Article

Adult Cardiac-Resident MSC-like Stem Cells with a Proepicardial Origin

James J.H. Chong,[1,10,12] Vashe Chandrakanthan,[1,2,10] Munira Xaymardan,[1,4,5,10] Naisana S. Asli,[1] Joan Li,[1,13]
Ishtiaq Ahmed,[1,14] Corey Heffernan,[1,15] Mary K. Menon,[1,16] Christopher J. Scarlett,[6,7] Amirsalar Rashidianfar,[1]
Christine Biben,[1,8] Hans Zoellner,[5] Emily K. Colvin,[6] John E. Pimanda,[2] Andrew V. Biankin,[6] Bin Zhou,[9,17] William T. Pu,[9]
Owen W.J. Prall,[1,8] and Richard P. Harvey[1,3,4,*]

[1]Victor Chang Cardiac Research Institute, Darlinghurst, New South Wales, 2010, Australia
[2]Lowy Cancer Research Centre, Prince of Wales Clinical School
[3]St. Vincent's Clinical School, Faculty of Medicine
University of New South Wales, Kensington, New South Wales, 2052, Australia
[4]Australian Research Council Special Research Initiative in Stem Cell Science – Stem Cells Australia
[5]Cellular and Molecular Pathology Research Unit, Department of Oral Pathology and Oral Medicine, Faculty of Dentistry,
University of Sydney, New South Wales 2006, Australia
[6]Cancer Research Program, Garvan Institute of Medical Research, Darlinghurst, New South Wales, 2010, Australia
[7]School of Environmental & Life Sciences, University of Newcastle, Ourimbah, NSW, 2258, Australia
[8]Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia
[9]Harvard Stem Cell Institute and Department of Cardiology, Children's Hospital, Boston, MA, 02115, USA
[10]These authors contributed equally to this work
[12]Present Address: Centre for Cardiovascular Biology, University of Washington, Seattle, WA98109 USA
[13]Present Address: Institute for Molecular Bioscience, University of Queensland, 4067, Australia
[14]Present Address: Department of Cardiothoracic Surgery, James Cook University Hospital, Middlesbrough, TS43BW, UK
[15]Present Address: Centre for Reproduction and Development, Monash Institute for Medical Research, Monash University,
Clayton 3168, Australia
[16]Present Address: Merck Research Laboratories, Boston, Massachusetts, 02115, USA
[17]Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
*Correspondence: r.harvey@victorchang.edu.au
DOI 10.1016/j.stem.2011.10.002

SUMMARY

Colony-forming units – fibroblast (CFU-Fs), analogous to those giving rise to bone marrow (BM) mesenchymal stem cells (MSCs), are present in many organs, although the relationship between BM and organ-specific CFU-Fs in homeostasis and tissue repair is unknown. Here we describe a population of adult cardiac-resident CFU-Fs (cCFU-Fs) that occupy a perivascular, adventitial niche and show broad trans-germ layer potency in vitro and in vivo. CRE lineage tracing and embryo analysis demonstrated a proepicardial origin for cCFU-Fs. Furthermore, in BM transplantation chimeras, we found no interchange between BM and cCFU-Fs after aging, myocardial infarction, or BM stem cell mobilization. BM and cardiac and aortic CFU-Fs had distinct CRE lineage signatures, indicating that they arise from different progenitor beds during development. These diverse origins for CFU-Fs suggest an underlying basis for differentiation biases seen in different CFU-F populations, and could also influence their capacity for participating in tissue repair.

INTRODUCTION

Building on advances in the stem cell biology of renewable tissues, research over the last decade has shown that most and probably all adult organs contain multipotent stem or progenitor-like cell populations. However, for all but a few adult systems, stem cell lineage origins, descendants, and dispersal remain unexplored.

Perivascular cells of the bone marrow (BM) sinusoids form a key component of the hematopoietic stem cell (HSC) niche. However, they also have stem-like properties—they appear to be the in vivo correlate of BM colony-forming cells (colony-forming units – fibroblast, or CFU-Fs; Friedenstein et al., 1970) which grow in vitro as multipotent mesenchymal stem cells (MSCs), and have the ability when freshly isolated and transplanted to heterotopic sites to form a bone-encased vascularized stroma and ectopic microenvironment for HSCs (Méndez-Ferrer et al., 2010). In vitro, MSCs are capable of clonogenic passage, long-term growth, multilineage mesodermal differentiation, homing to sites of injury, and immunomodulation (Caplan, 2007). That CFU-Fs have an ability to replenish bone in vivo is strongly suggested by transplantation studies, as well as the osteoporotic phenotype of mice mutant for Sca1, which supports MSC self-renewal (Bonyadi et al., 2003). Even the origin of these long-studied stem cells is controversial: they lack hematopoietic markers and early studies of sex-mismatched allograft patients suggest a non-HSC origin (Simmons et al., 1987). However, more recent data have placed BM CFU-Fs and HSCs in the same hierarchy (Ebihara et al., 2006) or point to a neural crest origin for fetal although not adult MSCs (Takashima et al., 2007).

MSC-like colony-forming cells have been isolated from several adult solid organs, and some of these have been shown
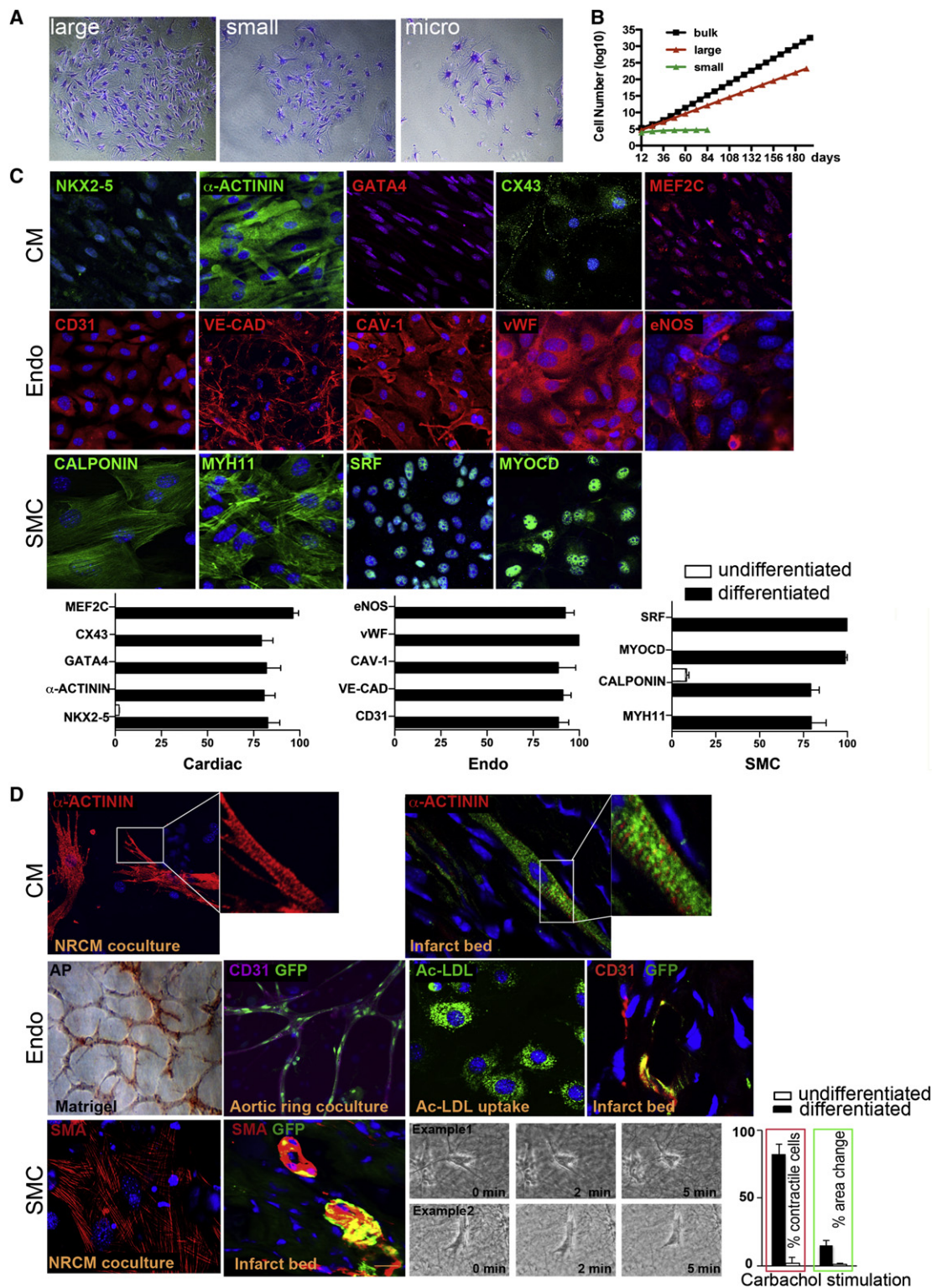
Figure 1. cCFU-F Growth and Differentiation Assays

