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REVIEW

Developmental origins and lineage descendants of endogenous adult cardiac progenitor cells

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Abstract Mammalian hearts carry a number of primitive stem cell-like populations, although the magnitude of their contribution to tissue homeostasis and repair remains controversial. Recent CRE recombinase-based lineage tracing experiments suggest only a minor contribution to the formation of new cardiomyocytes from such cells, albeit one that might be augmented therapeutically. As the field explores clinical translation of cardiac stem cells, it will be important to understand the biology of these cells in great detail. In this review we document the various reported stem and progenitor cell populations in mammalian hearts and discuss the current state of knowledge on their origins and lineage capabilities.

Contents

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Introduction

In this review we focus on documenting and evaluating the origins and fate of primitive cell populations (cardiac progenitor cells; CPCs) that have been identified in post-natal mammalian hearts and credited with possessing stem cell-like properties in vivo or after ex vivo expansion. Characterization of CPCs is driving forward a new era in CV biology and stem and progenitor cell therapies that regenerate myocardium lost in the wake of ischemic or other injurious stimuli would have enormous benefit to humanity. Clinical data acquired thus far from cell therapy trials using bone marrow (BM) and cardiac-derived cell fractions (Abdel-Latif et al., 2007; Bolli et al., 2011; Clifford et al., 2012; Makkar et al., 2012) appear to be safe, although the therapeutic effect is modest at best (Nowbar et al., 2014) and in most cases the observed benefits likely involve paracrine effects on endogenous repair mechanisms rather than exogenous stem cell deployment (Laflamme and Murry, 2011). Thus, there is much to learn about the biology and therapeutic potential of these cells.

Lineage tracing tools for investigating stem cell biology

The investigation of cell lineage involves labeling cells of interest, then tracing the destiny of their progeny. The reliance on the presence of cell markers alone to infer the origin or fate of a cell population is fallible, as marker expression may change as cellular context changes; for example, markers on BM cells can be down-regulated after their relocation to solid organs (Rota et al., 2007; Spees et al., 2008). Evolving techniques for lineage tracking have used "vital" dyes, stable isotopes, radioactive compounds, inter-species chimeras and lineage-specific molecular markers in normal or genetically modified organisms (Steinhauser et al., 2012; Stern and Fraser, 2001). Transgenic animals expressing indelible genetic lineage tracers have allowed the fate of specific cell populations to be followed over very long periods during homeostasis, aging and under various disease conditions such as myocardial infarction (MI). A useful type of surrogate tracing is achieved if a genetic tracer protein, such as GFP, is more stable than the endogenous protein it replaces, the latter often down-regulated during differentiation (Kikuchi et al., 2010; Lepilina et al., 2006).

Genetic CRE-Lox lineage tracing: strengths and limitations

Indelible genetic cell tagging using DNA recombinase-based technology has been the mainstay for investigating progenitor cell origins in the current era. CRE recombinase catalyzes site-specific DNA deletion between short recognition sequences termed *loxP* sites (Branda and Dymecki, 2004). Specificity of cell labeling is achieved by genetically combining a CRE gene cassette, expressed under control of an appropriate cis-regulatory element, and a "reporter" cassette (Fig. 1A). The reporter may be a "two color" system in which *loxP* sites flank one marker gene cassette ("STOP"), which blocks expression of a second marker cassette until the first one is deleted via CRE recombination (Lobe et al., 1999; Novak et al., 2000; Soriano, 1999) Among the several additional transgenic platforms used for lineage tracing are the tetracycline-sensitive Tet-Off/Tet-On systems (Gossen and Bujard, 1992; Jaisser, 2000) (Fig. 1B). In Tet-OFF, tetracycline or an analog (e.g. doxycycline: Dox) inhibits the activity of a tetracycline-sensitive transcriptional activator, tTA, which otherwise activates expression of transgenes via a synthetic tetracycline response cis-regulatory element (*TRE*) positioned upstream of the transgenic cassette. In Tet-On, a mutant version of tTA (rtTA) can only activate the transgene (via the *TRE*) if it is itself bound by a tetracycline.

There are a number of important issues to consider in interpreting CRE-based lineage data. Although CRE-mediated genetic recombination is a binary and permanent event in each cell, it is influenced by the threshold levels of CRE required to induce recombination, which in turn is influenced by the accessibility of CRE to chromatin. As a consequence, different reporter mice vary in their sensitivity to CRE (Ma et al., 2008).

CRE efficacy can also depend on the genetic background of CRE or reporter alleles (Nakamura et al., 2006). Furthermore, transgenes integrate into the host genome approximately randomly and, since most cloned cis-elements are incomplete, they can fall under the influence of endogenous chromosomal regulatory elements that positively or negatively impact the penetrance, expressivity or specificity of transgene expression. In knock-in mice, the insertion site may unintentionally disrupt regulatory elements including intronic enhancers, non-coding RNA binding sites, or domains important for chromosome architecture. Thus, the absence of

Table 1 Proposed adult cardiac-resident stem/progenitor cell populations.

Progenitor cell population	Possible origin	In vitro potency	In vivo fate			References
			Post-transplantation to injury models	Constitutive CRE or surrogate lineage tracing	Inducible CRE-lineage tracing	
ISL1+	SHF (suggested)	Multipotent (CV lineages)	N/A	Embryonic progenitors contribute to CM, SM and ECs.	Fetal ISL1+ cells give rise to CM, SA node, SM, EC in neonatal heart (SHF regions).	Laugwitz et al. (2005); Moretti et al. (2006); Genead et al. (2010)
C-KIT+	Uncertain; observed in early cardiogenic mesoderm (E6.5); also possible blood/ bone marrow origin	Multipotent (CV lineages)	CM, EC, SM	Debated — CRE: EC (>fibroblast and SM); few CM. cKit-BAC-GFP tg or cKit-GFP lentivirus: CM (robust), EC, SM	Induction post-natal — mostly EC cell fate (>fibroblasts and SM); few CM — most result of cell fusion; labeled CM increase after MI; also rare CM with ISO cardiopathy model	Bearzi et al. (2007); Beltrami et al. (2003); Kajstura et al., 2012; Rota et al. (2007); Tang et al. (2010); Jesty et al. (2012); Tallini et al. (2009); Ellsion et al. 2013, van Berlo et al. (2014);
Cardiospheres/ cardiosphere-derived cells	Heterogeneous population; likely cardiac origin without contribution from circulating cells	Multipotent (CV lineages)	CM, EC, SM	N/A	N/A	Chimenti et al. (2010); Malliaras et al. (2012); Davis et al. (2009); White et al. (2011).

SCA1+	Heterogeneous population; origins poorly defined	CM, chondrocytes, osteocytes	CM (most the result of cell fusion)	N/A	SM; Rare CM; possibly also ECs (difficult to score)	Matsuura et al. (2004); Oh et al. (2003); Wang et al. (2006); Uchida et al. (2013)
Side Population	Heterogeneous population; in part BM derived with possible NC contribution	CM	CM, EC, SM (injury); peripheral nervous tissues and cardiac OFT (chick model)	N/A	N/A	Pfister et al. (2005); Oyama et al. (2007); Mouquet et al. (2005); Tomita et al. (2005)
Adult epicardium/ epicardium-derived progenitor cells	Pro-epicardial organ/epicardium	SM,fibroblasts/ myofibroblasts, EC	EC, SM (no CM)	SM, fibroblasts, EC and possibly CM (reflecting fat of embryonic epicardium)	SM,EC, fibroblasts, rare CM	Winter et al. (2007); Zhou et al., 2008a; Smart et al. (2007).
Cardiac CFU-F/MSCs	Pro-epicardial organ/epicardium	Broad mesodermal and trans-germ layer potency	CM, SM, EC (injury); broad range of lineages from different germ layers (teratoma assay)	N/A	N/A	Chong et al., 2011.
Mesoangioblasts	Uncertain: suggested vessel-associated mesodermal progenitors; potential origins from endothelial cells	SM, CM	Fib, CM	N/A	N/A	Galli et al. (2005).

Abbreviations — CM: cardiomyocytes; ES: endothelial cells; ISO: isoproterenol; N/A: not applicable; NC: neural crest; OFT: outflow tract; SHF: second heart field; SM: smooth muscle cells; tg: transgene.

CRE-mediated recombination may not necessarily indicate lack of lineage association, unless other work supports this.

Conversely, CRE expression may be detected in cells other than those whose lineage is being traced due to unexpected (or unrecognized) activation of the driving regulatory elements. Recent lineage tracing studies in the heart using *Tbx18* and *Wt1* CRE drivers suggested that the developing epicardium gives rise to CMs as well as vascular and interstitial cells (Cai et al., 2008; Zhou et al., 2008a). However, these CRE drivers were shown to be either normally expressed in CMs, or expressed unfaithfully in some circumstances (Christoffels et al., 2009; Rudat and Kispert, 2012), demanding careful interpretation of results (Zhou and Pu, 2012).

Inducible forms of CRE have been valuable for gaining additional temporal specificity of CRE recombination and may limit artifacts caused by unwanted activation of CRE. This approach employs fusion proteins composed of CRE linked to either one or two modified estrogen receptor ligand binding domain/s (CRE-ER or merCREmer), making them responsive to synthetic, exogenously-delivered estrogens, such as tamoxifen (Hayashi and McMahon, 2002) (Fig. 1A). The need for an exogenous drug inducer imposes kinetic limitations on the system, often leading to decreased frequency of CRE recombination. Tamoxifen toxicity toward the conceptus (Danielian et al., 1998) is also a limitation.

Cell transplantation as a cell fate mapping tool

In vivo transplantation of progenitor cells has been used commonly for discerning lineage potential and in vivo fate. Using this approach, stem/progenitor cells indelibly labeled using either a dye, virally delivered fluorescent protein, or genetic tag, are transplanted into the organ in which function is to be studied. Importantly, however, this method necessarily removes the population from its natural cellular context and therefore, as for in vitro studies, may instruct how a cellular population *might* behave without necessarily informing on how it actually *does* behave in its natural context. In fact, the outcomes may be different (Lescroart et al., in press; Richardson et al., 2011). Cellular plasticity likely involves permissive genome states that can be readily modified under non-physiological conditions (Zipori, 2004). Cell fusion between host and donor cells after transplantation can also confound the picture of lineage potency (Wagers and Weissman, 2004).

