cardiomyocytes, endothelial cells, smooth muscle cells	None	These cells can only be identified after removal from the heart so *in vivo* identification is not possible

endo, endocardium; epi, epicardium; ESC, embryonic stem cell; interstitium, cardiac interstitium; n/a, not applicable.
*Excludes adoptive transfer studies.

from mouse embryos at E8.5 (Wu et al., 2006), and show downregulation of KIT expression upon differentiation. Various studies aimed at pin-pointing the emergence of KIT$^+$ cells during early murine cardiac development have been performed using fluorescent reporter techniques. In one study, a transgenic reporter carrying enhanced green fluorescent protein (EGFP) expressed under the control of *Kit* regulatory elements showed that EGFP$^+$ cells were found in cardiogenic mesoderm as early as E6.5 and scattered throughout the myocardial wall at later stages (Ferreira-Martins et al., 2012). Cardiac *Kit*-EGFP$^+$ cells isolated from fetal stages had stem cell-like properties *in vitro*, similar to adult KIT$^+$ cells. These properties included a lack of lineage markers,

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

clonogenicity, the ability to undergo symmetric and asymmetric cell divisions and the presence of Ca^{2+} oscillations. Furthermore, the authors demonstrated that, as for adult clonogenic KIT^+ cells, transplantation of these fetal KIT^+ cells following *in vitro* expansion promoted heart repair after injury (Ferreira-Martins et al., 2012). Using a different *Kit*-EGFP transgenic reporter, $EGFP^+$ cells have also been detected in fetal and neonatal myocardial walls, mostly at the epicardial boundary (Tallini et al., 2009). The studies described above raise the question of whether adult cardiac-resident KIT^+ cells originate from within the embryonic cardiogenic fields or, alternatively, from an extra-cardiac compartment (Balmer et al., 2014; Klotz et al., 2015; Nakano et al., 2013; Stanley et al., 2002). A recent lineage-tracing study suggests that the KIT^+ cells in the fetal heart might in fact be of neural crest origin (Hatzistergos et al., 2015). However, a direct lineage relationship between KIT^+ or *Kit*-$EGFP^+$ cells in the developing heart and those in the adult is unproven and requires further exploration using alternative lineage-tracking systems. KIT^+ cells with different identities and lineage origins might co-exist in the heart and in assessing the lineage fates of KIT^+ cells researchers need to be mindful that *Kit* expression might be induced in KIT^- cells after injury or explant culture (Keith and Bolli, 2015).

Regenerative potential in cell transplantation studies

The regenerative capacity of endogenous KIT^+ cardiac cells is hotly debated. Early studies following sex-mismatched heart transplantation in humans (male recipients receiving female donor hearts), showed that host cells migrated into the donor heart and a fraction of these cells were positive for the stem cell markers MDR1 (also known as ABCB1), SCA1 (also known as LY6A) and KIT (Quaini et al., 2002). These presumed precursors were also positive for cardiac markers GATA4, FLK1 (also known as KDR) and MEF2C, suggesting a cardiac field origin and/or cardiac lineage commitment (Quaini et al., 2002). The same authors later defined an endogenous KIT^+ population in human hearts that increased in number after ischemic injury, albeit balanced by stress-induced senescence and death (Urbanek et al., 2005).

In considering the properties of cardiac KIT^+ cells it is important to distinguish between their *in vivo* function and the pro-regenerative capacity of transplanted culture-expanded KIT^+ cells, either as bulk cultures or clonal derivatives. When culture-expanded LIN^-/KIT^+ cells derived from rat were injected into the injured heart, a proportion of the cells was shown to survive and expand, giving rise to large swathes of cardiomyocytes as well as endothelial cells and smooth muscle cells, and replacing lost tissue (Beltrami et al., 2003). Numerous studies have reported similar properties for cultured KIT^+ cells from adult mice, dogs and humans, with reported salutary roles including cardiac functional recovery after injury (Anversa et al., 2013). Expanded clonogenic murine KIT^+ cells from fetal hearts have also been reported to have such properties (Ferreira-Martins et al., 2012). However, not all studies confirm such findings in the mouse model (Keith and Bolli, 2015). KIT^+ progenitor cells co-expressing FLK1 have been defined within coronary vessels, and in transplant studies these show preferential formation of vascular structures, including patent large caliber vessels (Bearzi et al., 2009; Kajstura et al., 2011). Interestingly, only a small subset (2.4%) of KIT^+ cells freshly isolated from the neonatal hearts of mice acquired a cardiomyogenic phenotype when co-cultured *in vitro* with fetal cardiomyocytes (Zaruba et al., 2010). Adult KIT^+ cells, however, failed to undergo cardiomyogenic differentiation in this assay or when transplanted into normal or infarcted hearts. These data suggest that KIT^+ cells with cardiomyogenic potential are very rare in adult hearts, and selective cell culture favors the expansion of rare self-renewing progenitors with multi-lineage plasticity.

In vivo lineage descendants

Several attempts to trace the fate of endogenous KIT^+ cells have been reported. As noted above, Tallini and co-workers used a *Kit*-EGFP transgenic reporter in a surrogate lineage-tracing approach, relying on the inherent stability of EGFP to mark KIT^+ progeny (Tallini et al., 2009). After cryo-injury of adult hearts, EGFP was found in endothelial cells, smooth muscle cells and fibroblasts. In some cardiomyocytes, EGFP was detected at the injury border zone, but in general $EGFP^+$ cells showed no indication of prior cell division; no mitotic figures or phospho-histone H3 staining were observed, and neither were immature cardiomyocytes observed. The authors concluded that differentiated $EGFP^+$ cells could not have arisen by self-renewal or division of stem cells. A subsequent study suggested that cells carrying the same reporter construct could in fact form some new cardiomyocytes and vascular cells in cryo-injured neonatal hearts; however, in injured adult hearts only vascular cells formed (Jesty et al., 2012). It is important to note that these studies are limited by the indirect nature of the lineage-tracing methods.