(A) Colony morphology.

(B) Growth curves of bulk cCFU-F colonies and clonally isolated large and small colonies.

(C and D) Marker expression and cell conversion graphs after differentiation of cCFU-F colonies into cardiomyocyes (CM), endothelial cells (Endo), and smooth muscle cells (SMC) for in vitro and in vivo differentiation assays as indicated. All immunofluorescence panels were costained with Hoechst to detect nuclei. Graphs show percent conversion to marker-positive cells (black bars) relative to undifferentiated cells (open bars). Right SMC panels in (D) show cCFU-F-derived

to occupy a perivascular location (Armulik et al., 2011; Crisan et al., 2008; da Silva Meirelles et al., 2008). Indeed, there is a growing view that MSCs are broadly distributed and equate to pericytes (Armulik et al., 2011; da Silva Meirelles et al., 2008; Méndez-Ferrer et al., 2010). Furthermore, MSCs isolated from different locations show biases with respect to lineage differentiation, suggesting organ-specific functions (Kern et al., 2006). A global view is that CFU-Fs may be a population of progenitors dedicated to maintaining the integrity of matrix, stroma, and vessels of multiple organs, while retaining an ability to contribute to parenchyma in an organ-specific manner, especially in injury and disease.

The longstanding dogma that the mammalian heart is a postmitotic organ with limited regenerative reserve has been challenged by the discovery of a number of multipotent stem-like cell populations and their likely contribution to new cardio-myocytes (CMs) and vascular lineages after injury. Retrospective carbon dating suggests that about half of the CMs in the healthy human heart are replaced during life (Bergmann et al., 2009), albeit from an unknown source. Lineage tracing in mice found no CM replacement from a non-CM (potentially stem cell) source during aging, although it did yield evidence for significant replacement (5%–15%) after injury (Hsieh et al., 2007). However, a much more dynamic view of CM turnover and renewal based on stem cell deployment in normal and diseased hearts has also emerged using different prospective and retrospective lineage tracking techniques (Kajstura et al., 2010).

Primitive cardiac stem/progenitor cells (CPCs) bearing the capacity to differentiate into CMs, endothelial cells (ECs), and smooth muscle (SM) cells have been defined based on expression of markers of HSCs (Beltrami et al., 2003; Oh et al., 2003), cardiac precursors (Laugwitz et al., 2005), and neural stem cells (Takashima et al., 2007; Tomita et al., 2005); efflux of Hoechst nuclear dye (Mouquet et al., 2005); and the ability to be propagated as adherent, sphere, or outgrowth cultures (Galvez et al., 2008; Messina et al., 2004; Shenje et al., 2008). Long-term in vitro growth and multilineage differentiation potential have been demonstrated in a variety of settings. However, the origins and relationships between these populations are largely unknown. ISL1^{+} CPCs in the neonatal heart are likely to have their origins in the cardiac progenitor fields during development, although a formal lineage link to such cells has not been established (Laugwitz et al., 2005). Cardiac-resident cKIT[+] CPCs may also have their origins in the early heart or its progenitor fields (Wu et al., 2006), or in immature or dedifferentiated CMs (Li et al., 2008; Zhang et al., 2010), although other data is consistent with a BM or HSC origin (Fazel et al., 2008).

Cardiac CFU-Fs have not yet been defined. In this paper we document CFU-Fs as an additional stem-like population in the developing and adult heart, and provide evidence for their epicardial origin in development and long-term persistence in vivo.

RESULTS

Colony-Forming Assay Defines a Cardiac-Resident Stem Cell Population

We employed an in vitro colony-forming assay first used to characterize BM MSCs (Friedenstein et al., 1970) to define a cardiac MSC-like population from the adult murine heart (Pelekanos et al., 2011). Colonies that formed in 20% fetal calf serum had a mesenchymal morphology and showed a range of sizes (Figure 1A), with larger colonies predominating in primary as well as secondary and subsequent assays performed clonally (Figure S1A available online and data not shown). Limit dilution assays also showed that colonies were clonal (Figure S1B), and we therefore refer to the parent cell for each colony in vivo as a cCFU-F (Friedenstein et al., 1970). cCFU-F colonies expressed markers of MSCs including CD44, CD90, CD29, and CD105, and the microarray signature of colonies correlated very highly with that of BM CFU-F cultures (r = 0.88) (Pelekanos et al., 2011), suggesting analogous function, common origin, or both.

Bulk cultures of freshly isolated cCFU-Fs grew exponentially for around 11 months (∼40 passages) before senescence (Figure 1B; Figure S1C), indicating their long-term growth potential. Bulk cCFU-F cultures and those clonally derived from large colonies exhibited equivalent multipotency for a range of mesodermal lineages (CMs, ECs, SM, adipocytes, cartilage and bone) in vitro using multiple markers (Figure 1C, Figure S1D). PCR and western blotting showed that the cardiovascular lineages formed in vitro expressed, for the most part, robust levels of lineage markers comparable to that seen in cognate tissues (Figures S1E–S1G). The functionality of derivative cardiovascular lineages was shown in a number of contexts (Figure 1D)—CMs formed α-actinin[+] striated sarcomeres after cCFU-F coculture with neonatal rat cardiomyocytes (NRCMs); ECs formed tubular networks in matrigel and aortic ring cocultures, and showed acetylated low density lipoprotein uptake; and SM-like phenotypes were seen as myofibroblast-like cells displaying abundant SM-α-actin[+] stress fibers after membrane coculture with NRCM, and SM cells contracted after stimulation of the muscarinic acetylcholine receptor with carbachol, which was not seen in undifferentiated cells. The progeny of 2×10^5 green fluorescent protein (GFP)-tagged colony cells injected into the ischemic zone of hearts with induced myocardial infarction (MI) also included α-actinin[+] striated CMs, as well as ECs and SM cells assembled into luminal vessels (Figure 1D). CMs have not been observed to beat in the NRCM coculture assay, as an inhibitory factor is expressed by the majority non-CM cCFU-F derivatives (NRCMs are also inhibited). However, the presence of striated sarcomeres in CMs produced in culture and after injection of cCFU-F colony cells into infarct beds indicates an advanced state of cellular differentiation and specialized macromolecular synthesis.