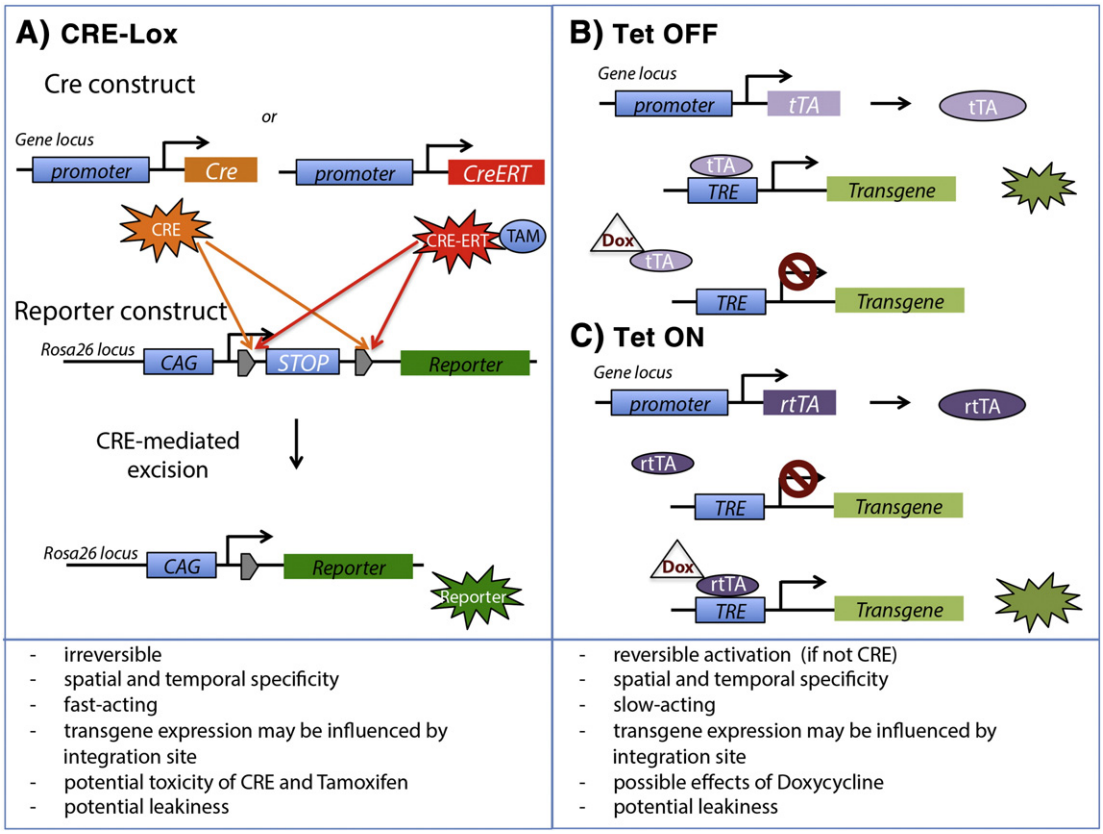


Figure 1 Genetic lineage tracing strategies. A. Schematic representation of the CRE-Lox system consisting of two main elements: a construct in which the gene for CRE or a modified tamoxifen-dependent form (e.g. CreERT) is placed under the control of an appropriate *cis*-regulatory element (promoter) to gain lineage specific expression; a reporter construct from which the expression of a marker protein occurs after CRE-mediated excision of a "STOP" cassette flanked by two LoxP sites. Without CRE, the STOP cassette inhibits expression of the marker. B. TetOFF system: tTA (tetracyclcline-sensitive transcriptional activator) is expressed under the control of an appropriate promoter sequence and activates the expression of a reporter transgene downstream the TRE (Tetracycline Response *cis*-regulatory Element). The expression of the reporter is inhibited in presence of Dox (doxycycline). C. TetON system: the rtTA variant of tTA can bind the TRE and activate the expression of a reporter gene only in presence of Dox. The main advantages and disadvantages of the systems are summarized.

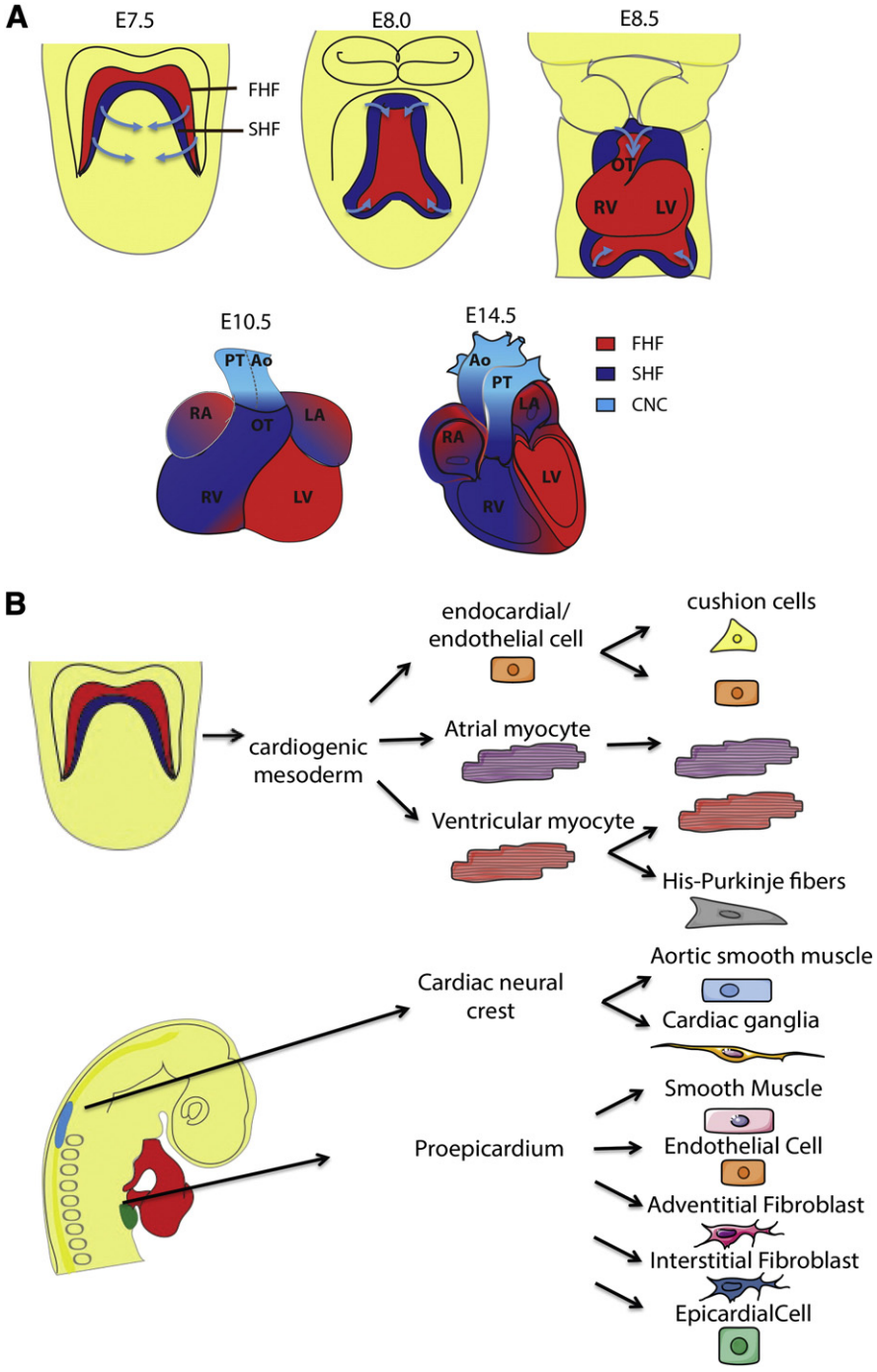


Figure 2 Embryonic cardiogenesis and origin of cardiac cell types. A. Mouse heart development: E7.5 — appearance of mesodermal myocardial progenitors at the anterior margins of the embryo divided in two distinct fields, the "first heart field" (FHF, red) and the "second heart field" (SHF, blue). E8 — the FHF undergoes morphogenesis to form the early tubular heart. By E8.5, the heart tube has begun looping and cells from the SHF migrate into the heart from the inflow and outflow poles (arrows). E10.5 — convergence of the inflow and outflow poles; neural crest progenitors (CNC, light blue) migrate to the heart contributing to the outflow tract, atrioventricular septum, cardiac valves and ganglia. E14.5 — the four chambers are well defined and septation of the outflow tract is observed. Color shades represent the contributions of the FHF, SHF, CNC to different regions of the heart.; Ao, aorta; LA, left atria; LV, left ventricle; OT outflow tract; PT, pulmonary trunk RA, right atria; RV, right ventricle. B. Different cardiac cell types originating from cardiogenic mesoderm (top panel); extra-cardiac lineages cardiac neural crest and pro-epicardium contribute to the heart and coronary vessels (bottom panel).

Clonal readout of lineage tracing

In the stem cell field, it is vital to probe stem and progenitor cell potency at a single cell level. For example, the epicardium is often regarded as a multipotent cardiac progenitor cell population because population-based lineage tracing studies have ascribed multi-lineage outputs (see below). However, the few studies that have addressed potency at a clonal

level suggest that epicardium is in fact a highly heterogeneous population of mostly unipotent progenitors (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996). Various methods allow clonal analysis of lineage progeny, including homotypic or ectopic transplantation of individual genetically-tagged cells, often in the presence of untagged (helper) cells (C.K. Chan et al., 2013) that promote a "community effect" (Gurdon, 1988). Conditional or retrospective CRE lineage tracing strategies can also be used for tracking lineage fate at a single cell level when conditions favor recombination in only rare cells (Meilhac et al., 2004). A recent important development has been the use of multi-color CRE lineage tagging, whereby recombination is induced in reporter strains bearing multiple CRE-dependent cassettes with reporter readouts of different colors. Different progenitor cells, even those in close proximity, will have a very high probability of undergoing a different CRE-mediated recombination event, leading to different colored markers being expressed, thus enabling their progeny to be individually tracked (Gupta and Poss, 2012; Livet et al., 2007; Snippert et al., 2010; Uchida et al., 2013).

Diverse origins for cardiac progenitors in development

Understanding the progenitors that shape the heart in development (Moorman and Christoffels, 2003; Vincent and Buckingham, 2010) is necessary to appreciate the recent findings on the characteristics and origins of CPCs within the adult heart.

A progenitor population termed the "first heart field" (FHF) contributes to the formation of a crescent-shaped epithelium of splanchnic mesoderm at the anterior–lateral boundaries of the early embryo (Fig. 2A). The crescent begins to express cardiac sarcomeric genes and undergoes morphogenesis to form the early tubular heart, which serves as a scaffold for continuing growth. Progenitor cells of the "second heart field" (SHF) are deployed at both the inflow and outflow portals of the primary heart tube and contribute almost exclusively to the right ventricle myocardium and endocardium, as well as outflow tract myocardium, endocardium and smooth muscle (Buckingham et al., 2005) (Fig. 2A). They also contribute extensively to the atria, inflow vessel and nodal elements. Lineage tracing suggests that FHF and SHF cells arise from a common progenitor but become geographically separated during gastrulation (Devine et al., submitted for publication; Lescroart et al., in press; Meilhac et al., 2004).