In a recent study, Ellison and co-workers followed the activation, expansion and differentiation of endogenous KIT^+ cells in a murine model of diffuse cardiomyopathy induced by bolus injection of isoproterenol (ISO) (Ellison et al., 2013). ISO kills ∼10% of sub-endocardial and apical cardiomyocytes, although the resultant anatomical and functional defects are reversed within 28 days, ostensibly aided by preservation of the coronary circulation and endogenous stem and progenitor cell compartments. These authors used an established, although indirect, CRE-based lineage-tracing method (Senyo et al., 2013) and showed that many new bromodeoxyuridine-positive mononuclear cardiomyocytes formed apparently from endogenous primitive KIT^+ cells replacing those lost by ISO treatment. Using a more direct lineage-tracing method that relied on lentivirus delivery of a CRE recombinase transgene, albeit one driven by only the most proximal *Kit* cis-regulatory elements, KIT^+ cells were identified as the source of abundant new cardiomyocytes. Ablation of dividing endogenous KIT^+ cells (and other dividing interstitial cells) using 5-fluorouracil abolished regeneration and functional recovery after ISO; however, this was reversed by transplantation of the culture-expanded progeny of a single endogenous KIT^+ cardiac stem cell, accompanied by an abundance of newly differentiated cardiomyocytes, smooth muscle cells, endothelial cells and myofibroblasts. This study provided the most convincing evidence at that time for the cardiomyogenic capacity of endogenous KIT^+ cells in a regenerative setting.

In stark contrast to the study of Ellison and colleagues, van Berlo and colleagues used knock-in mice constitutively or conditionally expressing CRE under the control of endogenous *Kit* regulatory elements to irreversibly label immature KIT^+ cells with EGFP and trace their *in vivo* fate (van Berlo et al., 2014). During aging of non-conditional *Kit*-Cre mice, immature KIT^+ cells contributed to cardiomyocyte replacement only at a very low level, just ∼0.03% of all cardiomyocytes. Instead, most of the descendants appeared to be endothelial cells, with rare stromal fibroblasts, hemopoietic ($CD45^+$) cells and smooth muscle cells also detected. These results were confirmed using conditional (tamoxifen-dependent) *Kit*-CRE mice. After myocardial infarction, the number of $EGFP^+$ cardiomyocytes was also low, reaching 0.016% close to the infarct

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

border, although with 80-88% of these arising by fusion of KIT$^+$ cells and existing cardiomyocytes. A similarly low level of cardiomyocyte formation was seen in the ISO-induced cardiomyopathy model used by Ellison et al. and discussed above (Ellison et al., 2013). The authors concluded that rare KIT$^+$ cells do represent cardiomyocyte progenitors, but their ability to replace lost cardiomyocytes after diverse cardiac injuries is functionally insignificant (van Berlo et al., 2014).

Vigorous debate has ensued in the wake of the contrasting Ellison et al. (2013) and van Berlo et al. (2014) findings. Arguments focus mainly on the inherent limitations of the respective CRE-based lineage-tracing strategies, including the possibility of precocious re-expression of the transgenic *Kit*-CRE alleles in mature cardiomyocytes, and also the possibility of abnormal progenitor phenotypes in *Kit*-CRE knock-in mice (Molkentin and Houser, 2013; Nadal-Ginard et al., 2014; Torella et al., 2014). In an attempt to reconcile contrasting findings, Bolli and colleagues proposed that populations of KIT^{high} and KIT^{low} progenitors with differing origins and lineage potentialities might co-exist in the adult heart, and that only KIT^{high} cells, possibly an epicardial subfraction, are marked by the *Kit*-CRE knock-in lineage-tracing tools of van Berlo et al. (Keith and Bolli, 2015).

Attempting to shed light on these issues, Cai and Zhou and colleagues recently reported the generation of more sensitive knock-in alleles inserting conditional CRE and/or marker cassettes directly into the ATG start codon of the first *Kit* coding exon (Liu et al., 2016; Sultana et al., 2015). With these tools, in contrast to previous studies, KIT$^+$ cells were found to be abundant in fetal hearts and throughout postnatal life into adulthood. The cellular identity was consistent with that of endothelial cells, being PECAM1$^+$ and located within the endocardium and/or coronary endothelium. They maintained their endothelial identity after myocardial infarction and, consistent with the van Berlo et al. study discussed above, only very rare cells were identified in healthy or injured hearts that co-expressed KIT and a marker of cardiomyocyte precursors (NKX2-5) or differentiated cardiomyocytes (troponin T). With these new lineage tools, some cardiomyocytes could be labeled immediately after lineage tracing, suggesting that those traced in the adult heart did not derive from myocardial stem cells (Liu et al., 2016). These studies claim to have revealed the identity and behavior of the majority population of KIT$^+$ cells within the heart, most of which were not detected previously owing to the insensitivity of antibodies and lineage-tracing strategies.

Pre-clinical and clinical studies

A number of pre-clinical studies have reported the potent myogenic and vessel-forming potential of injected or infused culture-expanded KIT$^+$ cells in different mammalian models (Anversa et al., 2013; Bearzi et al., 2009; Ellison et al., 2013; Tillmanns et al., 2008). This prompted Phase I clinical trials, which subsequently demonstrated the safety and positive preliminary clinical efficacy of these cells (Box 2). However, other groups have not replicated the reported myogenic activity of exogenous KIT$^+$ cells in animal models, but suggest a largely transient paracrine function for these cells before they are cleared from the injection sites or, in the case of systemic infusion, from the vasculature of the lungs and other organs in which they become embolized (Keith and Bolli, 2015). Indeed, concern has arisen that the clinical trials involving KIT$^+$ cells were premature, based on the false assumption of bona fide stem cell functionality. However, in the course of the animal pre-clinical studies and human trials, perhaps a new clinical rationale has arisen through the discovery of the paracrine effects of KIT$^+$ cell delivery stimulating endogenous cardiac stem cells and repair processes. Understanding the differences between the animal cell therapy studies is obviously important for how clinical studies should unfold in the future. The results of larger efficacy trials are awaited and, as noted, the door is still open to the possibility of targeting KIT$^+$ cells *in vivo* to enhance their myogenic potential.