smooth muscle contraction after treatment with carbachol. Graphs show percentage of cells contracting and percentage of contraction of cell area. For (C), error bars = SEM between independent experiments. For (D), error bars = SD between independent experiments.
Marker abbreviations: CX43: CONNEXIN 43; VE-CAD: VE-CADHERIN; CAV-1: CAVEOLIN 1; vWF: VON WILLEBRAND'S FACTOR; eNOS: ENDOTHELIAL NITRIC OXIDE SYNTHASE; SRF: SERUM RESPONSE FACTOR; MYOCD: MYOCARDIN; AP: ALKALINE PHOSPHATASE; Ac-LDL: acetylated low density lipoprotein; MYH11: SMOOTH MUSCLE MYOSIN HEAVY CHAIN; SMA: SMOOTH MUSCLE ACTIN (bar = 50 μm).
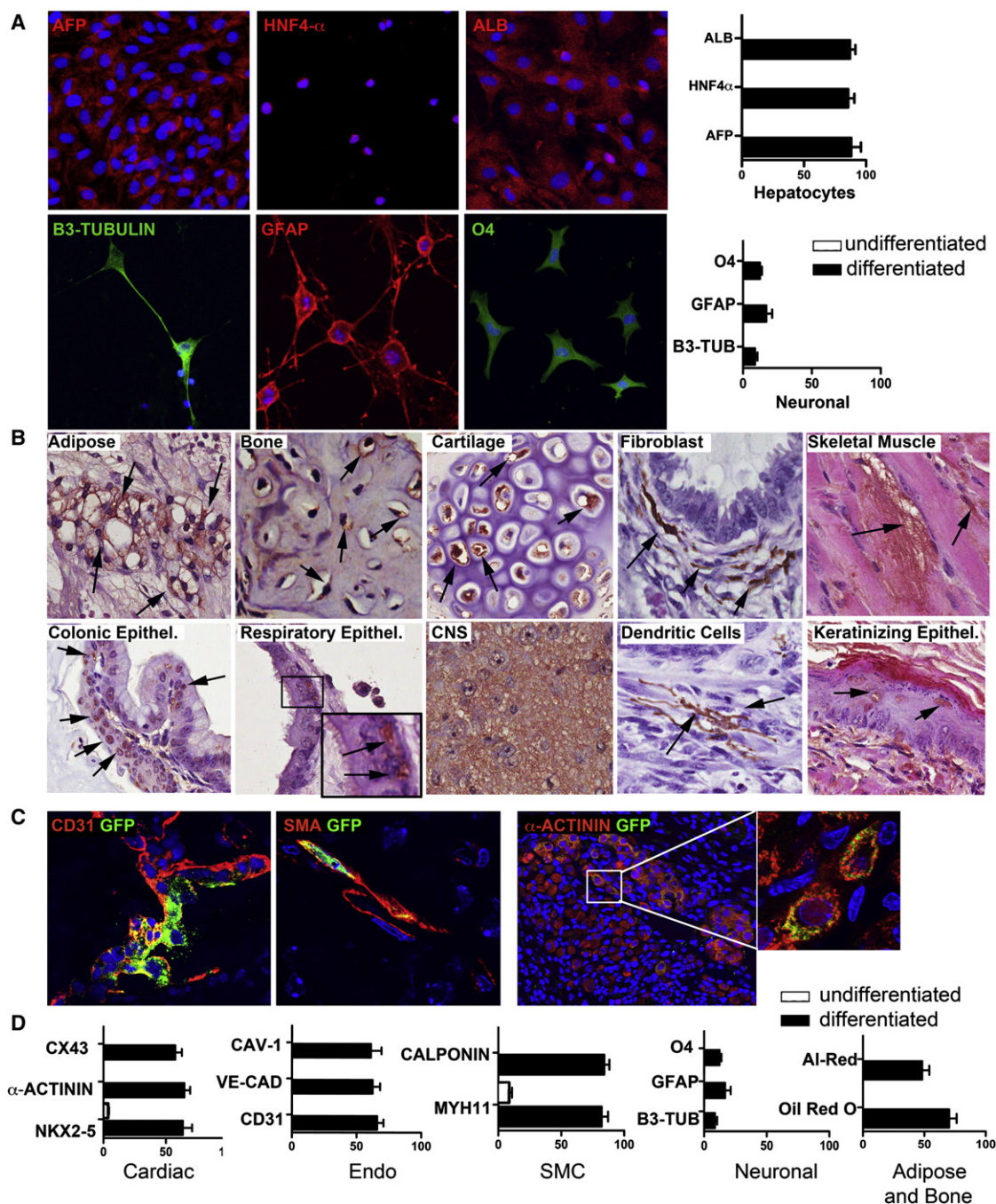See also Figure S1.

Figure 2. cCFU-F Trans-Germ Layer Differentiation

(A) Marker expression and cell conversion graphs for direct differentiation into hepatic, endoderm, and neuronal lineages. Graphs in (A) and (D) below show percentage conversion to marker-positive cells (black bars) relative to undifferentiated cells (open bars). (B) Descendant lineages of cCFU-F colony cells in ESC coculture teratomas. Brown staining indicates GFP expression in cCFU-F lineage descendants (arrows). (C) Immunostaining (red) and GFP fluorescence (green) for endothelial cells and smooth muscle (left panels) and cardiomyocytes in ESC coculture teratomas. (D) Percent conversion graphs for in vitro differentiation of freshly isolated S+P+ fraction. Marker abbreviations: AFP: ALPHA-FETOPROTEIN; ALB: ALBUMIN; β3-TUB: B3 TUBULIN; GFAP: GLIAL FIBRILLARY ACIDIC PROTEIN; O4: OLIGODENDROCYTE MARKER O4.

Error bars = SEM between independent experiments. See also Figure S2.

A limited number of adult stem cells display trans-germ layer plasticity in vitro. cCFU-F colony cells showed efficient conversion to an endodermal (hepatic) fate in vitro, as well as to neurectodermal fates (neuronal, glial, and oligodendrocyte), albeit with reduced efficiency (Figure 2A). However, colony cells did not form teratomas when transplanted under the kidney capsule