Neural crest progenitor cells are known to contribute to the outflow tract, atrioventricular septum, cardiac valves and ganglia of the developing heart (Creazzo et al., 1998; Nakamura et al., 2006) (Fig. 2B). Additional progenitors are contributed from the proepicardium (PE; Fig. 2B), a convoluted epithelial organoid with a mesenchymal core that comes to be situated adjacent to the atrioventricular canal of the forming heart (Asli and Harvey, 2013). It has its origins in the early cardiac fields (Lescroart et al., in press; Zhou et al., 2008b). The PE seeds progenitors that spread over the myocardium to create the epicardium. A subset of these cells, termed epicardial-derived cells (EPDCs), enter the sub-epicardial matrix and heart interstitium and differentiate into the mural elements of the coronary vasculature, as well as cardiac fibroblasts, mesenchymal stem/progenitor cells and possibly myocardium (Asli et al., 2014; Carmona et al., 2010; Winter and Gittenberger-de Groot, 2007).

Markers that distinguish cells within the cardiac progenitor fields have been extensively explored, although few if any definitively define these populations. For example, TFs *Mesp1* and *Mesp2* are expressed in the anterior primitive streak, then in an anterior embryonic territory that contains progenitors of the cardiac lineages, as well as other lineages (Bondue et al., 2008; S.S. Chan et al., 2013; Kitajima et al., 2000; Saga et al., 1999). Likewise, TFs associated with myocardial development, such as ISL1, NKX2-5, GATA4 and MEF2C, are expressed in the first and second heart progenitor fields as well as at different phases of epicardial, endocardial and/or neural crest development (Engleka et al., 2012; Mommersteeg et al., 2010; Nakano et al., 2013; Peng et al., 2013; Watt et al., 2004; Zhou et al., 2008a).

The identification of progenitor populations in the adult heart adds increased complexity to heart development, and raises many interesting questions – for example, are fetal or adult CPCs remnants of earlier embryonic progenitors, have they migrated in from other sources, or have they arisen de novo after dedifferentiation of CMs or other more differentiated cells? A fascinating possibility is that stem cell states can exist in latent form, to be stimulated under certain conditions (Asli and Harvey, 2013). Indeed, the concept of "facultative" stem cells, which are thought to arise from more differentiated cells under non-physiological conditions, is gaining traction (Ziv et al., 2013). Fate mapping strategies have been crucial for developing our current understanding of embryonic progenitors and will continue to be important for forging our views on newly identified adult CPC populations.

Diversity of adult cardiovascular lineages

CMs, stromal fibroblasts, vascular and lymphatic endothelial, mural and adventitial cells, myofibroblasts, neurons and immune cells comprise the major cell types of the heart. The relative proportions of these cells vary significantly throughout development and between species. The adult mouse heart is thought to comprise ~56% CMs, 27% fibroblasts, 7% endothelial cells and 10% vascular smooth muscle cells (Banerjee et al., 2007).

Cardiac stromal cells, generically termed *fibroblasts*, remain one of the most poorly characterized cell types in the heart (Davis and Molkentin, 2014). The distinction between fibroblast subtypes based on markers expression, physiologic function or origin is not well advanced (Krenning et al., 2010). The same applies to perivascular cell compartments, which include the autonomic nerves (Hogan and Bautch, 2004; Majesky, 2007; Majesky et al., 2011). There are many studies over the previous decades suggesting multi-lineage potency within the pericyte population and mesenchymal stem/progenitor cell activity within BM and other organs has been claimed to lie within the pericyte fraction (Chen et al., 2013). The vascular adventitia has been proposed to provide a niche for stem/progenitor cells that contribute to more mature vascular cell types (Campagnolo et al., 2010; Chong et al., 2011; Passman et al., 2008). Vascular ECs may also be subdivided based on function; for example, the endocardium

and coronary vessels are thought to harbor or nurture cells that give rise to blood cells (Nakano et al., 2013; Zape and Zovein, 2011) and mesenchymal stem-like cells (Azzoni et al., 2014). Stem cell-like plasticity has also been suggested for coronary endothelium (Bearzi et al., 2007; Zheng et al., 2007).

We are yet to completely understand the complex lineage relationships of the heart, although this surely remains one of the cardinal aims of the cardiac stem cell field.

Lineage tracing and cardiac physiological turnover

As extensively reviewed elsewhere in this Special Issue of *Stem Cell Research*, the long-standing dogma of the mammalian heart as a terminally differentiated organ with little regenerative reserve has been revised over the past decades. Despite poor regenerative capacity after ischemic injury, numerous studies now show a certain degree of CM turnover in adult mammalian hearts, albeit the extent of turnover and the origin of physiological cell replacement are still highly debated topics. The estimated homeostatic CM turnover ranges from 1 to 4% (Bergmann et al., 2009; Malliaras et al., 2013b; Senyo et al., 2013) to over 40% per year (Kajstura et al., 2010), and both proliferative CMs (Bersell et al., 2009; Malliaras et al., 2013b; Senyo et al., 2013) and cardiac-resident adult progenitor cells (Anversa et al., 2013; Ellison et al., 2013; Uchida et al., 2013; van Berlo et al., 2014) have been cited as the source of regenerative reserve. Needless to say, the degree and mechanisms of heart regeneration in mammals and other species are under intense scrutiny, as is the potential for awakening lost regenerative potential.

Proposed resident adult cardiac progenitor cell populations

One of the first reports of a putative cardiac stem cell/progenitor population came from the analysis of human cardiac transplants in which there was a sex mismatch between donor and host (Quaini et al., 2002). The authors described "primitive" cells expressing stem cell-associated cell surface proteins such as C-KIT, MDR-1 or SCA1. Since then, a variety of cell populations that possess one or more characteristic found in other stem cell populations (long-term self renewal, clonogenicity, multipotency) have been identified in adult hearts of mammalian species, including humans.

Isl1$^+$ cardiac progenitors

ISL1 is a TF of the LIM-homeodomain family that is expressed in early cardiac progenitor cells, with transcription from the *Isl1* locus down-regulated as progenitors differentiate. *Isl1* expression has often been cited as a marker of the SHF in development (Cai et al., 2003), although it is also expressed in FHF cells, as well as numerous other lineages (Castagnaro et al., 2013; Engleka et al., 2012; Ma et al., 2008; Peng et al., 2013; Prall et al., 2007; Zhou et al., 2008b). Genetic studies show that ISL1 is important for survival, proliferation and migration of early cardiac progenitors (Laugwitz et al., 2008). CRE-Lox lineage tracing has demonstrated that cardiac ISL1$^+$ progenitors contribute to a number of cardiac lineages (Laugwitz et al., 2005; Moretti et al., 2006) and in vitro differentiation assays suggested that some progenitors may be multipotent (Bu et al., 2009; Laugwitz et al., 2005; Moretti et al., 2006), although this has not yet been demonstrated in vivo.

Rare ISL1-expressing undifferentiated cells were found within the myocardial layer of mouse, rat and human hearts, with a distribution that suggested an origin within the SHF (Genead et al., 2010; Laugwitz et al., 2005). Relatively more cells were found in human hearts in late gestation and postnatally, proposed to be involved in the greater expansion of cardiac mass in human relative to rodent hearts (Bu et al., 2009). Conditional lineage tracing, performed in mice harboring a tamoxifen-inducible CRE cassette introduced into the *Isl1* locus, showed that ISL1-expressing cells present in the fetal heart gave rise as a population to some CM in the postnatal heart in a distribution consistent with a SHF origin, as well as sinoatrial node, and SM and ECs of the proximal aorta and pulmonary trunk (Laugwitz et al., 2005; Moretti et al., 2006). Further studies showed that ISL1$^+$ CPCs can be maintained and/or expanded in undifferentiated state during in vitro culture in the presence of co-isolated total cardiac mesenchymal fraction, and were able to differentiate into CMs, a rare subset of which generated action potentials similar to neonatal CMs (Laugwitz et al., 2005), and to SM cells (Moretti et al., 2006). Such in vitro progenitors expressed TF genes *Isl1*, *Nkx2-5* and *Gata4*, indicative of a cardiac identity, but did not express *c-Kit* or *Sca-1*, or a Side-Population phenotype, characteristics of other adult CPC populations (see below) (Laugwitz et al., 2005). Mouse and human embryonic stem (ES) cells harboring *Isl1* reporter genes have been used to isolate and expand ISL1-expressing cells (Bu et al., 2009; Moretti et al., 2006; Qyang et al., 2007). These express cardiac TFs NKX2-5, GATA4 and TBX20, as well as VEGF receptor and the EC surface protein CD31, and some 12% of clonally isolated cells showed multi-CV lineage differentiation (Moretti et al., 2006).

In summary, a SHF origin for postnatal ISL1$^+$ cells would appear likely, although this is not formally proven (Laugwitz et al., 2005). Their relative scarcity in the adult heart, their largely non-proliferative state in the postnatal period, even when CMs are still dividing (Genead et al., 2010), and their lack of substantial expansion in the human heart after MI with only focal expansion after ischemia–reperfusion (Genead et al., 2012), casts doubt on whether they could have any role in cardiac repair and regeneration in the adult. Cardiac *Isl1* expression in adults is mainly confined to the sinoatrial node (Weinberger et al., 2012).

C-KIT$^+$ cardiac progenitors in the adult

Anversa and colleagues explored whether there were circulating stem cells capable of homing to the heart and differentiating into cardiac lineages, concentrating on cells expressing the tyrosine kinase receptor C-KIT, a known marker of hematopoietic stem cells (Kondo et al., 2003; Morrison et al., 1997; Weissman et al., 2001). In a highly influential paper, flow-sorted BM-derived lineage-negative (LIN$^-$) c-KIT$^+$ cells were found to restore myocardium when injected into the boarder zone after MI surgery (Orlic et al., 2001a). Some 68% of CMs within the infarct zone were of exogenous origin. BM cells mobilized to the peripheral circulation with cytokines

were also found to home to infarcted hearts and differentiate into CMs (Orlic et al., 2001b). The premise of these papers – that hemopoietic cells can trans-differentiate into CMs after cell therapy procedures or peripheral mobilization – was emphatically contested (Balsam et al., 2004; Murry et al., 2004; Nygren et al., 2004) and then equally emphatically defended through additional experimental validation (Anversa et al., 2013; Dawn et al., 2006; Kajstura et al., 2005; Rota et al., 2007). In the first of the subsequent studies, Kajstura et al. found 4.5 million newly differentiated CMs formed in the absence of cell fusion after injection of C-KIT$^+$ BM cells into the infarct border zone of mice (Kajstura et al., 2005). Other groups have reported differentiation of BM or blood-derived cell fractions into CMs in vitro or after transfer to an infarct bed, although only at very low levels (Badorff et al., 2003; Xaymardan et al., 2009; Yoon et al., 2010). At the present time, this highly contentious issue remains unresolved, as the majority of work in the area has shifted toward the C-KIT$^+$ cardiac-resident population.