PDGFRα$^+$ cardiac mesenchymal progenitor cells

Identification and developmental origins

Mesenchymal stem/stromal cells (MSCs) were first identified in the bone marrow almost 50 years ago (see Box 3) based on their ability to form clonal colonies under certain culture conditions. This property of 'clonogenicity' is one of the hallmarks of stem cells, reflecting their ability to self-renew. Bone marrow cells with colony-forming ability were originally called colony-forming units-fibroblast (CFU-F). Bone marrow-derived CFU-F are regarded as skeletal stem cells that can generate multiple bone lineages, organize bone architecture and also condition the hemopoietic stem cell niche (Bianco and Robey, 2015). Recently, a population of MSC-like cells in the adult heart has been characterized that express

Box 2. Clinical studies of cardiac stem and progenitor cells

KIT$^+$ cells

In the Phase I Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO) trial, intracoronary delivery of autologous culture-expanded KIT$^+$ cells reduced infarct size after 4 months and improved left ventricular contractility after 1 year in a subset of patients (Bolli et al., 2011). The mechanisms of these beneficial effects require clarification. In addition, a Phase II study investigating the Combination of Mesenchymal and KIT$^+$ Cardiac Stem Cells as Regenerative Therapy for Heart Failure (CONCERT-HF) is presently underway (NCT02501811).

MSCs and PDGFRα$^+$ cells

Bone marrow-derived MSCs have been used in several clinical trials that fall outside the scope of this work (see recent reviews, Boyle et al., 2010; Williams and Hare, 2011). We are not aware of any adult human clinical trials using endogenous cardiac PDGFRα$^+$ cells.

ISL1$^+$ cells

A Phase I clinical trial (ClinicalTrials.gov Identifier: NCT02057900) was initiated in 2013, in which human ESC-derived ISL1$^+$/CD15$^+$ (also known as FUT4) progenitors embedded in a fibrin patch gel was placed on the epicardium of patients with severe left ventricular dysfunction. The results of this study are awaited.

Cardiospheres

In the Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction (CADUCEUS) trial, patients received intracoronary injection of autologous cardiosphere-derived cells 1.5-3 months after myocardial infarction (Makkar et al., 2012). No safety concerns were reported after 6 months and treated patients showed a reduction in scar mass, and an increased viable heart mass and function (Makkar et al., 2012). These broadly positive findings have led to a follow-up Phase II clinical trial – Allogenic Heart Stem Cells to Achieve Myocardial Regeneration (ALLSTAR).

Bone marrow-derived cell therapy

Many clinical trials have been conducted using EPCs or bone marrow mononuclear fractions. A recent and rigorous meta-analysis of these studies showed no beneficial effects (Gyongyosi et al., 2015; Kovacic and Fuster, 2015). A multicenter Phase III study of bone marrow mononuclear cell therapy is currently underway that should definitively address the utility of this therapy in patients that have suffered large myocardial infarctions.

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

Box 3. Mesenchymal stem/stromal cells – a brief history and definition

In the 1960s, osteogenic stem cells were identified in the bone marrow by Alexander Friedenstein and colleagues as clonal colony-forming units-fibroblasts (CFU-F) (Friedenstein et al., 1970, 1968). Cultured CFU-F could give rise to several mature skeletal lineages in the form of an 'ossicle' when transplanted at ectopic sites *in vivo*, and this continues to be a defining assay in CFU-F biology. More recently, bone marrow MSCs have been transplanted together with a mineralized scaffold into ectopic sites (Chan et al., 2013; Sacchetti et al., 2007), where they give rise to organized trabeculated bone that can attract the host vasculature and organize it into sinusoids that sustain hemopoietic stem cells and hemopoiesis. Single labeled cells give rise to osteocytes, chondrocytes and multiple stromal lineages, and can self-renew, as demonstrated in serial transplantation experiments. A number of terms have been used to describe CFU-F, including 'stromal stem cells' in 1988 by Maureen Owen to indicate their residency in the stroma rather than the hemopoietic compartment (Owen and Friedenstein, 1988), and 'mesenchymal stem cells' in 1991 by Arnold Caplan to emphasize the self-renewal and differentiation potential of such cells *in vitro* (Caplan, 1991). Despite criticism (Bianco, 2014), the term MSC is widely used in reference to skeletal stem cells in the bone marrow and CFU-F in other organs.

platelet-derived growth factor receptor alpha (PDGFRα) and SCA1, and possess qualities of stem cells including clonal colony formation (Chong et al., 2011). These cells were termed cardiac colony-forming units-fibroblast (cCFU-F), consistent with the nomenclature of bone marrow-derived CFU-Fs.

In the murine embryo, PDGFRα is expressed in the early mesoderm, including cardiac mesoderm (Chong et al., 2011; Kattman et al., 2011), and in cardiac neural crest (Hoch and Soriano, 2003). In *Pdgfra* knockout mice, the outflow tract, septal, chamber and coronary vessel defects reflect functions for PDGFRα in different aspects of early cardiogenesis (Soriano, 1997). However, in the fetal heart, *Pdgfra* expression is largely restricted to the proepicardium and epicardium, and is maintained in epicardium-derived cardiac stromal cells (also known generically as cardiac fibroblasts) as they undergo EMT to enter the interstitial spaces of the myocardial chamber walls and form valve tissue (Chong et al., 2011). Epicardium-specific *Pdgfra* knockout mice show defective EMT and a dearth of cardiac stromal fibroblasts, whereas smooth muscle cells, also derived from the epicardium, were unaffected (Smith et al., 2011). Most cardiac interstitial cells become committed to their fate while within the epicardial layer (Acharya et al., 2012). The role of PDGF signaling in cCFU-F and stromal cell biology is not well understood, although in addition to being necessary for EMT and formation of stromal fibroblasts in development, it might play a role in maintaining the stem cell phenotype (Ball et al., 2014).