(10^6 cells transplanted; n = 9), and therefore cannot be considered pluripotent by this criterion. Consistently, early passage cCFU-F colonies, while expressing pluripotency genes Oct4 and cMyc, lacked expression of Sox2 and Rex1 and showed extremely low levels of Klf4 and Nanog mRNAs (Figure S2A). We tested whether cCFU-F colony cells (ubiquitously expressing cytoplasmic GFP) would persist in teratomas formed by co-transplantion with ESCs (n = 10). Indeed, mononuclear cCFU-F derivatives, detected with anti-GFP antibody or by GFP fluorescence, were seen in a range of mesodermal, ectodermal, neuroectodermal, and endodermal lineages (Figure 2B; Figures S2B and S2C), supporting the broad plasticity of cCFU-Fs. The specificity of the anti-GFP antibody was confirmed using sections of heart from platelet-derived growth factor receptor-α (*Pdgfra*)-GFP$^+$ mice (see below), which express nuclear GFP (Figure S2D). Colony cells contributed to striated CMs and vessels in this assay (Figure 2C). Finally, a freshly isolated enriched cCFU-F fraction (SCA1$^+$/PDGFRα^+ fraction; see below) also formed mesodermal and neuroectodermal lineages (hepatocyte conditions were not tolerated) when directly transferred to low serum differentiation conditions (Figure 2D), arguing that the broad potency of cCFU-Fs is not an artifact of high serum culture.
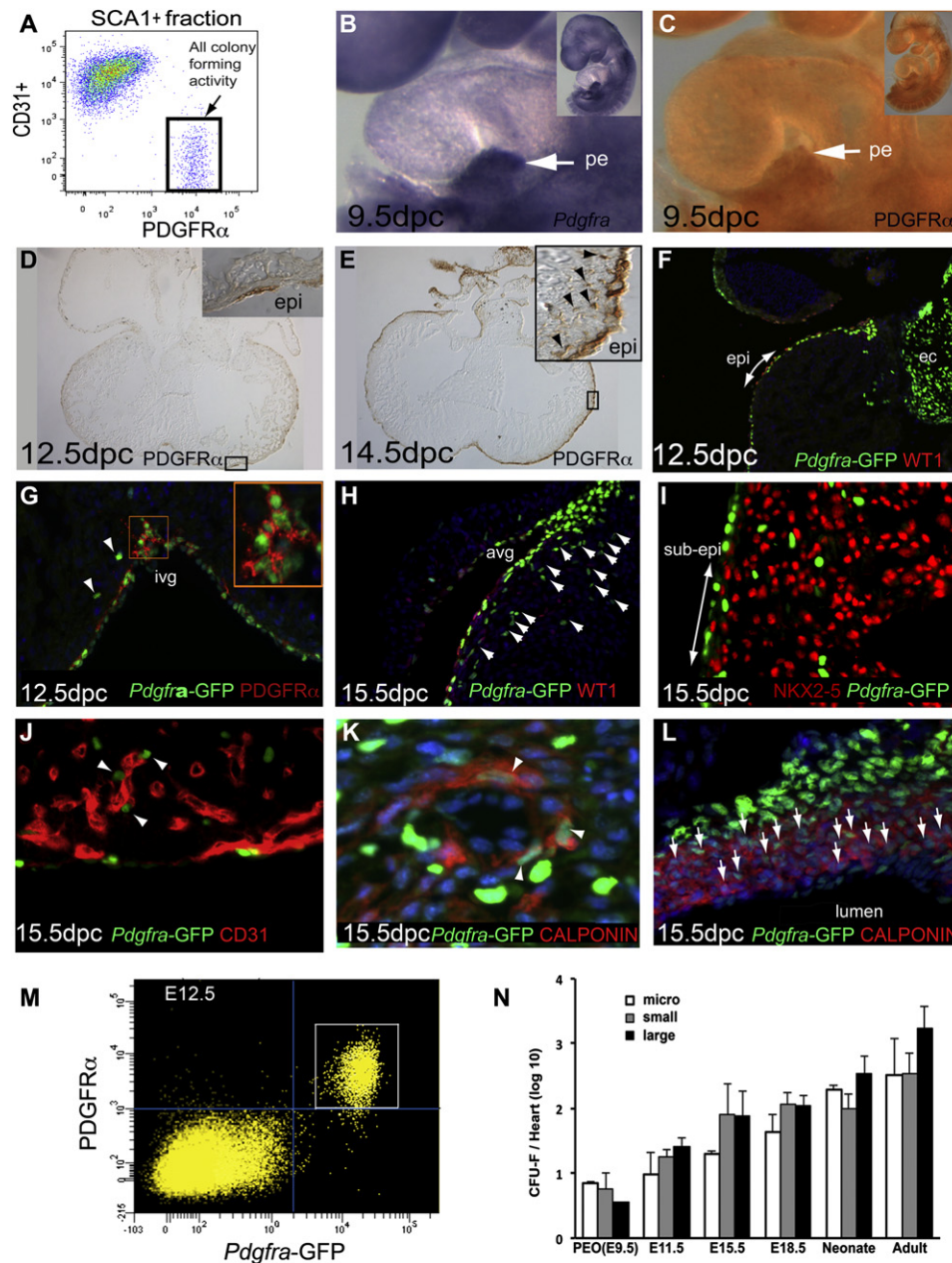
Fluorescence-activated cell sorting (FACS) of freshly isolated cardiac interstitial cells showed that cCFU-Fs were negative for the panhemopoietic marker CD45 and EC markers CD31 and FLK1, but expressed the HSC marker SCA1 and PDGFRα (Figure 3A). All CFU-Fs from the adult heart and ascending (supra-valvular) aorta were found within the SCA1$^+$/PDGFRα^+/CD31$^-$ mesenchymal fraction, hereafter referred to as S+P+ fraction.

Based on studies in other stem cell systems in which colony size has been used to distinguish stem from progenitor cells (Friedenstein et al., 1970; Louis et al., 2008), we explored the stem-like properties of isolated colonies after scoring them as micro (5–24 cells), small (>25 cells, <2 mm), or large ($\geq$2 mm) at 12 days postplating. It was only large colonies that contained clonogenic cells capable of long-term growth in vitro (>6 months/17 passages from clonal isolation) (Figure 1B). Large colonies also possessed the broadest in vitro lineage capacity, as defined above for bulk cultures (data not shown). Isolated large colonies (n = 3) showed slower growth than bulk cultures, likely due to selection favoring a minority of faster-growing colonies with the initial isolate. Isolated large colonies gave rise to all colony types (Figure S1A), reminiscent of BM or umbilical cord CFU-F in which clonogenic cells produce more committed progeny in a hierarchical fashion (Sarugaser et al., 2009). The S+P+ fraction without further enrichment contained ~1:140 cells with large colony-forming ability, while in secondary and subsequent clonal assays of isolated large colonies, ~1:7 cells (14%−17%) had large colony-forming ability (Figure S1A). Small colonies, in contrast, contained clonogenic cells that could also give rise to the spectrum of colony types (Figure S1A), but were capable of only slow and short-term growth (~2 months; four passages; Figure 1B) and represented more committed progenitors with potency restricted to the cardiovascular lineages (CM, EC, and SM) (data not shown). Micro colonies possessed no replating ability.

PDGFRα Defines Proepicardial and Epicardial Cells with CFU-F Ability

In situ hybridization (ISH) and immunostaining of embryos revealed dynamic and widespread expression of *Pdgfra* and PDGFRα protein (Figures 3B and 3C and data not shown). In hearts at 9.5 days postcoitum (dpc), however, high expression was seen only in proepicardium, the progenitor structure for the epicardium, and components of the coronary vasculature and interstitial fibroblasts, with the latter lineages formed from epicardium by epithelial-to-mesenchymal transition (EMT) (Carmona et al., 2010). In 12.5 dpc embryos, PDGFRα protein was evident in the epicardium, but not myocardium (Figure 3D), and at 14.5 dpc most cells expressing the highest levels of PDGFRα were seen in the subepicardium, with some isolated cells within the myocardial interstitium (Figure 3E, inset). We also analyzed GFP expression in a mouse knockin line in which a nuclear-localizing GFP cassette was inserted into the *Pdgfra* locus (Table S1 available online). FACS sorting for GFP fluorescence was equally efficacious compared to PDGFRα antibody in enriching for cCFU-F (Figure S1H). At 12.5 dpc, high GFP was seen in a mosaic pattern in epicardium (marked by Wilm's Tumor gene, WT1) and subepicardium, as well as endocardial cushions (Figure 3F). Perdurance of GFP allowed a surrogate fate tracking of the PDGFRα^+ lineage. At 12.5 dpc, a few *Pdgfra*-GFP$^+$ cells were present in the subepicardium of the atrioventricular and interventricular grooves (Figures 3F and 3G), while at 15.5 dpc many interstitial cells were evident, some at a considerable distance from the epicardium (Figure 3H arrows). They did not express the myocardial transcription factor NKX2-5 (Figure 3I) or the endothelial marker CD31 at this stage (except for some double-positive cells within endocardial cushions), although they appeared closely associated with CD31$^+$ coronary vessels (Figure 3J). In coronary arterioles already invested with SM at 15.5 dpc, GFP^{high} cells were seen in a perivascular, largely adventitial, location, with GFP^{weak} cells located within the media and coexpressing the SM marker CALPONIN1 (Figure 3K). Likewise, in the fetal aorta, GFP^{high} cells localized to the perivascular adventitia, and GFP^{weak} cells, to the SM medial layer (Figure 3L). These localizations are consistent with the perivascular location of MSC-like cells from other tissues (da Silva Meirelles et al., 2008) and suggest that in development, PDGFRα^+ epicardial cells undergo EMT and give rise to a minor subpopulation of cardiac interstitial cells that retain *Pdgfra*-GFP expression, the descendants of which include SM and other perivascular cells of the coronary vessels.