In a landmark study, a rare population of LIN$^-$ C-KIT$^+$ cells was identified in young adult rat hearts (present at ~1 per 10^4 myocytes), sometimes organized within interstitial niches consisting of clusters of undifferentiated as well as primitive CM marker-positive cells (Beltrami et al., 2003) (Fig. 2B). Some of these cells were clonogenic and self-renewing in culture, and were shown to differentiate into ECs, SM cells and immature CMs in vitro. Cardiac-resident C-KIT$^+$ cells have been shown to have other stem cell-like properties, including long-term retention of nucleotide analogs and proliferation in vivo (Urbanek et al., 2006), long-term growth in culture (Beltrami et al., 2003), ability to undergo asymmetric cell divisions giving rise to one daughter CPC and one lineage committed cell through differential regulation of NOTCH (Boni et al., 2008; Urbanek et al., 2006), and biased DNA segregation (Kajstura et al., 2012). After transfer to infarcted hearts, C-KIT$^+$ cells contributed to a cardiac regenerative response involving survival and expansion of injected cells, and differentiation into large swathes of CMs as well as SM and ECs within the infarct zone (Beltrami et al., 2003). C-KIT$^+$ cells with similar properties have been isolated from the hearts of dogs (Linke et al., 2005), mice (Urbanek et al., 2005) and humans (Bearzi et al., 2007), and cell therapy procedures after ischemic injury result in salutary effects on cardiac repair and functional recovery (Anversa et al., 2013), providing a rationale for their first use in cell therapies in humans (Bolli et al., 2011).

Origin of C-KIT$^+$ progenitors. Tracking the origins and fate of C-KIT$^+$ CPCs in vivo is complicated by a number of factors. Firstly, studies have used different C-KIT antibodies often with unproven specificity. C-KIT expression has also been reported in neonatal and dedifferentiating adult CMs (Kubin et al., 2011; Li et al., 2008; Tallini et al., 2009; Zhang et al., 2010)Li et al., 2008), coronary endothelial cells (Bearzi et al., 2009), and epicardial cells (Castaldo et al., 2008; Limana et al., 2007; Tallini et al., 2009). Thus, C-KIT is not a marker of cell lineage. Even though cardiac-resident C-KIT$^+$ CPCs were described as LIN$^-$ (Beltrami et al., 2003; Ellison et al., 2013), this does not exclude a BM origin (Rota et al., 2007; Spees et al., 2008). As in other solid organs, the heart contains a population of resident C-KIT$^+$, CD45$^+$, Tryptase$^+$ mast cells (Kubo et al., 2008; Patella et al., 1998; Pouly et al., 2008) and in the injured heart there is a significant influx of additional C-KIT$^+$ cells from BM (Chong et al., 2011; Fazel et al., 2006). These cells are thought to provide a pro-angiogenic cytokine milieu and are essential for cardiac reparative processes (Fazel et al., 2006).

Cell surface C-KIT antigen appears to mark a sub-population of cardiogenic progenitors in mammalian embryos prior to and after heart tube formation (Wu et al., 2006) and in differentiating ES cell cultures (Christoforou et al., 2008; Wu et al., 2006). Using a *c-Kit* promoter-*GFP* transgene, which marks most C-KIT immune-positive cells in E18.5 mice, Ferreira-Martins et al. identified GFP$^+$ and C-KIT$^+$ cells in a region of the early mesoderm at ~E6.5 that may overlap with cardiogenic mesoderm (Fig. 3A), and subsequently within the growing heart tube (Ferreira-Martins et al., 2012). A related study using a distinct bacterial artificial chromosome (BAC)-based *c-kit*-GFP transgene also found clusters of GFP$^+$/C-KIT$^+$ cells in fetal and neonatal hearts at various stages of immaturity and differentiation, some of them capable of clonal growth and multipotency in vitro (Tallini et al., 2009). Fetal and neonatal *c-Kit*-GFP$^+$ cells were negative for markers of blood cells and mesenchymal stem cells (MSCs), and mostly negative for CM and SM TFs (Ferreira-Martins et al., 2012). However, these early C-KIT$^+$ cells have not been well characterized. Some were capable of clonal propagation in vitro, after which they had properties identical to similarly expanded adult C-KIT$^+$ cells, including an ability to augment repair of infarcted adult myocardium after injection into the MI border zone (Ferreira-Martins et al., 2012). Based on these properties, the authors proposed a major role for c-KIT$^+$ CPCs in myocardial growth during embryonic and fetal development (see Lineage tracing to determine the fate of C-KIT$^+$ cells in vivo section), although this idea has not been further developed.

Lineage tracing of engrafted BM carrying both a *c-kit*-GFP BAC transgene and another transgene constitutively expressing dsRed (a red-fluorescent protein) in all engrafted cells, has recently been performed (Jesty et al., 2012). Many dsRed$^+$ BM cells entered the heart after infarction, although none of these were also *ckit*-GFP$^+$. The finding suggested that C-KIT$^+$ cells present in the heart after MI do not have a BM origin, although results are not consistent with other studies showing a florid influx of C-KIT$^+$ cells from BM after MI (Chong et al., 2011; Fazel et al., 2006), perhaps revealing the limitations of the *c-kit*-GFP transgene.

Taken at face value, the above studies provide suggestive evidence that adult cardiac-resident LIN$^-$ C-KIT$^+$ cells arise from self-maintenance of a population of progenitors present in early cardiogeneic mesoderm. However, this is far from proven. The studies are potentially limited by the surrogate nature of lineage tracing methods and uncertain expression characteristics of *ckit* transgenes (Molkentin and Houser, 2013). It is not until the models are applied that conditionally label C-KIT$^+$ cardiac progenitors in the gastrula and trace their progeny to multipotent C-KIT$^+$ cells in post-natal and adult hearts, can this be formally established. Since C-KIT is not a lineage marker, there remains the possibility that C-KIT$^+$ CPCs in developing and adult hearts are distinct.

In vitro and in vivo lineage potential of C-KIT$^+$ CPCs. As noted, in vitro-expanded rat or human C-KIT$^+$ CPC can differentiate into cells expressing markers of CMs, ECs and

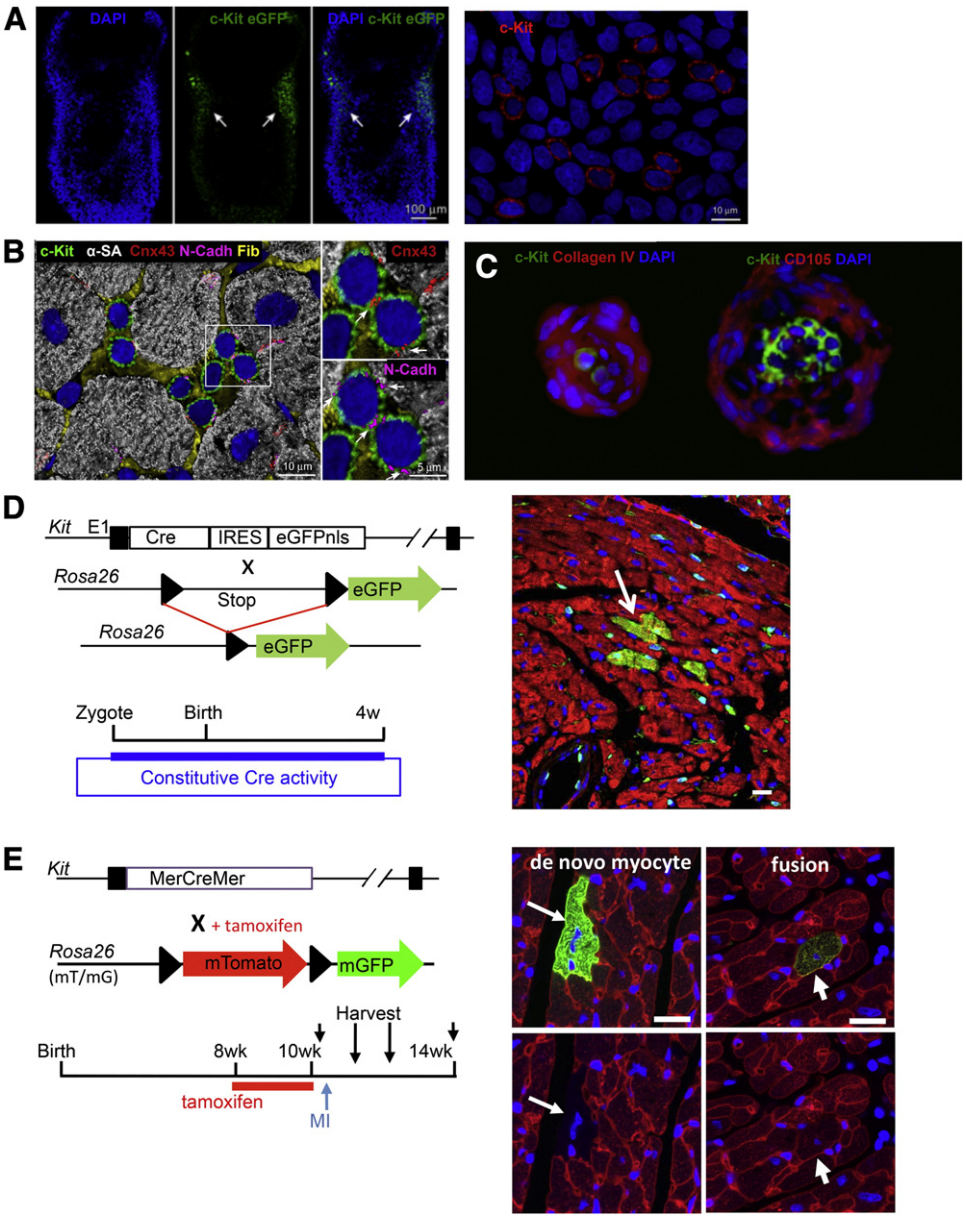


Figure 3 C-KIT$^+$ progenitor cells. A. Representative images showing C-KIT$^+$ cells in a E6.5 *c-Kit*-GFP embryo identified by GFP fluorescence (left panel) or antibody staining (right panel) in a region that may overlap with cardiogenic mesoderm. Republished with permission of Lippincott Williams and Wilkins from Ferreira-Martins et al. (2012); permission conveyed through Copyright Clearance Centre, Inc. B. Cluster of human C-KIT$^+$ cells in a presumed *niche* expressing Connexin-43 (Cnx43, red), N-Cadherin (N-Cadh, purple), surrounded by Fibronectin (Fib, yellow) in the interstitial space, among cardiomyocytes positive for α-sarcomeric actin (α-SA, white). Republished with permission of American Society for Clinical Investigation from Anversa et al. (2013); permission conveyed through Copyright Clearance Centre, Inc. C. Representative stainings of human cardiospheres, showing c-KIT$^+$ cells in the center (green) surrounded by stromal/supporting cells positive for CD105 (red, left panel) and extracellular matrix positive for CollagenIV (red, right panel). Republished with permission of John Wiley & Sons from Li et al. (2010); permission conveyed through Copyright Clearance Centre, Inc. D. *c-Kit*-CRE lineage tracing. A mouse carrying a bicistronic CRE-IRES-nuclear GFP (eGFPnls) cassette knocked in to the *cKit* locus is crossed with a CRE-dependent cytoplasmic GFP reporter strain. Only rare cardiomyocytes are tagged with cytoplasmic GFP at 4 weeks (right panel). Republished with permission of Nature Publishing Group from van Berlo et al. (2014); permission conveyed through Copyright Clearance Centre, Inc. E. Conditional lineage tracing using a *c-Kit* –MerCreMer knock-in mouse crossed to the mT/mG reporter line constitutively expressing membrane Tomato or membrane GFP after CRE deletion. Eight-week old mice were treated with Tamoxifen for two weeks, subjected to MI after three days and sacrificed after two more weeks for analysis. Approximately 0.01% of cardiomyocytes were mGFP positive, 80–88% of which retained membrane Tomato labeling, indicating that they result from fusion of GFP$^+$ cells with cardiomyocytes (right panels). Republished with permission of Nature Publishing Group from van Berlo et al. (2014); permission conveyed through Copyright Clearance Centre, Inc.