In the adult mouse and human heart, PDGFRα can be detected in the epicardial-derived stromal cells occupying both the interstitial spaces and the perivascular, adventitial niche (Fig. 4) (Chong et al., 2011, 2013). In mice, a minority fraction of PDGFRα$^+$/SCA1$^+$/PECAM1$^-$ (also known as CD31) cells represent cCFU-F and form clonal mesenchymal colonies *in vitro* that express markers typical of bone marrow MSCs, including CD44, CD90 (also known as THY1), CD29 (also known as ITGB1) and CD105 (also known as ENG) (Chong et al., 2011; Pelekanos et al., 2012). Colonies and the larger stromal cell population also express some cardiac developmental transcription factors, including GATA4, TBX5, HAND1 and MEF2C, suggesting a cardiac identity and/or lineage-committed state (Furtado et al., 2014; Noseda et al., 2015). Cultured cCFU-F show long-term growth through serial passage, reflecting their ability to self-renew, and display multipotency *in vitro* and in surrogate *in vivo* assays, including co-culture with ESCs to form teratomas and after injection into infarcted hearts (Chong et al., 2011; Noseda et al., 2015). Using CRE lineage-tracing methods and bone marrow transplantation, cCFU-F in both healthy and infarcted hearts were shown to have their lineage origins in the epicardium during development and were not derived from circulating bone marrow cells (Chong et al., 2011; Noseda et al., 2015). Thus, adult cCFU-F may be considered true endogenous cardiac stem cells in that they have their origins in the proepicardium and epicardium, which derive from the early cardiac progenitor fields (Zhou et al., 2008b).

Developmental fate

cCFU-F and the cardiac PDGFRα$^+$ stromal cell fraction may share properties with the epicardium and other mesothelia that give rise to stromal and smooth muscle lineages during development (Asli et al., 2014; Rinkevich et al., 2012). In the adult heart, cCFU-F have been proposed to give rise to the diverse variety of stromal cells as well as perivascular cells, adipocytes, myofibroblasts, coronary endothelial cells and (controversially) cardiomyocytes in homeostasis and disease (Asli et al., 2014). Two CRE lineage-tracing studies that may shed light on the fate of cCFU-F *in vivo* have been performed. In the first, Braun and colleagues mapped the lineage descendants of the adult cardiac interstitial cells expressing the stem cell marker SCA1 (Uchida et al., 2013). The SCA1$^+$ population is heterogeneous and includes coronary microvascular endothelial cells, KIT$^+$ progenitor cells (Urbanek et al., 2006) and PDGFRα$^+$ stromal cells, including cCFU-F (Chong et al., 2011). Previous studies had ascribed robust cardiomyogenic potential to the SCA1$^+$ fraction after transplantation to infarcted hearts (Oh et al., 2003). Furthermore, some SCA1$^+$ cells appear to be located beneath the basal lamina of cardiomyocytes, reminiscent of skeletal muscle stem cells (Uchida et al., 2013). In the SCA1 lineage-tracing experiment, the differentiated progeny of SCA1$^+$ cells were mostly smooth muscle cells, although coronary endothelial cells and rare cardiomyocytes were also identified. Interestingly, clonal lineage-tracing studies showed that most SCA1$^+$ cells were committed unipotent progenitors that differentiated without expansion. The study is limited in that it did not specifically discriminate between KIT$^+$, PDGFRα$^+$, cCFU-F or other SCA1$^+$ subpopulations, and rare multipotent stem cells might not have been marked efficiently.

Abnormal or persistent PDGF signaling has been implicated in fibrosis in multiple organ systems, including heart (Olson and Soriano, 2009). In a second lineage-tracing study, the descendants of GLI1$^+$ perivascular cells were traced using a tamoxifen-inducible *Gli1*$^{CRE-ERT2}$ knock-in allele in models of cardiac pathology (Kramann et al., 2015). GLI1 is an effector of hedgehog signaling and is thought to mark a population of PDGFRβ$^+$ perivascular MSCs with colony-forming ability in diverse organs, including heart. GLI1 and related protein GLI2 are markers of fibrosis, and sonic hedgehog signaling appears to contribute to the self-renewal of MSCs *in vitro* (Kramann et al., 2015). The lineage-tracing experiment identified myofibroblasts (expressing smooth muscle α-actin; SMA, also known as ACTA2) as the main cellular descendants of tissue-resident *Gli1*$^{CRE-ERT2}$-tagged cells in hypertensive and pressure overload models of cardiac fibrosis. After myocardial infarction, the scar area was replete with GLI1$^+$/SMA$^+$ myofibroblasts and NG2$^+$ (also known as CSPG4) pericytes were also observed, although whether GLI1$^+$ cells have bi-lineage potential was not examined. Specific ablation of GLI1$^+$ cells using

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

the inducible diphtheria toxin approach in the setting of pressure overload reduced fibrosis, preserved left ventricular function and prevented heart failure.

In summary, adult cCFU-F are likely to represent one type of cardiac lineage progenitor, possibly maintaining the stromal, matrix, vascular and adipocyte compartments of the heart during homeostasis, and contributing to vascular cells and myofiboblasts during heart repair. cCFU-F also probably contribute to fibrosis and fibro-fatty infiltration that accompanies certain cardiac pathologies (Sommariva et al., 2015). Cardiac stromal cells probably also act as sentinels of cardiac stress and autocrine function, and mediate local cellular dialogs with other cardiac-resident cells, including cardiomyocytes and immune cells, through paracrine mechanisms (Amoah et al., 2015; Ieda et al., 2009). However, additional markers that distinguish cCFU-F from the larger PDGFRα$^+$ stromal population and more specific lineage-tracing tools are needed to understand better the cardiac interstitial lineage hierarchy and individual cellular function in health and disease. Whether the proliferative capacity of cCFU-F cells *in vitro* reflects their self-renewal and progenitor status *in vivo* requires investigation, and it remains to be demonstrated whether any PDGFRα$^+$ cells in the adult mammalian heart have cardiomyogenic potential that could be augmented in a therapeutic setting. Furthermore, the notion of a 'living scar' and the many roles of cardiac stromal cells and descendant myofibroblasts is well established and targeting different aspects of fibrogenesis is an active area of research (Furtado et al., 2016).