To explore this concept further, we confirmed the expression of *Wt1* in epicardium and subepicardium at 15.5 dpc scoring GFP expression from *Wt1$^{GFPCre/+}$* embryos (Table S1), and we confirmed that both *Pdgfra* and *Wt1* transcripts were restricted to *Wt1*-GFP$^+$ cells (Figures S3A and S3B). We then analyzed the lineage descendants of *Wt1*-expressing epicardial cells from 10.5 to 15.5 dpc. This was achieved by treating dams with embryos carrying both a sensitive CRE-dependent GFP reporter allele (*Rosa26$^{mTmG/+}$*) and a conditionally activated *Wt1-Cre* allele (*Wt1$^{CreERT2/+}$*) (Table S1) with tamoxifen at 10.5 dpc. At 15.5 dpc, in addition to epicardium and subepicardium, there was extensive GFP expression in interstitial lineages, including vessel-associated cells (Figures S3A and S3B), contrasting with the sparse pattern of *Pdgfra*-GFP$^+$ cells at this
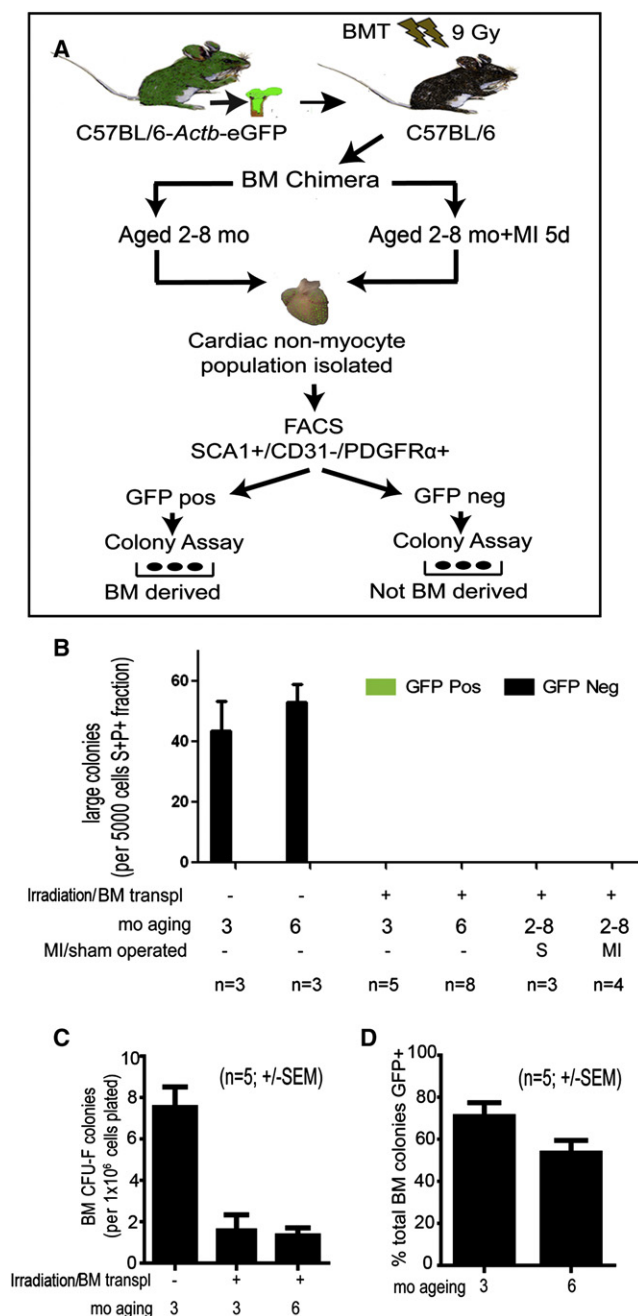
Figure 3. PDGFRα Expression and cCFU-Fs in the Developing Heart

(A) Colony forming cells were derived from the SCA1⁺/CD31⁻/PDGFRα⁺ fraction of adult cardiac nonmyocytes (S^+P^+ fraction, gate).

(B and C) Whole-mount ISH (B) and immunohistochemistry (C) of PDGFRα expression at 9.5 dpc (arrows show proepicardium; pe).

(D and E) Immunohistochemistry at 12.5 dpc (D) and 14.5 dpc (E) showing increasing PDGFRα expression in the epicardium (epi) and subepicardium.

(F–J) *Pdgfra*-GFP-expressing cells at 12.5–15.5 dpc relative to indicated markers showing expression in epicardium, the interstitium, and interventricular and atrioventricular grooves (ivg and avg, respectively), as well as in endocardial cushions (ec), at 12.5 dpc. Arrowheads in (G) and (H) indicate interstitial cells, and in (J), *Pdgfra*-GFP⁺ cells in a perivascular position.

(K and L) Smooth muscle fate of some *Pdgfra*-GFP⁺ cells is suggested by CALPONIN coexpression with GFP^low cells in coronary vessels and aorta at 15.5 dpc (arrows).

(M) Colony formation in the 12.5 dpc heart is limited to *Pdgfra*-GFP⁺/PDGFRα⁺ cells (gate). Although not shown on this plot, some PDGFRα⁺/*Pdgfra*-GFP⁻ cells appear variably at fetal stages and may be nonspecific. (N) Cardiac CFU-F colony formation is found in the proepicardium (pe), and embryonic, early postnatal (free ventricular walls), and 8-week-old adult heart.

Error bars = SD between independent experiments. See also Figure S3.

Figure 4. cCFU-Fs Are Lost after Irradiation and Cannot Be Rescued by Whole BM Transplantation

(A) Overview of BM transplant experimental design. All transplants were on mice 8 weeks of age.

(B) Large colony formation was completely lost in BM transplanted mice. Colony formation was not rescued after 3 months or 6 months aging, or after MI.

(C) Reduced BM CFU-F colonies from BM-GFP chimeric mice, which do not recover after 6 months.

(D) Majority of remaining BM CFU-F colonies are GFP⁺, indicating donor origin. Error bars = SEM between independent experiments. See also Figure S4.

stage (Figures 3H–3J). *Pdgfra* and *Wt1* transcripts were again enriched in GFP⁺ cells, confirming the association between *Pdgfra*⁺ cells and the epicardial lineage tree.