SM cells when exposed to defined growth and differentiation factors or after co-culture with neonatal CMs (Bearzi et al., 2007; Kubo et al., 2008; Miyamoto et al., 2010; Tallini et al., 2009; Zaruba et al., 2010). Zaruba et al. tested the lineage potential of freshly-sorted (non-expanded) C-KIT$^+$ cells in vitro after co-culture with neonatal rat CMs as a function of age and found only a low-level of CM differentiation in neonatal preparations (2.4% of cultured cells), while negligible differentiation was seen in adult preparations (Zaruba et al., 2010). These studies suggest that among neonatal C-KIT$^+$ population only few cells have CM differentiation potential. While this property is even rarer among adult C-KIT$^+$ cells, it is likely that it could be selected or enhanced by clonal isolation and in vitro expansion, consistent with findings that populations of self-renewing C-KIT$^+$ cells can be readily isolated from adult human biopsies (Bolli et al., 2011), even those from octogenarians (Kajstura et al., 2012). Indeed, multi-CV lineage differentiation of in vitro-expanded adult rat or human GFP-tagged C-KIT$^+$ CPCs has also been seen after direct injection or intracoronary infusion into an MI border zone in animal models (Bearzi et al., 2007; Beltrami et al., 2003; Kajstura et al., 2012; Rota et al., 2007; Tang et al., 2010) and their fate can be swayed toward vascular lineages by prior culture in insulin-like growth factor 1 and hepatocyte growth factor (Tillmanns et al., 2008).

Lineage tracing to determine the fate of C-KIT$^+$ cells in vivo. Surrogate lineage tracing experiments using the *c-kit*-GFP BAC transgenic mice mentioned above, have been performed to determine the fate of C-KIT$^+$ cells in neonate and adult mice (Jesty et al., 2012; Tallini et al., 2009). In cryo-infarcted neonatal hearts, numerous GFP$^+$ CMs as well as ECs and SM cells were seen in the infarct zone at 21 days, suggesting that C-KIT$^+$ cells participate in the neonatal regenerative response. Some 42% of BrdU$^+$ CMs in the infarct zone were C-KIT$^-$ GFP$^+$ at day 5 post-infarction. This contrasts with previous work reporting that CM replacement during postnatal heart regeneration after ischemic injury arises predominantly from dedifferentiation and proliferation of existing CMs (Mahmoud et al., 2014; Porrello et al., 2011). When *c-kit*-GFP$^+$ cells negative for expression of an *αMHC*-mCherry transgene were purified from neonates 6 h after infarction, they activated the mCherry reporter and formed beating CMs after cultured in differentiation medium (Uchida et al., 2013). Further indirect lineage tracing studies in neonatal mice suggested that about one third of new CMs in the infarct zone at 5 days post-infarction were of non-CM origin. However, when the above surrogate lineage tracing methods were applied to adult infarcted hearts, C-KIT$^+$ cells gave rise only to vascular cells but not CMs.

In a recent detailed study, Ellison et al. explored the behavior of putative C-KIT$^+$ CPCs in rat and mouse models of diffuse (Takotsubo-like) myocardial damage induced by a single dose of isoproterenol (ISO) (Ellison et al., 2013). The model is associated with death of 8–10% of ventricular CMs in the presence of a patent coronary circulation, and potentially has less impact on cardiac-resident CPCs than ischemic myocardial infarction, with the damage reversed spontaneously and completely without fibrosis by 28 days. The response may involve both a regenerative component as well as reversal of myocardial metabolic stunning (Molkentin and Houser, 2013; Shao et al., 2013). Ellison et al. document the presence and expansion of small C-KIT$^+$ BrdU$^+$ or C-KIT$^+$ Ki67$^+$ cells that, with time, acquired expression of cardiac TFs such as NKX2-5 and GATA4, and markers of CM differentiation and sarcomere assembly. The immature CMs increased in size and integrated into spared myocardium. In the first of two lineage-tracing experiments, tamoxifen-dependent *αMHC*-MerCREMer mice were crossed with *Z/EG* CRE-dependent reporter mice providing readout of myogenic status (expressing LACZ in the absence of CRE; GFP in its presence). When tamoxifen was delivered for a short window before ISO-induced CM damage, immature C-KIT$^+$ BrdU$^+$ CMs were LACZ$^+$ GFP$^-$, suggesting that they did not arise from existing CMs. The assumption here is that the C-KIT$^+$ cells are CPCs and not dedifferentiated CMs. A remaining worry is that founder cells might express *αMHC*-MerCREMer at sub-threshold levels with respect to CRE activity. In a second surrogate lineage tracing approach, a lentivirus carrying *ckit* promoter elements driving CRE expression was deliver to the apical myocardium of ISO-treated and control mice carrying a CRE reporter which expresses yellow fluorescent protein (YFP) in the presence of active CRE. In ISO-treated mice, 73% of newly formed apical BrdU$^+$ CMs were YFP$^+$, compared to $<$0.06% in ISO-naïve controls. Although concerns have been raised about the proper regulation of truncated *ckit* cis-regulatory elements (Cairns et al., 2003; Molkentin and Houser, 2013), and notwithstanding concerns about CRE thresholds and possible C-KIT expression during CM dedifferentiation (Kubin et al., 2011), the sum of data support the view that in the ISO model a C-KIT$^+$ CPC population of non-CM origin survives the injury, proliferates, and differentiates into functional CMs.

Remarkably, this conclusion was not supported by the first formal genetic lineage tracing study of cardiac C-KIT$^+$ cells (van Berlo et al., 2014). Using a mouse strain carrying a bicistronic CRE-IRES-nuclear GFP cassette inserted into the *ckit* locus, nuclear GFP was found in ~76% of the non-CM fraction of the heart at 4–12 weeks of age, but not in CMs. When mice with this CRE allele were crossed with a CRE-dependent cytoplasmic GFP (cGFP) reporter strain, most C-KIT antibody-positive cells in the heart were also cGFP$^+$. Using this constitutive genetic lineage tracing system, only rare CMs were tagged at birth or at 4 weeks (0.027% of all CMs were cGFP$^+$) (Fig. 3D). Most GFP tagged cells were ECs (77% of all cGFP$^+$ cells), while fibroblast and SMs were tagged only rarely. van Berlo et al. also devised a conditional lineage tracing scheme in which a tamoxifen-dependent merCREmer cassette was knocked-in to the *ckit* locus and these mice were crossed to different GFP reporters (Fig. 3E). When tamoxifen was delivered postnatally, only rare CMs were marked with GFP (0.0055%). This frequency increased 4-fold after MI, irrespective of whether tamoxifen was delivered before or across the time of MI surgery, with most GFP$^+$ CMs located in the boarder zone. Only rare CMs were traced in the ISO model of diffuse cardiomyopathy discussed above. In the context of MI, most GFP$^+$ CMs arose as a result of fusion of C-KIT$^+$ CPCs to existing CMs.

This study confirms that some C-KIT$^+$ cells represent lineage progenitors, albeit that their ability to form CMs at any time in development or postnatal life, and after injury, is extremely limited and most likely functionally trivial. The dominant potency is toward ECs. However, the results are not easily reconciled with previous genetic and surrogate

lineage tracing studies reporting abundant CM formation from C-KIT$^+$ cells after injury in the neonate and adult (Ellison et al., 2013; Jesty et al., 2012; Leri et al., 2011). They are also incompatible with reports of C-KIT expression in neonatal CMs and function in their terminal differentiation (Li et al., 2008), and expression of C-KIT in dedifferentiating CMs (Kubin et al., 2011; Tallini et al., 2009; Zhang et al., 2010). In future studies, resolving this rift will require the application of the most rigorous standards to confirm C-KIT antibody specificity, validate the integrity of expression from *c-kit*-GFP transgenic and knock-in alleles, and confirm the ability of CRE in transgenic and knock-in strategies to reach thresholds that capture all functionally relevant cells that are C-KIT$^+$ or positive for sarcomeric markers such as αMHC (Molkentin and Houser, 2013; Nadal-Ginard et al., 2014). These findings have key relevance to our understanding of the biology of CPCs and for the initial and ongoing rationale for C-KIT$^+$ CPC human clinical trials (Bolli et al., 2011).

Cardiosphere-derived progenitors

Messina et al. first described the in vitro isolation of a cardiac-derived phase bright cell population which could be further cultured as 3D clusters termed "cardiospheres" (CSs) (Messina et al., 2004). Such spheres are heterogeneous clusters thought to recapitulate a *niche*-like microenvironment for CPCs (Li et al., 2010; Smith et al., 2007). Cells expressing c-KIT reside at the core, while more differentiated stromal/supporting cells occupy the peripheries and these have mixed expression of SCA1 and CD34, and markers of the three main CV lineages (Li et al., 2010; Messina et al., 2004) (Fig. 3C). When injected into murine, rat or porcine MI models, CS-derived cells (CDCs) improve cardiac function (Malliaras et al., 2012, 2013a; Messina et al., 2004). Human CDCs consistently expressed C-KIT and MSC markers CD105 and CD90 (Davis et al., 2009; Smith et al., 2007) and could be stimulated to form contractile CMs by co-culture with neonatal rat CMs (Smith et al., 2007). LACZ-labeled human CDCs transplanted in immune-compromised mice after MI resulted in occasional donor derived CMs and ECs and a global improvement in LV function. CDC therapy also enhanced the expansion of small TROPONIN T$^+$ cells thought to be immature cardioblasts (Malliaras et al., 2014a; Xie et al., 2014). In a comparative study, human CDCs were shown to be superior to other adult extra-cardiac cell populations (BM-mononuclear cells, BM-MSCs, adipose-derived MSCs and C-KIT$^+$ cells) with respect to their ability to stimulate angiogenesis, CM proliferation, ischemic tissue preservation and functional benefit (Li et al., 2012), and the CD90$^-$ sub-fraction had the highest therapeutic efficacy (Gago-Lopez et al., 2014).