ISL1$^+$ progenitor cells

Developmental origins and location in the adult heart

The LIM-homeodomain transcription factor ISL1 binds and controls *cis*-regulatory elements of the insulin gene (Karlsson et al., 1990). ISL1 is also expressed in cardiac mesodermal progenitors of the FHF and SHF, with expression downregulated as progenitors enter the heart and differentiate into cardiac lineages (Cai et al., 2003; Peng et al., 2013; Prall et al., 2007). Expression in cardiac neural crest cells has also been detected using a lineage-tracing approach, although the number of cells highlighted by both neural crest and ISL1 lineage-tracing tools was modest (Engleka et al., 2012). ISL1 deletion affects the survival and proliferation of heart progenitors and their deployment to the forming heart tube (Cai et al., 2003; Laugwitz et al., 2008). Explant cultures revealed that ISL1$^+$ cells can differentiate into cardiomyocytes and smooth muscle cells *in vitro*, and CRE lineage-tracing studies showed that ISL1$^+$ progenitors contribute cardiomyocytes, endothelial cells and smooth muscle cells to the developing heart (Laugwitz et al., 2005; Moretti et al., 2006).

Conditional CRE lineage-tracing studies in mice have revealed that immature ISL1$^+$ cells persist beyond early heart development into fetal, neonatal and adult life, distributed in a pattern that is consistent with an SHF origin (Bu et al., 2009; Genead et al., 2010; Laugwitz et al., 2005), although this has not been formally proven. At fetal stages, some ISL1$^+$ cells were negative for cardiac lineage markers (Bu et al., 2009), but most expressed the cardiomyocyte marker troponin T, suggesting a cardioblast identity, and only a minority were proliferating (Genead et al., 2010). Conditional lineage tagging of fetal ISL1$^+$ cells showed that they do give rise to a minor proportion of cardiomyocytes in the postnatal murine heart (Laugwitz et al., 2005). Relatively more ISL1$^+$ cells have been observed in the postnatal human heart, potentially reflecting the need for greater proliferative expansion of cardiomyocytes before birth in humans (Bu et al., 2009). However, regardless of the species – mouse, rat or human – ISL1$^+$ cells are rare in the adult, and in the mouse they are largely confined to the sinoatrial node (Fig. 4) (Weinberger et al., 2012).

Regenerative capacity

It is unclear whether ISL1$^+$ cells represent a compelling stem cell population in the adult heart that can be drawn upon for repair. Not only are they very rare, but there is evidence against their involvement in the regenerative post-injury response. No ISL1$^+$ cells were found in the infarct region after myocardial infarction (Weinberger et al., 2012) and only focal expansion of cells was seen after ischemia-reperfusion injury (Genead et al., 2012). However, human and mouse ESCs bearing *Isl1* reporters have been used to enrich for ISL1$^+$ progenitors, which were able to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells *in vitro* (Bu et al., 2009; Moretti et al., 2006; Qyang et al., 2007). A proportion of clones expressing ISL1 and NKX2-5 showed tri-lineage differentiation potential that could be maintained on feeder layers expressing WNT3A (Qyang et al., 2007). Therefore, human pluripotent stem cell-derived cardiac progenitors marked by ISL1$^+$ and/or other cardiac markers might be suitable in cell therapy procedures after cardiac injury (Chong et al., 2014b). Interestingly, treatment of human ESC-derived ISL1$^+$ progenitors with modified vascular endothelial growth factor (VEGF) mRNA switched their fate to an endothelial phenotype (Lui et al., 2013), whereas injection of *VEGF* mRNA into infarcted hearts activated migration and proliferation of epicardium, biasing its differentiation towards endothelial cells and improving cardiac vascularity and functional recovery (Zangi et al., 2013). Injection of an *Isl1* expression cassette as naked DNA into the peri-infarct region in mice subject to myocardial infarction also increased left ventricular function and tissue revascularization, and reduced fibrosis (Barzelay et al., 2012). Although the exact mechanism is unknown, the authors did note that proangiogenic cytokines were increased. ISL1 has been identified as a regulator of EMT in epicardial cells (Brønnum et al., 2013), suggesting that promotion of stem cell characteristics in epicardium (Asli and Harvey, 2013) might be a possible regenerative target for ISL1 therapy.

SCA1$^+$ cells, side population cells and cardiospheres

Other cardiac-resident progenitor populations with cardiac lineage potential have been described: SCA1$^+$ cells, cardiac side population (SP) cells and cardiospheres. The developmental origins of these populations have not been rigorously studied, although, to a certain extent it may be possible to speculate on their origins based on marker expression (Table 1 and see below).

SCA1$^+$ cells

SCA1 is a cell surface protein often used to identify or enrich for hemopoietic stem cells (Holmes and Stanford, 2007). SCA1$^+$ cells can also be found in the heart; these are highly heterogeneous and include microvascular endothelial cells, KIT$^+$ progenitor cells, PDGFRα$^+$ cCFU-F and stromal cells, as well other interstitial and perivascular cells (Fig. 4). The SCA1$^+$ fraction expresses the cardiac transcription factors GATA4, MEF2C and TEF1 (also known as TEAD-1), but lacks other cardiomyocyte lineage markers including NKX2-5 (Oh et al., 2003). Although many cells are positive for PECAM1, they do not express more mature endothelial or hemopoietic cell markers. Interestingly, transcriptome analysis comparing several cardiac and bone marrow progenitor fractions showed that the total cardiac SCA1$^+$ fraction was closest to cardiomyocytes, with KIT$^+$ cells being the most immature (Dey

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

et al., 2013). SCA1$^+$ cardiac interstitial/vascular fractions from humans and mice can be differentiated into cardiomyocytes *in vitro* using 5-azacytidine, and in adoptive cell transfer procedures in infarcted mice, which improved cardiac repair and function (Oh et al., 2003; Smits et al., 2009; van Vliet et al., 2008; Wang et al., 2006). Two weeks after engraftment, ∼18% of cardiomyocytes in the infarct boarder zone were donor derived, although about half of these were produced by fusion between donor cells and existing cardiomyocytes (Oh et al., 2003). As also noted above, CRE lineage tracing has shown that a rare subset of SCA1$^+$ cells give rise to cardiomyocytes during normal aging, with most forming smooth muscle cells and potentially also endothelial cells (Uchida et al., 2013). Deletion of *Sca1* results in decreased cardiac function and a hypertrophic response in older mice (Bailey et al., 2012), which is likely to be the collective effect of a reduced SCA1$^+$ stromal fraction and/or reduced SCA1$^+$ coronary microvasculature. A systematic dissection of the nature, roles and origins of cardiac SCA1$^+$ cells remains to be performed. Recent single-cell expression profiling confirmed that the SCA1$^+$/PDGFRα$^+$ fraction of cardiac interstitial cells, probably derived from the epicardium (Chong et al., 2011; Furtado et al., 2014), is enriched in clonogenicity and multi-lineage differentiation after *in vivo* engraftment (Noseda et al., 2015). However, cell retention after engraftment was low and it remains to be determined whether heterogeneity per se in the total SCA1$^+$ fraction provides a survival or functional advantage in cell therapies.