Using FACS, we next purified a *Pdgfra*-GFP⁺ cell fraction from dissected and pooled proepicardia (9.5 dpc), the free ventricular walls of progressively older fetal hearts (devoid of endocardial cushions), and whole adult hearts. The majority of *Pdgfra*-GFP⁺ cells were positive for PDGFRα protein (Figure 3M and data not shown). Colony-forming ability was present in the proepicardium and at all stages in the ventricular free wall (Figure 3N). As in the adult, cCFU-Fs arose exclusively from the *Pdgfra*-GFP⁺ fraction (note that the cardiac mesenchymal fraction does not express SCA1 until midgestation). The number of cCFU-Fs rose approximately exponentially throughout fetal life and peaked in adulthood (Figure 3N), with 1705 ± 183 (SD) large cCFU-Fs/heart (n = 3).

Consistent with an important role for adult epicardium in heart regeneration in multiple species (Kikuchi et al., 2011; Smart et al., 2011), it was of interest to determine if cCFU-F persisted in the epicardial layer into adulthood. Immunofluorescence revealed that ∼5% of total heart *Pdgfrα*-GFP⁺ cells were found in the epicardial layer, marked by expression of pan-CADHERIN (Figure S3D). However, when adult epicardial cells were isolated by FACS using E-CADHERIN antibody (E-CAD⁺S⁺P⁺), only small and micro colonies were detected (Figure S3D), suggesting that epicardium contains only committed progenitors and is devoid of cCFU-Fs of the highest rank.

Since both endocardial cushions and valve leaflets in the adult contained *Pdgfra*-GFP⁺ cells (Figure 3E), we also explored whether cushions and valves represent an alternative niche for cCFU-Fs, which is also of interest in light of studies showing that some valve cells have their origins in epicardium (Lie-Venema et al., 2008; Zhou et al., 2009). Dissected 13.5 dpc cushions yielded only few micro colonies (Figure S3E). S⁺P⁺ cells FACS-purified from adult mitral and tricuspid valve leaflets also yielded mostly micro colonies, although there were also some small and even a very few large colonies (Figure S3D), albeit with altered morphology (not shown). Large CFU-Fs within the valve leaflets represented only ∼0.006% of all large CFU-Fs detected in the adult heart.

Finally, in the adult, *Pdgfra*-GFPᴴⁱᵍʰ cells did not express the pericyte and SM marker NG2 (Figure S3C). This is at odds with claims for the equivalence of CFU-Fs and perivascular cells or pericytes in BM and other organs (Armulik et al., 2011; Méndez-Ferrer et al., 2010) (see Discussion).

Adult BM Cannot Rescue cCFU-Fs after Irradiation Injury

Blood-born progenitors mobilized from the BM are a credible source of adult tissue stem cells, particularly after ischemic injury. To explore a possible BM origin for some or all cCFU-Fs, total BM cells from transgenic mice expressing GFP ubiquitously (Table S1) were engrafted into lethally irradiated mice. BM transplants were performed on 8 week C57BL/6 recipients, with subsequent aging from 2 to 8 months. We also induced MI by ligation of the anterior descending coronary artery in BM engraftment mice as a further stimulus for BM flux (Figure 4A). In engrafted mice, FACS confirmed the presence of GFP⁺ expressing cells in peripheral blood and BM at 4–6 weeks post-transplantation (86.4% ± 5% and 65.0% ± 15% of total cells, respectively; Figures S4A and S4B). However, in cCFU-F assays, no large colonies were seen in either the BM-derived (GFP⁺)
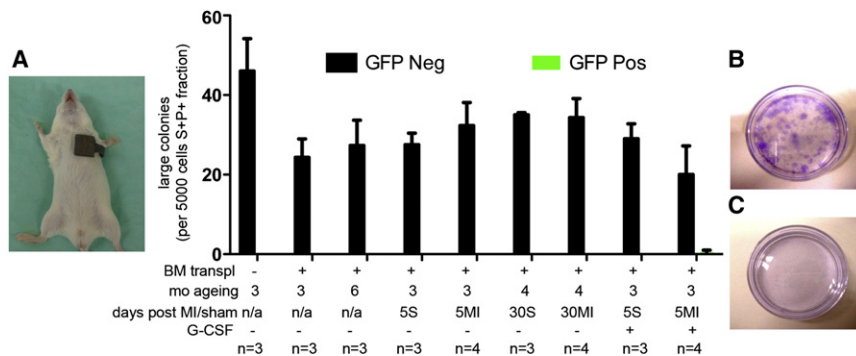
Figure 5. Large cCFU-Fs Are Not BM Derived

(A) Cardiac lead shielding during irradiation preserved large colony formation after BM transplantation. Large colony formation was from the non-BM-derived fraction after 3 and 6 months aging, MI (6 weeks posttransplant; cells isolated either 5 or 30 days after MI), or MI with G-CSF administration for 6 days.

(B and C) Crystal-violet-stained colonies from GFP$^-$ and GFP$^+$ fractions.

Error bars = SEM between independent experiments. See also Figure S5.

or non-BM-derived (GFP$^-$) fractions (Figure 4B). Induction of MI had no effect on colony formation (analysis at 5 days post-MI). To investigate the immediate effects of irradiation, C57BL/6 mice were subjected to 9 Gy with cCFU-F assays performed 6 hr later. Again, no large colonies were formed (Figure S4E). We also assessed the effects of irradiation on BM CFU-Fs (bmCFU-Fs), which yielded only ~20% of the total colony numbers at both 3 months and 6 months (Figure 4C). Of these, ~70% were GFP$^+$ (and therefore of nonirradiated, donor origin) at 3 months, diminishing to 50% at 6 months (Figure 4D). These data show that CFU-Fs residing in BM and heart are radiation sensitive. Importantly, engrafted total BM cells can re-establish the niche for bmCFU-F, but cannot rescue ablated cCFU-F in the irradiation injury setting, even after 8 months of aging and MI.

BM Does Not Contribute to cCFU-Fs after MI or HSC Mobilization

To avoid irradiation damage to the heart, we protected it by lead shielding (Figure 5A). Successful engraftment of GFP$^+$ cells was again confirmed by FACS analysis of blood and BM (Figures S4C and S4D). GFP$^+$ cells infiltrating the heart 5 days postsham or MI surgery in shielded (and unshielded) mice were all CD45$^+$ (>99%), with majority subfractions positive for SCA1 and CD31 (Figure S5A). Within the S+P+ population from shielded mice, ~4% of cells were GFP$^+$, although no cells were positive for the perivascular marker CD146. Shielded mice showed a decrease in cCFU-F to ~50% of nonirradiated controls (Figure 5A). After 3 months and 6 months, and at 5 days and 30 days post-MI (performed at 6 weeks posttransplantation), all large cCFU-Fs were derived from the non-BM, GFP$^-$ fraction (Figures 5A–5C). Granulocyte colony stimulating factor (G-CSF) mobilizes HSCs and endothelial progenitor cells, and potentially also MSC-related cells (Lund et al., 2008). However, in mice that had received daily G-CSF administration for 5 days after MI, virtually all cCFU-F were from the non-BM-derived fraction, with only a single colony from donor cells (Figure 5A).

cCFU-Fs Are Distinct from cKIT$^+$ CPCs

CPCs have been previously defined on the basis of expression of cKIT and a SCA1-like epitope (Linke et al., 2005). The majority of such cells are from BM and require cKIT function for mobilization and involvement in cardiac repair after MI (Fazel et al., 2006). We examined the relationship between cKIT$^+$ cells and cCFU-F

activity in the SCA1$^+$ and S+P$^+$ fractions in healthy, sham-operated and MI hearts (Figure S5B). There was a strong influx of cKIT$^+$ cells after MI, the vast majority segregating to the SCA1$^+$/PDGFRα$^-$/CD45$^+$ population, meaning they were therefore of BM origin. cKIT$^+$/CD45$^-$ cells were absent or extremely rare within the SCA1$^+$ and S+P$+$ fractions in healthy, sham-operated, and MI hearts, and neither cKIT$^+$/CD45$^+$ nor cKIT$^+$/CD45$^-$ fractions in MI hearts formed colonies. All large colonies came from the S+P$+$/cKIT$^-$/CD45$^-$ population (quadrant 3; Figures S5B and S5C). However, within cCFU-F colonies expanded in vitro, some 3.3% of cells were cKIT$^+$, and these cells were lost as cultures were induced to differentiate in low serum (Figure S5D). Therefore, while cCFU-Fs are distinct from the majority BM-derived cKIT$^+$ population, it remains possible that there is some overlap between the cCFU-F and cKIT$^+$ lineage trees in vivo, but only within a rare subset of the cKIT$^+$ population.