The CS concept has led to a novel cardiac autologous cell therapy with the results of a Phase I clinical trial and 1 year follow-up on a small number of patients recently reported (Makkar et al., 2012; Malliaras et al., 2014b). Intra-coronary delivery of human autologous CDCs to patients with recent MI appears to be safe, decreases scar size and increases regional cardiac function.

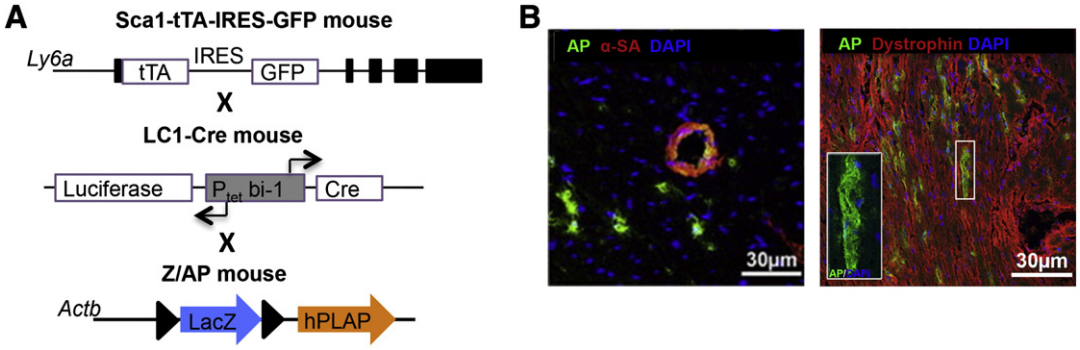
The origins and fate of CDCs. The identity, origin and fate of the therapeutic component of CDCs have been difficult to determine given the method of isolation and heterogeneous composition. Furthermore, while CSs appear to harbor cells that are clonogenic and capable of limited self-renewal (Davis et al., 2010), and are able to contribute to CV lineages in vivo (Chimenti et al., 2010), the mechanism of benefit in cell therapy is likely indirect, with few cells surviving after 3 weeks in the rat model (Chimenti et al., 2010; Malliaras et al., 2012). Controversies have arisen over whether CDCs harbor progenitor cells at all, and their origins and fate (Andersen et al., 2009; Davis et al., 2009; Shenje et al., 2008; Ye et al., 2013). One study using CRE genetic fate mapping methods concluded that explant-derived cells (first stage of CS isolation) were phagocytic, had a non-CM (possibly blood-born) origin, and had no spontaneous cardiomyogenic activity (Shenje et al., 2008). The possible blood origin was not supported by subsequent studies (Davis et al., 2009; Ye et al., 2013).

Davis et al. also devised a lineage tracing strategy to explore possible CM origins of CSs (Davis et al., 2009). Here, explant-derived cells were generated from tamoxifen-treated adult *MHCα*-merCREmer x Z/EG transgenic mice, but these were exclusively GFP$^-$, including the ~6.5% that were C-KIT$^+$. Recently, human recipients of sex-mismatched cardiac transplants were used to show that CDCs were exclusively of cardiac donor origin (White et al., 2011). Another recent study showed that TGFβ-mediated epithelial-to-mesenchymal transition (EMT) accompanied by up-regulation of WT1 (an epicardial TF) is required for CS formation (Forte et al., 2012). WT1 is essential for epicardial EMT during heart development (von Gise et al., 2011) and is re-expressed in adult epicardium after ischemic injury (Zhou et al., 2011). Thus, at least one component of CSs may be of epicardial origin.

In summary, it appears that CSs have a cardiac but non-CM origin. An attractive hypothesis is that CSs are composed of C-KIT$^+$ CPCs and proliferative supporting cells derived from the endogenous cardiac MSC lineage, which is known to arise from the epicardium (Asli et al., 2014; Chong et al., 2011).

SCA1$^+$ cardiac progenitors

Stem cell antigen-1 (SCA-1) is an 18-kDa mouse GPI-anchored cell surface protein and one of the most common markers used to enrich adult murine HSCs (Spangrude et al., 1988). Although a *SCA1* gene is not present in humans, there are related genes of the *LY6* family involved in cell adhesion, migration, and ECM degradation. In mice, SCA1 is expressed in the microvasculature as well as in CPC populations (Chong et al., 2011; Linke et al., 2005), including some C-KIT$^+$ cells (Bailey et al., 2012; Linke et al., 2005). In 2003, Oh et al. used this marker to identify a subset of murine interstitial cells, approximately 14% of the non-CM population, that contained cells co-expressing early cardiac-specific TF genes *Gata4*, *Mef2c* and *Tef-1*, but lacked cells expressing most endothelial or blood lineage markers (Oh et al., 2003). A small percentage of cells underwent differentiation in vitro into CM, chondrocytes and osteocytes (Matsuura et al., 2004; Oh et al., 2003; Wang et al., 2006), and in a mouse model of myocardial ischemia–reperfusion, some cells of the SCA1$^+$ fraction administered intravenously homed to the site of injury, engrafted and formed abundant new CMs, although 50% of these were the product of cell fusion (Oh et al., 2003). Direct injection of SCA1$^+$ CD31$^-$ cells into MI hearts also improved cardiac function albeit likely through paracrine effect (Wang et al., 2006).



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Lineage tagging	% of AP+ cells		
	EC	SMC	CM
Before Birth	77.46	8.39	4.21
After Birth	68.77	12.78	8.76

Figure 4 SCA1$^+$ progenitor cells. A. Conditional lineage tracing of SCA1$^+$ cells (Uchida et al., 2013). The *Sca1*-tTA-IRES-GFP mouse was crossed with a Luciferase1-CRE (LC1) mouse, in which expression of CRE is controlled by the Tet-system (P_{tet} bi-1 promoter), as well as the Z/AP reporter mouse. The resulting tri-allelic mouse expresses CRE in the absence of doxycycline (Tet-OFF), permanently activating human placental alkaline phosphatase (hPLAP) expression in SCA1 expressing cells. B. Representative images showing SCA1$^+$ cell derivatives (AP positive, green), smooth muscle cells (αSA, red) and cardiomyocytes (dystrophin, red). C. Table 1 shows percentage of SCA1$^+$ CPC contribution to different cell lineages in the adult heart when labeling is conducted before birth (+doxycycline from birth) or after birth (+doxycycline from conception until birth).

Even though humans do not carry a *SCA1* gene, a population of CPCs has been isolated from human adult and fetal biopsies using the murine SCA1 antibody (van Vliet et al., 2008). Such cells differentiated into CMs in vitro with high efficiency (80–90%) using the 5-AZA method (Smits et al., 2009; van Vliet et al., 2008). *Sca1* knockout mice develop early-onset contractile deficiency and an impaired response to pressure overload associated with compromised proliferation and reduced numbers of SCA1$^+$ C-KIT$^+$ putative CPCs (Bailey et al., 2012; Rosenblatt-Velin et al., 2012), although coronary EC function may also be affected.

The heterogeneous nature of the SCA-1$^+$ cell population means that its lineage origins are poorly documented (see below). However, a recent lineage tracing study, using a triple transgenic mouse based on the Tet-Off CRE system (see Fig. 1C), has traced the fate of the total SCA1$^+$ cell population (Uchida et al., 2013) (Figs. 4A–C). Using continuous expression of CRE, SCA-1$^+$ CPCs were shown to give rise to a low and probably functionally irrelevant number of CMs during aging independently of cell fusion, as well as to SM cells. EC fate was difficult to score in this study since SCA1 is expressed in coronary ECs. Clonal lineage tracing using the Tet-Off system combined with the multi-color R26R-Confetti CRE-dependent reporter showed that SCA-1$^+$ cells were largely uni-potent and that most CPCs forming CMs did not undergo clonal expansion. The proportion of CMs increased 3-fold after trans-aortic constriction (TAC), although not after MI. The presence of tagged CMs in adults with induction of CRE expression after birth indicated that some CMs derive from SCA1$^+$ cells present in the neonate or adult (Fig. 4C). As discussed above, genetic lineage tracing systems reach their limit of performance when used to demonstrate rare cellular outcomes, as it is difficult to exclude a low extent of transgene leakage or stochastic activation. Nevertheless, this carefully performed CRE lineage-tracing study suggests that a yet-to-be-defined SCA1$^+$ CPC sub-population can form vascular SM cells and CMs, although their potential to form CM is extremely limited during both aging and injury (see Discussion).

Side-Population cardiac progenitors

Cells possessing the ability to efflux the dye Hoeschst 33342 from their nucleus are termed Side-Population (SP) cells and are detected by flow cytometry. When applied to BM cells, the SP is greatly enriched for HSCs (Scharenberg et al., 2002). Using this strategy, SP cells were detected within post-natal hearts (~1% of all cells) that were SCA1^{high} but they lacked markers of mature hematopoietic cells (Hierlihy et al., 2002). They may potentially overlap with SCA1$^+$ CD31$^-$ CPCs (Martin et al., 2004; Pfister et al., 2005). ABCG2 is the transporter responsible for dye efflux and co-culture of GFP-labeled cardiac SP with neonatal CMs demonstrated their myogenic potential (Martin et al., 2004). Intravenous infusion of GFP-tagged cardiac SP cells into cryo-injured hearts led to homing of cells to the injury site and differentiation of a proportion of surviving cells into CM, SM and ECs (Oyama et al., 2007).

The depletion of SP after MI was rapidly replenished by BM-derived cells, suggesting a BM origin (Mouquet et al., 2005). Some cells expressed CD31, suggesting conversion to ECs in situ. However, a separate study has suggested that cardiac SP CPCs are derived from embryonic neural crest (Tomita et al., 2005). Here, murine SP cells were isolated from the non-CM fraction and expanded as free-floating spheres (Reynolds and Weiss, 1992). Early in culture cardiac spheres expressed markers of undifferentiated neuronal precursors (*nestin* and *musashi*-1) and then by two weeks markers of CMs and SM. A fraction of cells exhibited spontaneous contraction and action potentials. After labeling with a vital-dye and transplantation into the head region of chick embryos, some

cardiac SP cells or their descendants integrated into the peripheral nervous system as well as the cardiac outflow tract and conotruncus, a pattern reminiscent of neural crest cell contribution to the heart. Lineage tracing using a neural crest-specific *P0*-CRE transgene revealed rare neural crest-derived CMs.