Cardiac side population cells

SP cells were first detected in the bone marrow, and are greatly enriched in hemopoietic stem cells (Goodell et al., 1996). SP cells are identified using flow cytometry, based on their ability to efflux DNA-binding dyes through an ATP-binding cassette transporter. In the adult heart, SP cells are immature and represent ∼1% of all cells (Hierlihy et al., 2002; Martin et al., 2004; Pfister et al., 2005). They significantly overlap with the SCA1$^+$ population (Noseda et al., 2015) and as such they express the cardiac transcription factors NKX2-5 and GATA4, although not hemopoietic or myofilament markers (Pfister et al., 2005). Co-culture of SP cells with neonatal or adult cardiomyocytes induces differentiation of SP cells to cardiomyocytes with rhythmic contraction and calcium transients (Pfister et al., 2005). Cardiac SP cells are also able to form cardiomyocytes when cultured alone, or with trichostatin A and oxytocin (Oyama et al., 2007). The rapid reconstitution of the cardiac SP fraction from bone marrow after myocardial infarction suggests a bone marrow origin for these cells (Mouquet et al., 2005). However, a neural crest cell origin has also been suggested, based on the expression of neuronal markers in SP-derived cardiospheres, as well as their behavior in transplant assays in chick embryos (Tomita et al., 2005). The specific enrichment of clonogenic cells expressing cardiac transcription factors in both cardiac SP and SCA1$^+$/PDGFRα$^+$ populations (Noseda et al., 2015) challenges these views. As for SCA1$^+$ cells, both intravenous and intramyocardial injection of cardiac SP cells in mice following cardiac injury demonstrated their potential to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells *in vivo* (Liang et al., 2010; Oyama et al., 2007). In summary, SP cells have progenitor properties but are likely to be composed of a heterogeneous mix of resident cardiac cell populations with unresolved identity and origins.

Cardiospheres

Cardiospheres are cellular aggregates derived from the phase-bright, poorly adherent cells that appear in outgrowths of cardiac tissue (Messina et al., 2004). These heterogeneous clusters are grown in suspension and are thought to create a niche-like environment that allows propagation of KIT$^+$ cardiac progenitor cells at their core (Li et al., 2010; Messina et al., 2004), with diverse stromal and more differentiated cells occupying the periphery. Given the technique used to derive cardiospheres, their origins are difficult to map. They appear to be of cardiac but not cardiomyocyte origin (Davis et al., 2009; White et al., 2013; Ye et al., 2013) and might actually be a composite of KIT$^+$ progenitors and cells from the MSC lineage and/or epicardium (Chong et al., 2014a). TGFβ-dependent EMT appears to be important for their formation (Forte et al., 2012). Cardiosphere-derived cells can differentiate into cardiomyocytes in co-culture with neonatal rat cardiomyocytes (Smith et al., 2007), and when injected into murine, rat or porcine models of myocardial infarction, they improve cardiac function (Malliaras et al., 2012, 2013; Messina et al., 2004), although the beneficial effects are likely to be paracrine (Chimenti et al., 2010; Malliaras et al., 2012). Functional improvements were also seen where donor cells and host were from distinct inbred rat strains, potentially reflecting immunomodulatory roles for cardiosphere-derived cells (Tseliou et al., 2013). Of the three populations discussed in this section, only cardiosphere-derived cells are currently undergoing clinical evaluation (Box 2).

The adult epicardium as a progenitor population

The epicardium is the outermost mesothelial layer of the adult heart. During development, it undergoes EMT to give rise to epicardium-derived cells, which form smooth muscle cells and probably some endothelial cells of the nascent coronary vascular tree as well as interstitial stromal cells and cCFU-F. The ability of the epicardium to form significant numbers of cardiomyocytes during development remains contentious (Asli et al., 2014), although it is essential for promoting cardiomyocyte proliferation via a paracrine effect (Perez-Pomares and de la Pompa, 2011). Early studies showed that the adult epicardium is highly reactive to injury and is involved in immune surveillance (Gittenberger-de Groot et al., 2010; Yung and Chan, 2009). It is now clear that the adult epicardium, as for its developmental counterpart, acts as both a progenitor population and a source of pro-angiogenic and other paracrine signals after injury (Limana et al., 2010, 2007). In both zebrafish and mouse, the epicaridum of injured adult hearts undergoes global activation of its fetal epicardial program (Bollini et al., 2014; Kikuchi et al., 2011; Lepilina et al., 2006; Zhou et al., 2011), and experimental depletion of epicardium in fish leads to its rapid regeneration guided by signals from the outflow tract including Hedgehog ligand (Wang et al., 2015). In adult mouse hearts subjected to myocardial infarction, epicardial cells undergo EMT and migrate to the sub-epicardium, where they differentiate into myofibroblasts and smooth muscle cells (but not cardiomyocytes or endothelial cells), which incorporate into new vessels (Zhou et al., 2011). The epicardium is highly heterogeneous (Bollini et al., 2014), and a minority KIT$^+$/CD34$^+$/CD45$^-$ population has been observed to proliferate after injury and differentiate into smooth muscle cells and endothelial cells organized into vessels, with a potential role for pericardial fluid in maintaining their progenitor status (Limana et al., 2010, 2007). A population of glycolytic progenitors expressing HIF1α has also been described (Kocabas et al., 2012). Cultured human epicardium-derived cells have been transplanted into the hearts of mice following myocardial infarction, whereupon they engrafted and improved cardiac repair and function, most likely mainly though paracrine mechanisms (Winter et al., 2007).