Lineage Analysis of the Developmental Origins of cCFU-Fs

To investigate the embryonic origins of cCFU-Fs more formally, we used CRE-recombinase lineage tracing. A suite of lineage-specific CRE driver mice was crossed with the *Z/EG* transgenic reporter mouse that carries a ubiquitously expressed *lacZ* transgene (Table S1). After exposure to CRE, the *lacZ* cassette is lost, leading to expression from a *GFP* cassette. Lineage-CRE × *Z/EG* hearts were harvested at 8–12 weeks and FACS was used to isolate the cardiac S$^+$P$^+$ fraction. cCFU-F assays were performed with colonies scored at 12 days for both β-galactosidase (LACZ) and GFP (Figures 6A and 6B). In germline *CMV-Cre* × *Z/EG* progeny, 91.3% ± 1% of large colonies were GFP$^+$/LACZ$^-$, the remainder being GFP$^-$/LACZ$^+$, which is likely the result of insufficient CRE activity in rare cells (Figure 6C). Without CRE, 100% of the colonies were GFP$^-$/LACZ$^+$, demonstrating the lack of ectopic GFP expression in this system (Figures 6B and 6C). Importantly, no GFP$^-$/LACZ$^-$ colonies were observed in these or additional crosses, demonstrating a lack of transgene silencing.

In the *Mesp1-Cre* line (Table S1), CRE is active in nascent mesoderm, but not in neuroectoderm, including neural crest or endoderm. In *Mesp1-Cre* × *Z/EG* progeny, most large colonies (81.3% ± 7%) were GFP$^+$/LACZ$^-$, demonstrating a largely (if not exclusively) mesodermal origin (Figure 6C). Recent reports suggest that neural crest may give rise to some CPCs in the postnatal murine heart (Tomita et al., 2005) and may populate
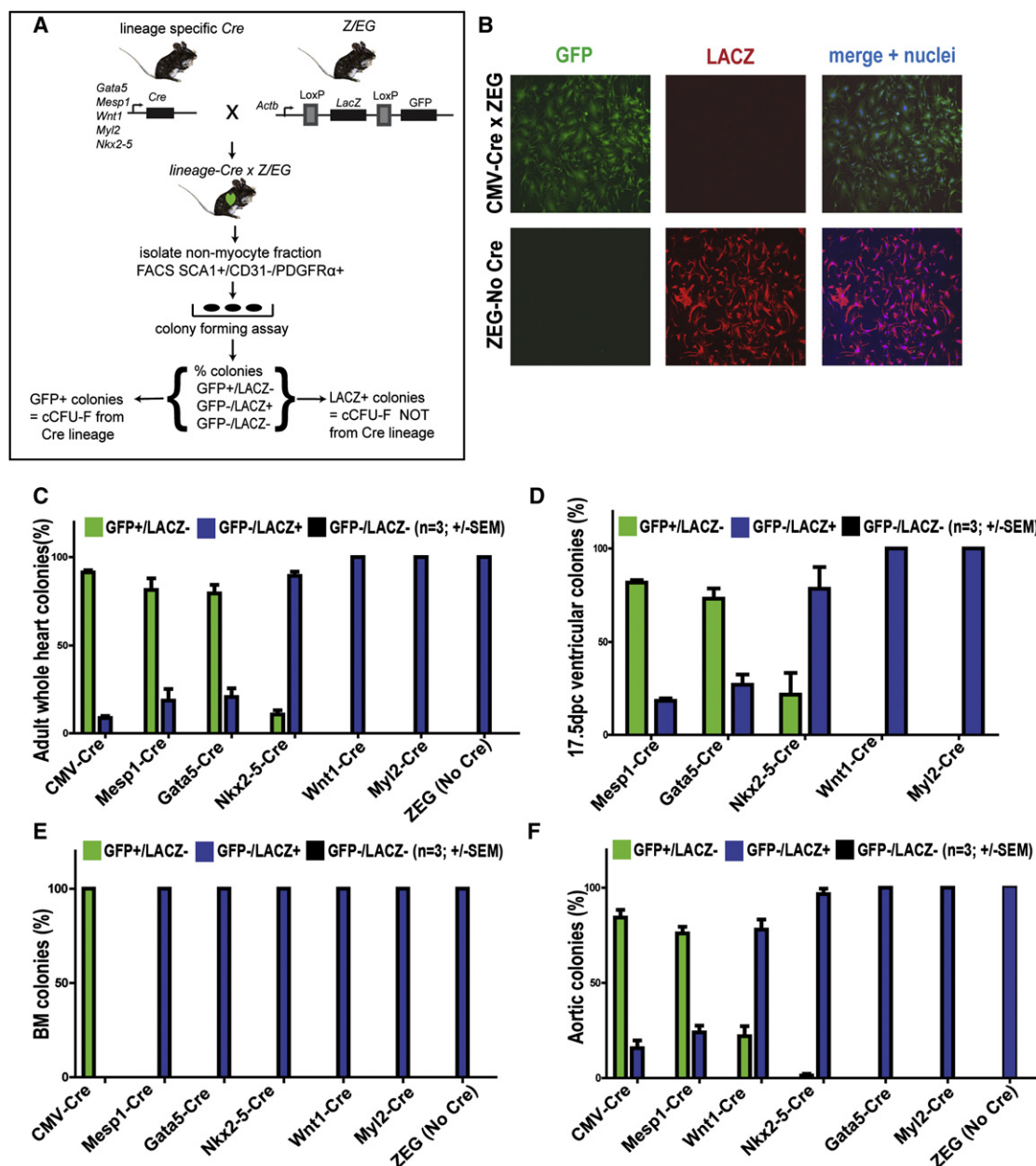
Figure 6. Lineage Tracing Studies Suggest an Epicardial Origin for cCFU-Fs

(A) Overview of lineage tracing strategy.

(B) Expression of GFP and LACZ in colonies from control mice.

(C and D) Percentage of GFP$^+$/LACZ$^-$ or GFP$^-$/LACZ$^+$ cCFU-F colonies from (C) adult whole hearts and (D) ventricles of 17.5 dpc lineage-CRE × Z/EG mice.

(E and F) Colonies derived from adult BM (E) or adult proximal aorta (F).

Error bars = SEM between independent experiments. See also Figure S6.

the proepicardium (Stottmann et al., 2004). We therefore used the *Wnt1-Cre* line in which CRE expression is neural crest specific (Table S1). However, all large colonies from *Wnt1-Cre × Z/EG* progeny were GFP$^-$/LACZ$^+$ (Figure 6C).