In summary, the cardiac SP fraction is a heterogeneous population that likely overlaps with SCA1$^+$ CPCs, and is at least in part derived from BM and potentially also neural crest. Tracking the origins of this population is made difficult because of the flow cytometry-based assay that defines it and lack of specific markers.

Epicardium-derived cardiac progenitors

As discussed in the Diverse origins for cardiac progenitors in development section, epicardial cells have their origin within a transient epithelial organoid present in the early looping heart termed the proepicardium (PE) (see Fig. 2B). During development, PE cells undergo EMT and migrate over the myocardium to form the mesothelial epicardial layer during which a subset of these cells (EPDCs) enters the sub-epicardial matrix and cardiac interstitium. Fate mapping studies using both classical and CRE recombinase-based methods have shown that EPDCs give rise to SM and perivascular cells of the coronary arteries, interstitial stromal cells, interstitial colony-forming cells and potentially some ECs (Asli et al., 2014; Carmona et al., 2010).

Several recent studies have shown that the adult murine epicardium, generally assumed to be quiescent, has latent stem cell-like properties. In both adult zebrafish and mammals, the epicardium undergoes a global activation of its fetal program after injury (Gittenberger-de Groot et al., 2010; Kikuchi et al., 2011; Lepilina et al., 2006; Zhou et al., 2011). Some adult mouse epicardial cells underwent EMT and gave rise to SM components of new vessels as well as fibroblasts and myofibroblasts (although no CMs or ECs) and activated cells also secrete pro-angiogenic cytokines (Zhou et al., 2011). Isolated epicardium transplanted into MI hearts showed differentiation into SM and ECs, but not CM (Winter et al., 2007). Smart et al. demonstrated that the actin monomer binding protein THYMOSIN β4 (Tβ4) enhanced the ability of adult murine epicardium to undergo migration and differentiation into vessels, fibroblasts and even a small number of CMs after injury (Smart et al., 2007). Limana et al. identified cells in the fetal and adult human epicardium that were C-KIT$^+$ CD34$^+$ CD45$^-$, and had the ability to differentiate into ECs in vitro (Limana et al., 2007). By injecting a tagging retrovirus into the pericardial space, tagged epicardial C-KIT$^+$ cells were shown to proliferate after MI and differentiate into SM and ECs arranged into vascular structures.

More recently, Zangi et al. showed that a single injection of modified (mod) RNA encoding VEGF-A into myocardium at the time of MI significantly increased expression of WT1, an epicardial TF, and expression of the VEGF-A receptor (FLK1) in WT1$^+$ cells (Zangi et al., 2013). ModRNAs evade the innate immune system and allow pulse-like delivery of secreted factors. Lineage tracing of WT1$^+$ cells was then performed using mice carrying the tamoxifen-dependent *Wt1-CRE^{ERT2}* allele (Zhou et al., 2011) crossed to a bi-color reporter (*R26^{mTmG}*), or by the application to the heart of a biocompatible gel carrying a modRNA encoding CRE. VEGF-A modRNA injection stimulated the mobilization of WT1$^+$ cells into the myocardium and enhanced differentiation into ECs and a low number of CMs (5% of traced cells). It also stimulated vascular density and perfusion of the infarct region, preserved ejection fraction and enhanced long-term survival. The pulse-like expression of VEGF-A avoided the adverse effects on vascular permeability that have been previously linked to persistent expression of this potent angiogenic molecule. In vitro clonal analysis suggested an increase in overall potency of isolated WT1$^+$ cells, with some single cells forming fibroblasts, SM and ECs (Zangi et al., 2013).

SCA1$^+$/PDGFRα^+ cardiac MSC-like cells derived from the epicardium

Our group has recently established a platform for studying multi-potent, self-renewing, colony forming cells resident within the non-CM fraction of embryonic and post-natal murine hearts (Chong et al., 2011). These cells, termed cardiac colony-forming units, fibroblast (cCFU-F), are isolated by mechanical and collagenase digestion, then FACS enrichment for SCA1$^+$ PDGFRα^+ CD31$^-$ cells (Fig. 5A), with subsequent growth on tissue culture plastic in 20% fetal bovine serum. This method is based on that used to isolate MSC cultures from BM by Freidenstein and colleagues in the 1970s (Friedenstein et al., 1974), and cCFU-F colonies are MSC-like in their cell surface marker and transcriptome profiles (Pelekanos et al., 2012). As for BM CFU-F, there is no single specific marker for cCFU-F in vivo, and markers used to enrich for cCFU-F (SCA1 and PDGFRα) are also expressed on the majority of cardiac stromal cells. Recent work has established the presence of cCFU-F in human hearts (Chong et al., 2013; Koninckx et al., 2013).

cCFU-F gave rise to colonies of different sizes ranging from a few cells to >100 cells when assessed 12 days after plating (Fig. 5B). Based on studies in other stem cell systems (Friedenstein et al., 1970; Louis et al., 2008), we found that colony size reflected founder cell status. Cells within micro colonies (5–24 cells) have a differentiated phenotype (Chong et al., 2011). In contrast, large colonies (>2 mm), while containing a minority of differentiated cells, also bear cells that are clonogenic and can be propagated for >40 passages without senescence. cCFU-F can be coaxed to differentiate into a broad range of lineages in a variety of in vitro and surrogate in vivo assays (Chong et al., 2011). Large colonies show broad mesodermal potency. Cloned large colonies give rise in subsequent passages to large, small and micro-colonies. Thus, distinct metastable progenitor states exist within the cCFU-F hierarchy and are amenable to study.

Lineage potency of CFU-F and MSCs. The initial MSC concept, rooted in the work of Friedenstein et al. on the skeletogenic properties of BM stroma when transplanted ectopically, has attracted much scientific attention as a stem cell system (Bianco et al., 2013). MSC can be derived from virtually all solid organs and soft tissues (da Silva Meirelles et al., 2006) and there are over 300 clinical trials using MSCs for a variety of indications (Hare, 2009). Controversies have arisen surrounding the generalization of the BM MSC concept to all tissues and the scientific justification for their use in cell therapies of non-skeletal origin (Bianco et al., 2013).

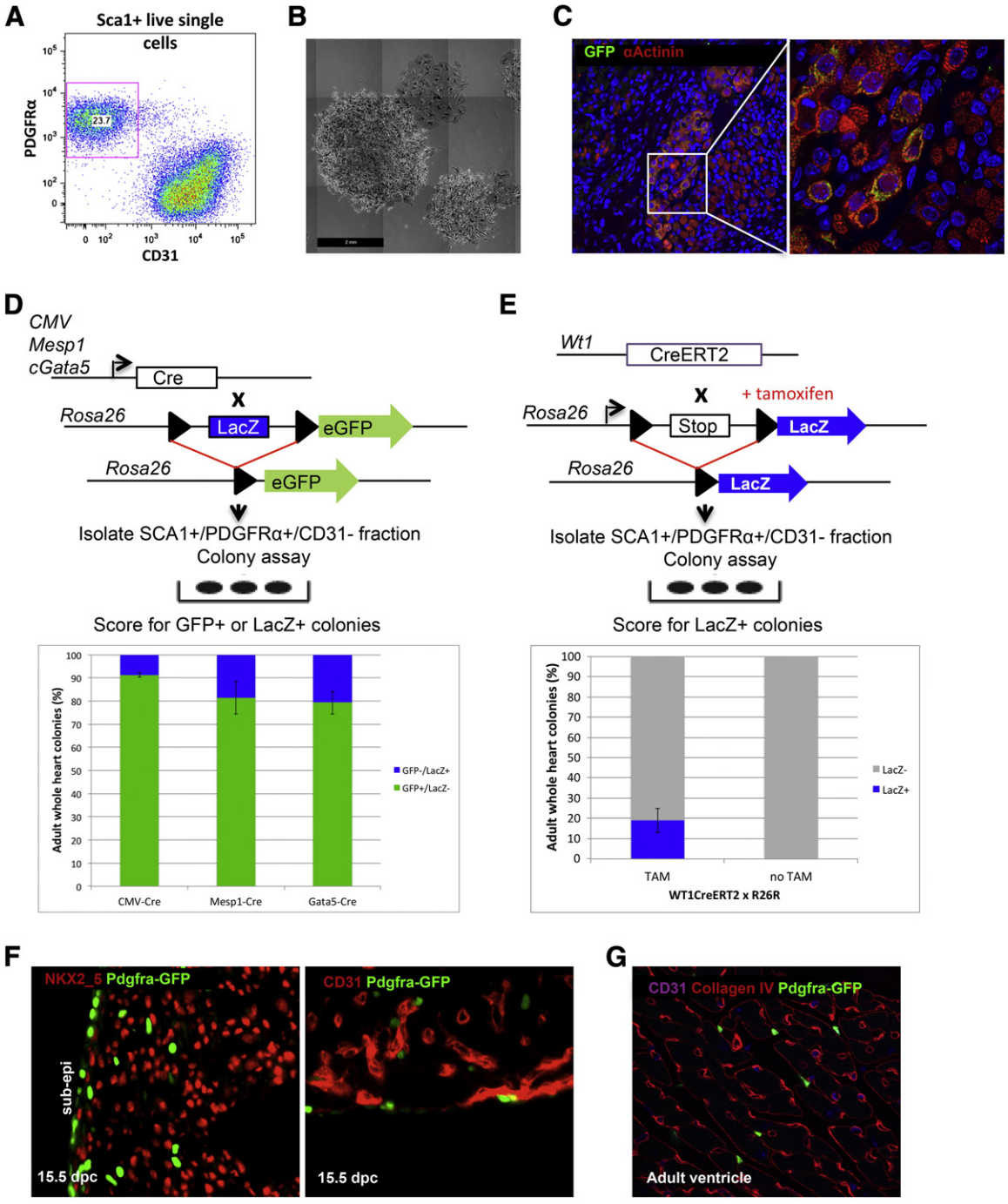

Figure 5 SCA1$^+$/PDGFRα^+ cardiac MSC-like cells derived from the epicardium. A. Representative FACS plot of the PDGFRα^+/CD31$^-$ population (boxed) within the SCA1$^+$ interstitial cell fraction. All colony-forming cells (cCFU-F) are derived from this fraction. B. Bright field image of a large (>2 mm), small (<2 mm) and micro (25 cells) colony. Image courtesy of Vaibhao Janbandhu. C. Teratoma assay – co-transplantion of cCFU-F constitutively expressing GFP (green) with mouse embryonic stem cells under the kidney capsule. Representative immunostaining for αACTININ (red). Republished with permission of CELL Press from Chong et al. (2011); permission conveyed through Copyright Clearance Centre, Inc. D. Lineage tracing strategies to determine the origin of cCFU-F (Chong et al., 2011). Mice expressing CRE under the control of different promoters (*CMV*, *Mesp1*, *cGATA5*) are crossed with the *Z/EG* reporter mouse. The percentage of LacZ$^+$/GFP$^-$ or LacZ$^-$/GFP$^+$ colonies obtained from the SCA1$^+$ PDGFRα^+ CD31$^-$ fraction was scored (graphs below). E. Lineage tracing strategy using a conditional (tamoxifen-dependent) *Wt1*-CRE-ERT2 mouse crossed with a *Rosa26R* reporter confirmed the epicardial origin of cCFU-F. Some 20% of adult colonies were tagged after tamoixifen delivery from E9.5-11.5, indicating self-maintenance of fetal cCFU-F parent cells into adulthood. F. Immunofluorescence on heart sections at E15.5 showing *Pdgfr*α-GFP $^+$ cells (green) in sub-epicardium and interstitium, non-overlapping with NKX2-5$^+$ cardiomyocytes (red, left panel) and CD31$^+$ endothelial cells (red, right panel). Republished with permission of CELL Press from Chong et al. (2011); permission conveyed through Copyright Clearance Centre, Inc. G. Section of adult heart showing *Pdgfr*α-GFP$^+$ cells (green) situated in the interstitium (basement membrane Collagen IV, red; CD31, pink). Image courtesy of Dr Henrik Reinhard.