Migration and differentiation of adult epicardial cells into vascular structures can be stimulated by the actin monomer-

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

binding protein thymosin β4 (Tβ4; also known as TMSB4X) delivered at the time of induction of myocardial infarction (Smart et al., 2007). In the same study, the authors also claimed that a small number of epicardial-derived cardiomyocytes formed under these conditions (Smart et al., 2007), although this was later disputed (Zhou et al., 2012). Tβ4 also influences cardiac repair indirectly through modulation of the immune response and signaling pathways in cardiac myofibroblasts and cardiomyocytes, providing resistance to oxidative stress and cell death (Bock-Marquette et al., 2009; Evans et al., 2013; Gupta et al., 2012).

Bioengineering methods for delivering therapeutic factors to the epicardial surface are being explored for clinical feasibility. For example, injection of modified *VEGFA* mRNA into the myocardium at the time of myocardial infarction led to increased expression of epicardium marker WT1, with lineage tracing showing mobilization of $WT1^+$ epicardial cells into the myocardium and sub-epicardium, and their enhanced differentiation to endothelial cells (5% of traced cells) with some cardiomyocytes also observed (Zangi et al., 2013). *In vitro* clonal analysis showed the increased lineage potency of single $WT1^+$ cells, some of which could form myofibroblasts, endothelial cells and smooth muscle cells. The secreted signaling inhibitor follistatin-like 1 (FSTL1) is upregulated after cardiac injury and has been flagged as a biomarker of acute coronary syndrome (Alteköester and Harvey, 2015). In a recent study, the epicardial isoform of FSTL1 was delivered to the heart after myocardial infarction via a collagen patch sutured to the infarct surface (Wei et al., 2015). This treatment increased animal survival, vascularization and cardiac function, while decreasing adverse remodeling. Proliferation of existing cardiomyocytes in the border zone was increased, and some cardiomyocytes had even migrated into the collagen patch. The above studies provide insight not only into the important role of the epicardium in adult homeostasis and repair, but also non-classical avenues for therapeutic intervention to enhance cardiac regeneration.

Origins and plasticity of cardiac fibroblasts

The origin of stromal fibroblasts and myofibroblasts is an important consideration in this Review, because in addition to providing a cardiac scaffold and structural integrity to the heart, stromal cells and myofibroblasts regulate organ development, wound healing, stem cell niches and inflammation (Furtado et al., 2016). An early CRE-based lineage-tracing experiment suggested that a subset of cardiac stromal fibroblasts arise *de novo* via endothelial-to-mesenchymal transition (EndMT) from adult endothelial cells (Zeisberg et al., 2007). However, more recent lineage-tracing studies have shown that the proliferation and expansion of resident fibroblasts during cardiac hypertrophy accounts for almost all cardiac fibroblasts (Ali et al., 2014; Moore-Morris et al., 2014). Around 80% of the fibroblasts had their origins in the epicardium, with the remainder derived from the endocardium via EndMT (Ali et al., 2014; Moore-Morris et al., 2014), although a limited number of adult fibroblasts in the right atrium and outflow tract region might derive from neural crest cells (Ali et al., 2014). Importantly, the developmental origin of adult stromal fibroblasts does not appear to affect the cellular fibroblast phenotype, transcriptional profile, response to apoptotic stimuli or proliferation rate (Ali et al., 2014). Based on the current literature, therefore, it appears likely that *de novo* EndMT makes a modest contribution to the myofibroblast population in the adult heart. However, studies performed in other organs, such as the kidney, suggest that *de novo* EMT gives rise to fibroblast-like cells that adopt a partially transitioned state intermediate between epithelial cells and fibroblasts (Grande et al., 2015; Lovisa et al., 2015). This possibility is unexplored in the adult heart but may reconcile the apparent differences between lineage-tracing studies on the origin of the cardiac fibroblast. In a final twist, evidence has recently emerged that adult myofibroblasts can undergo mesenchymal-to-endothelial transition (MEndT) and adopt an endothelial phenotype in an acute cardiac ischemia setting (Ubil et al., 2014). The transition appears to be complete, with myofibroblast-derived endothelial cells exhibiting the functional characteristics of native endothelial cells. MEndT was observed to be associated with increased vessel density in the injury border zone, but not remote regions of the heart, and was shown to be regulated by the p53 (TRP53) pathway.

Circulating endothelial progenitor cells: origins and roles in repair

It is generally accepted that circulating endothelial progenitor cells (EPCs) exist in the adult heart, and that they might be involved in restoration of cardiac vasculature after injury (Kovacic et al., 2008). This possibility gained credibility from the observation that $CD34^+$ cells from human peripheral blood localize to areas of neovascularization when injected into nude mice with hindlimb ischemia (Asahara et al., 1997). Further studies showed that CD133 (also known as PROM1) is expressed on EPCs but not mature endothelial cells, and thus this marker has been considered by some to be specific for EPCs (Miraglia et al., 1997; Yin et al., 1997).

To address the origin and function of circulating EPCs more precisely, numerous studies have analyzed the regenerative properties, specifically the restoration of cardiac function and new vessel formation, of enriched bone marrow progenitor cells (Murry et al., 2004; Nygren et al., 2004; Orlic et al., 2001) or other peripheral blood fractions (Hirschi et al., 2008). Despite early promising results (Asahara et al., 1997), animal studies with reconstituted genetically marked bone marrow concluded that bone marrow-derived cells were detectable only as perivascular cells supporting new vessel growth, but were not integrated in the endothelial lining (Rajantie et al., 2004; Ziegelhoeffer et al., 2004). Similarly, it was shown that circulating EPCs do not contribute to the endothelium overlying plaques in murine atherosclerosis (Hagensen et al., 2010). It has been suggested that the majority of EPC-like cells in the peripheral blood are inflammatory or monocytic cells, which may be required to activate resident EPCs to form vessels by releasing pro-angiogenic factors (Gulati et al., 2003; Rohde et al., 2007; Yoder et al., 2007). Indeed, putative EPCs have distinct monocytic features and can be cultured from $CD14^+$ cells (Urbich et al., 2003). A subset of monocytic cells expressing TIE2 (also known as TEK) and VEGFR2 (also known as FLK1 and KDR) was shown to play a pivotal role in tumor angiogenesis (De Palma and Naldini, 2009; De Palma et al., 2005), and monocytes expressing F4/80 (ADGRE1), CD31 and VEGFR2 can contribute to both tumor angiogenesis and revascularization following ischemia (Kim et al., 2009). Several studies showed that monocytes/macrophages can promote angiogenesis and possibly arteriogenesis (Heil et al., 2002; Pipp et al., 2003) by releasing pro-angiogenic factors, for example angiopoietin, VEGF and basic fibroblast growth factor (bFGF), or by transdifferentiating into non-phagocytic cells resembling mesodermal and neuroectodermal lineages (Kuwana et al., 2003). A primitive population termed monocyte-derived multipotential cells (MOMCs) can be derived *in vitro* from human peripheral blood mononuclear cells, and can be differentiated into distinct mesenchymal cell types, including bone, fat, and skeletal and cardiac muscle (Kuwana et al., 2003). MOMCs express several endothelial markers [VE-cadherin (cadherin 5),

Development (2016) 143, 1242-1258 doi:10.1242/dev.111591

VEGFR1 (FLT1)] and human MOMCs can incorporate into newly forming vessels as endothelial cells, suggesting that in a permissive environment monocytic cells can in fact differentiate into endothelial cells and that these cells may hold utility for therapeutic vascular formation (Kuwana et al., 2006).

During embryogenesis, endothelial and hemopoietic cells derive from a common ancestor, the hemangioblast (reviewed by Michelis et al., 2014). Specific environmental factors, such as a gradient of FGF expression, can induce hemangioblasts to differentiate preferentially to endothelial or hemopoietic fates. In the mouse embryo, it has been shown that neovascularization is influenced by monocytes and their mature derivatives, i.e. macrophages (Anghelina et al., 2004, 2006; Moldovan, 2002; Pipp et al., 2003), which are present in the angiogenic fields prior to the advancement of new capillaries (Goepfert et al., 2001; Levine et al., 2000; Takakura et al., 2000). Although the hemangioblast concept represents a possible link between the embryonic endothelial and hemopoietic compartments, the extent to which adult hemopoietic cells transdifferentiate into endothelial cells remains unclear. Furthermore, it is not certain whether monocytes can become fully functional endothelial cells or if their contribution during angiogenesis is restricted to the production of vascular growth factors. Moreover, the role of 'bona fide' EPCs that are committed to an endothelial cell fate but do not have any relationship to monocytes is not yet fully understood. Understanding these aspects will be important in order to understand the role of EPCs and monocytes in cardiac repair.

Therapeutic use and future perspectives

Organ regeneration is the re-establishment of correct tissue architecture and function following injury, and if this definition is adhered to, clinical cardiac regeneration by injection of precursor cells or other approaches has not yet been achieved. However, the clinical trials conducted to date have shown that cell transplantation is generally safe and might trigger beneficial effects, probably as a result of release of growth factors and cytokines/chemokines that favor endogenous tissue re-endothelialization and decreased apoptosis (Gnecchi et al., 2005, 2006). Indeed, the main function of cardiac stem cells might be to induce the re-awakening of cell proliferation, inhibition of apoptotic signaling, decreased inflammation and modulation of angiogenic pathways. Careful analyses have not excluded cardiac stem cell differentiation into cardiomyocytes, but this seems to be such a rare event under normal conditions (Smart et al., 2011; Uchida et al., 2013), that the large-scale regeneration of millions of cardiomyocytes in patients with cardiomyopathies or after myocardial infarction is hard to envision in the short to medium term. For these reasons, we believe that it may be necessary to reflect on the current data and form new hypotheses about the function of adult cardiac stem cells, and how these cells can be manipulated therapeutically. It has been shown, for example, that fully differentiated adult cardiomyocytes can dedifferentiate in response to specific stimuli (Kubin et al., 2011). Furthermore, the forced expression of specific developmentally regulated transcription factors can induce fibroblasts to directly transdifferentiate into cardiomyocytes both *in vitro* and *in vivo* (Ieda et al., 2010; Inagawa et al., 2012; Qian et al., 2013; Qian et al., 2012; Song et al., 2012). These experiments show that cellular phenotype is not necessarily fixed, but can be manipulated with appropriate stimuli, opening up new possibilities for manipulating cell fate for cardiac repair.

As mentioned previously, the generation of morphologically and functionally different cardiac cells during embryogenesis is achieved by EMT and by mesenchymal-to-epithelial transition (MET), the reverse mechanism (Kovacic et al., 2012). Although other processes are involved, EMT and MET are essential portals to stem cell identity (Asli and Harvey, 2013) and are fundamental for cellular diversity during development and in adulthood. Interestingly, in adult tissues, EMT is not only associated with wound healing and organ repair, but also with fibrosis and cancer progression (Kalluri, 2009; Kovacic et al., 2012). In this regard, a significant difference between the embryonic and the adult environment is the role of the immune system. Embryos develop in an immune-permissive environment, compared with adult tissues in which innate and secondary immune responses are robust. Therefore, it is possible to speculate that during tissue repair in the adult, inflammatory reactions preclude stem cell differentiation or perturb the signaling pathways controlling EMT and MET, or the related processes EndMT and MEndT. Hypothetically, it is possible that different inflammatory states could produce different outcomes, inducing cells to differentiate into fibroblasts, for example during post-myocaridal infarction remodeling, or instructing them to remain mesenchymal, as in cancer metastasis. Newt lens regeneration and tadpole-frog development are two examples of the latter (Brockes and Kumar, 2008). Whether evolution of the innate and adaptive immune responses led to tissue repair by fibrotic reactions at the expense of tissue regeneration remains a possibility to be explored in future analyses (Sattler and Rosenthal, 2016).

In conclusion, although controversies have arisen and many questions remain unanswered, there can be no question that the seminal discovery of adult cardiac stem/progenitor cells has left an indelible mark on cardiovascular biology and medicine. The need to understand the developmental origins and biology of these cells is paramount. Setbacks and controversies are to be expected, and the ensuing revision of ideas plays a key role in our overall advancement. The opportunity for developmental biologists, pre-clinical researchers and clinicians to work together towards the common goal of cardiac regeneration is almost without precedent, and remains one of the inspiring features of working in this field.

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Competing interests

The authors declare no competing or financial interests.

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