The ability of BM perivascular fraction to make bony parenchyma, cartilage, adipocytes, fibroblasts and stromal cells in ectopic organoids has been confirmed at a single cell level (C.K. Chan et al., 2013), although to our knowledge this has not been shown clonally in the native BM setting. In many studies, BM MSC lineage plasticity has extended beyond skeletal derivatives (Kopen et al., 1999; Makino et al., 1999; Pijnappels et al., 2008; Sato et al., 2005; Shake et al., 2002; Toma et al., 2002; Woodbury et al., 2000). Others, however, have found limited or no CM differentiation (Berry et al., 1992; Gallo et al., 2007; Noiseux et al., 2006; Rose et al., 2008).

The in vivo fate of cCFU-F is not known, although we can anticipate that CFU-F from all tissues act as progenitor cells for the perivascular, stromal and connective tissue elements, and potentially parenchyma. In vitro, using strong chemical drivers, cCFU-F cultures differentiated into CM, ECs, SM, adipocytes, chondrocytes and osteocytes as judged by expression of markers (Chong et al., 2011). They also had potential to form endoderm and, to a more limited extent, neuronal lineages, suggesting trans-germ layer potential in vitro. The functionality of ECs was demonstrated by acetylated-LDL uptake and network formation, and that of SM by contraction in the presence of the muscarinic receptor agonist carbachol. GFP-tagged bulk-cultured cCFU-F were also mixed with ES cells and transplanted under the kidney capsule to generate teratomas. The cardiac MSCs survived and GFP could be found in tissues of ectodermal, endodermal and mesoderm origin, including immature CMs showing striated sarcomeres (Chong et al., 2011) (Fig. 5C).

The breadth of such lineage outcomes should not be interpreted to reflect the natural lineage fate of cCFU-F. It most likely reflects the relatively open chromatin configuration of these primitive cells and their suggestibility in the face of strong extracellular chemical signals – a form of reprogramming (Bianco et al., 2013). Such plasticity is a likely contributor to the pathological fibrosis and fibro-fatty infiltration that accompanies cardiac pathologies (Judson et al., 2013; Liu et al., 2012). Whether cCFU-F-derived cells can form CMs in vivo is unknown, although the lineage tracing studies of SCA1$^+$ cells (Uchida et al., 2013) suggests that this does not occur at a compelling level.

Origin of cCFU-F. The clonal nature of CFU-F means that their origins can be determined using CRE lineage tracing methods. The origin of BM CFU-F is not settled, with evidence suggesting hemopoietic stem cell (Ebihara et al., 2006) or neural crest (Takashima et al., 2007) origins. In human ES cells, CFU-F arise from early multi-potent mesodermal progenitors that share a fate with hemoangioblasts (Slukvin and Vodyanik, 2011). We have also addressed the origin of cCFU-F. Reconstitution of the blood system after engraftment of GFP-tagged BM into irradiated mice, showed that adult cCFU-F were not in flux with BM during aging, after MI or when BM stem cells were mobilized with G-CSF (Chong et al., 2011). CRE lineage tracing using a number of lineage-restricted CRE drivers strongly indicated that the origin of cCFU-F was epicardium (Fig. 5D), while CFU-F from aorta and BM had distinct, non-epicardial CRE-driver signatures. These findings were strongly supported by embryo experiments. Expression of PDGFRα, or *Pdgfra*-GFP from a knock-in allele, have been useful tools for enriching cCFU-F in the adult (Chong et al., 2011) (Fig. 5G). These markers are also highly specific to embryonic epicardium (Fig. 5F), which gives rise to cardiac stromal cells in a PDGFRα-dependent manner (Smith et al., 2011). cCFU-F activity in the fetus occurred within the *Pdgfra*-GFP$^+$ population and activity could be traced back to the PE.

In summary, cCFU-F appear to represent a primitive mesodermal and epicardium-like stem cell type. While lineage tracing data suggest an epicardial origin, whether they arise as stem cells early in development and are able to self-renew within a supportive niche throughout fetal and adult life is still unknown. It is possible that cCFU-F represent a form of transient facultative stem cell generated in both the embryo and adult at a low level from stromal cells, epicardium or other more differentiated cells (Zipori, 2004; Ziv et al., 2013). More rigorous analysis of their origins and in vivo function are needed.

Cardiac mesoangioblasts

Mesoangioblasts were defined as clonal vessel-derived cells that have skeletal myogenic activity, with the highest concentration in the aorta (De Angelis et al., 1999). The presence of a vascular reservoir that can replenish satellite cells after transplantation has been supported by a number of studies (Bentzinger et al., 2013). Mesoangioblast cultures express stem cell markers C-KIT and SCA-1, and a number of endothelial cell markers, which may explain their ability to efficiently extravasate from vessels into damaged muscle (Bentzinger et al., 2013). When labeled quail or mouse aortas were grafted into host embryos, vessel-derived cells were found dispersed in a variety of mesodermal tissues, including blood, cartilage, bone, SM, and skeletal and cardiac muscle (Minasi et al., 2002). It has been suggested that mesoangioblasts represent broadly potent perivascular mesenchymal stem cells, potentially derived from endothelial cells, important for both developmental organogenesis and tissue repair in the adult via invading vascular networks (Bianco and Cossu, 1999). This concept is derivative of the idea that vessel walls provide a niche for a variety of stem cell populations.

Percutaneous injection of clonally-expanded, GFP-labeled, mesoangioblast cells into the left ventricle after induction of MI led to salutary effects on heart function and remodeling, and among the few surviving GFP$^+$ cells, perivascular cells and some CMs were evident (Galli et al., 2005).

Mesoangioblasts have also been isolated from hearts using an explant protocol (Galvez et al., 2008, 2009). They first appear as small round cells which adhere poorly to the fibroblast outgrowth, and are detached by gentle pipetting (see the Cardiosphere-derived progenitors section). Mouse mesoangioblasts proliferate slowly for over 25 passages before spontaneous differentiation, a feature that distinguishes them from MSCs. Ventricular clones are SCA-1$^+$ C-KIT$^+$ CD31$^+$ CD34$^+$ CD45$^-$, and express cardiac TFs and pericyte markers. Adult cardiac mesoangioblasts differentiated efficiently into CM in low serum or when co-cultured with neonatal rat CMs, showing action potentials similar to ventricular CMs and pacemaker cells (Barbuti et al., 2010; Galvez et al., 2008). They could also differentiate into SM, although not osteocytes, adipocytes or skeletal muscle. After injection into mouse hearts with MI, GFP-tagged cardiac mesoangioblasts improved

cardiac function and GFP was found in differentiated CMs, apparently in the absence of fusion.

The origin and in vivo lineage descendants of cardiac mesoangioblasts remain to be determined. Their SCA-1$^+$ C-KIT$^+$ profile gives only weak clues to their identity. Expression of both pericyte and EC markers, and cardiac TFs, and their strong CM potency, suggests a committed CM or CV progenitor, although their relationship to the cCFU-F hierarchy or C-KIT$^+$ cells is unclear.

Conclusions

Fetal, neonatal and adult mammalian hearts bear several stem and progenitor cell populations (CPCs) as defined by their immaturity, behavioral characteristics, lineage potency and expression of markers associated with stem cell states. However they remain poorly characterized and the relationships between them are not well understood. The developing epicardium and its descendant lineages, including stromal cells, coronary SM cells, cCFU-F, and the adult epicardium itself, appear to have differentiation and proliferative plasticity in common. These cells will continue to generate significant interest as potential targets of new therapies for heart ischemic injury and heart failure. The broader roles of CPCs as stress sensors, paracrine factories and immune modulators are also important areas of research, and the impact of organ function, aging, disease and environment are critical parameters for analysis.

A growing number of studies have attempted the difficult task of measuring CM division and turnover in normal and diseased human and rodent hearts (Bergmann et al., 2009; Hsieh et al., 2007; Mollova et al., 2013; Senyo et al., 2013). Most have suggested that while turnover does occur, it is extremely low. The most rigorous CRE recombinase-based lineage tracing experiments published to date suggest that C-KIT$^+$ and SCA1$^+$ CPC populations are capable of acting as reserve cells for CV lineages (Uchida et al., 2013; van Berlo et al., 2014). The vascular potential of these cells seems compelling. However, the finding that trivially low numbers of CMs are formed from these progenitors, some the result of cell fusion, is surprising in light of numerous other studies claiming a more robust role for CPCs in cardiac homeostasis and repair (Anversa et al., 2013; Ellison et al., 2013; Jesty et al., 2012).

As discussed above, lineage-tracing technologies and their surrogates have significant limitations when applied to rare progenitor cell populations, and their application has periodically led to misleading claims in heart biology and ensuing turmoil in the literature. Resolving the dichotomous results concerning the role of CPCs remains a high-level issue for the field. At stake, in addition to our general understanding of cardiac development, repair and physiology, is the rationale for human stem cell therapies and a way forward for heart rejuvenation. The first clinical trials using selectively expanded CPC cultures for cardiac repair have indicated safety and potential efficacy (Bolli et al., 2011; Makkar et al., 2012), although the mechanisms of action are still unclear. It is not until all functionally relevant CPCs can be labeled with demonstrable certainty, and their descendants traced under a variety of conditions, will these issues be resolved. This may require lineage analysis at a clonal level using markers that are not subject to dynamic cell states.

Despite these various uncertainties, a baseline for the contribution of CPCs to cardiac lineages in homeostasis and repair has been established, and this allows us to proceed optimistically with attempts to sustain and augment CPC-related processes in aging and disease. A deeper understanding of CPC biology should yield many new insights and possible avenues for therapy.

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