Some adult CMs are capable of reentering the cell cycle and contributing to heart repair (Bersell et al., 2009). To investigate a CM origin for cCFU-Fs, we used *Myl2-Cre* knockin mice in which CRE expression occurs in ventricular myocytes from

8.5 dpc (Table S1). However, all colonies from *Myl2-Cre × Z/EG* progeny were GFP$^-$/LACZ$^+$. *Nkx2-5* is expressed from 7.5 dpc in CPCs, and subsequently, at the birth of all CMs, save for a minor population caudally (Christoffels et al., 2006). Virtually all colonies resulting from a cross with an *Nkx2-5IRESCre* knockin line (Table S1) were GFP$^-$/LACZ$^+$ (89.3% ± 2%; Figure 6C), again arguing against a CM origin for cCFU-Fs. The few GFP$^+$/LACZ$^-$ colonies are discussed below.

Gata5-CRE Lineage Tracing Suggests an Epicardial Origin for cCFU-Fs

The *Gata5-Cre* transgenic line has been used previously to delete genes in the proepicardial/epicardial lineage (Martínez-Estrada et al., 2010; Mellgren et al., 2008; Merki et al., 2005; Zamora et al., 2007). In *Gata5-Cre × Z/EG* progeny, most large colonies (79.3% ± 5%) were GFP$^+$/LACZ$^-$ (Figure 6C). In our hands, using the Soriano *R26R* reporter (Table S1), CRE expression was first seen in proepicardium and in a few epicardial cells on the surface of the ventricles in 20 somite pair (sp) embryos (Figures S6A and S6B). Later (25sp–11.5 dpc), expression was seen more extensively in the epicardium, and also in substantial patches of myocardium and endocardium (Figures S6C–S6G). In the adult, expression was seen in myocardial patches, and in epicardium and its descendant lineages (Figures S6H–S6K).

As noted above, in *Nkx2-5IRESCre × Z/EG* progeny, virtually all colonies were GFP$^-$/LACZ$^+$, consistent with a nonmyocyte origin for cCFU-Fs (Figure 6C). However, 10.7% ± 2% of colonies were GFP$^+$/LACZ$^-$. This is most likely because *Nkx2-5*-driven CRE is also expressed transiently in a compartment of the early cardiac progenitor fields that encompasses precursors of the proepicardium (Ma et al., 2008; Zhou et al., 2008b).

The overall CRE lineage signature for cCFU-Fs seen above was also observed in isolated ventricles (devoid of endocardial cushion tissue) at 17.5 dpc (Figure 6D). We confirmed the lineage analyses using *R26R* as an independent reporter (Figure S6L) and also confirmed that *Gata5*-CRE was not activated as a result of in vitro culture (Figure S6M).

A Distinct CRE Lineage Signature for BM and Aortic CFU-Fs

CRE lineage signatures for bmCFU-F and aortic CFU-Fs (aCFU-Fs) were distinct from those of cCFU-Fs (Figures 6E and 6F). With CRE lines used here, all bmCFU-F colonies were GFP$^-$/LACZ$^+$. aCFU-F colonies had a multi-germ layer origin, mostly derived from mesoderm (76.0% ± 4% from a *Mesp1-Cre* lineage), with one fifth from neural crest (22.0% ± 5% from a *Wnt1-Cre* lineage), consistent with the known contributions of both heart field mesoderm and neural crest to the mural compartment of branchial vessels.

Confirmation of an Epicardial Origin of cCFU-Fs using a Conditional *Wt1-Cre* Line

To confirm an epicardial origin for cCFU-Fs, CRE lineage tracing was performed using a tamoxifen-inducible CRE driver (*Wt1^{CreERT2}*) regulated by *cis*-elements of *Wt1*, expressed in proepicardium and epicardium (Zhou et al., 2008a). We used a tamoxifen regime (2 mg intraperitoneally daily from 9.5–11.5 dpc) gauged to maximize CRE-ERT2 activity in the forming epicardium, and the moderately sensitive CRE-dependent reporter R26R (Table S1). LACZ expression in tamoxifen-treated *Wt1^{CreERT2} × R26R* progeny at 11.5–14.5 dpc was seen in epicardium, albeit in a patchy manner (Figures 7A–7E). Pericardium was also positive, as were interstitial cells including perivascular cells and fibroblasts (Figure 7E). However, in contrast to crosses with *Gata5-Cre*, endocardium, endocardial cushions, and myocardium were negative (Figures 7B–7E). LACZ$^+$ cells were also seen at a number of extracardiac sites (Figures S7A–

S7H), overlapping with the known expression sites of WT1 (Martínez-Estrada et al., 2010).

In *Wt1^{CreERT2}* crosses at 14.5 dpc, 19.3% ± 4% of cCFU-Fs were LACZ$^+$, and these were tamoxifen-dependent (Figure 7F). Using our tamoxifen regime, fetuses were reabsorbed from 15.5 dpc onward, although coinjecting tamoxifen and progesterone (used to offset tamoxifen toxicity) yielded nine pups that survived to adulthood after Caesarian section and cofostering, five of which were of the relevant genotype. In the two tamoxifen-treated mice, 19.5% ± 6% of colonies were LACZ$^+$ (Figure 7G). These data strongly support an epicardial origin for cCFU-Fs. While neither the *Gata5-Cre* nor *Wt1^{CreERT2}* lineage tags were epicardial specific, only the epicardium, pericardium, and liver capsule and mesenchyme were uniquely at the intersection of their respective expression domains (Figure S7H).

cCFU-F Progenitors Derive from the Epicardial Lineage after MI

We performed colony assays on *Gata5-Cre × Z/EG* progeny 5 days and 30 days after induction of MI at 8 weeks (Figure 7H). *Gata5-Cre* is activated in epicardium upon MI, although cCFU-F numbers only increased slightly in the post-MI period (data not shown). Importantly, we found no significant dilution of the proportions of GFP$^+$/LACZ$^-$ large colonies at either time point relative to sham-operated controls. These experiments do not exclude a minor contribution to adult cCFU-Fs from other lineages. However, they show that the epicardium makes the major contribution in both health and disease.

DISCUSSION

We define here a population of MSC-like CPCs in the developing and adult heart. As for other MSC-like populations, cCFU-Fs are multipotent and capable of long-term in vitro growth. This putative stem cell population likely occupies a perivascular, adventitial niche, and is distinct from the majority cKIT$^+$ CPC population, which is CD45$^+$. cCFU-Fs are also distinct from cardiac mesoangioblasts, which are a perivascular progenitor seemingly related to MSCs but with much more limited potency (Galvez et al., 2008). Embryo studies and CRE-lineage tracking strongly point to a proepicardial/epicardial origin for cCFU-Fs. cCFU-Fs also showed a CRE lineage signature distinct from that of bmCFU-Fs and aCFU-F, demonstrating the diverse postgastrulation lineage origins of CFU-F populations in different organs. We detected no flux with adult BM-derived cells in health or after MI. Conditional CRE experiments defined a population of epicardial cells at 9.5–11.5 dpc that are present as cCFU-Fs in the adult heart, supporting the long-term persistence of these cells in vivo, likely by self-renewal.

CFU-Fs have been isolated from the brain, spleen, liver, kidney, lungs, skeletal muscle, thymus, adipose tissue, pancreas, skin, and BM (Crisan et al., 2008; da Silva Meirelles et al., 2008). Our study shows that CFU-Fs can also be isolated from heart. Their long-term growth and multipotentiality in vitro, and similarities in transcriptome and cell surface antigen profile with BM MSCs (Pelekanos et al., 2011), strongly suggest that solid organ CFU-Fs are related to BM MSCs in evolution and function.
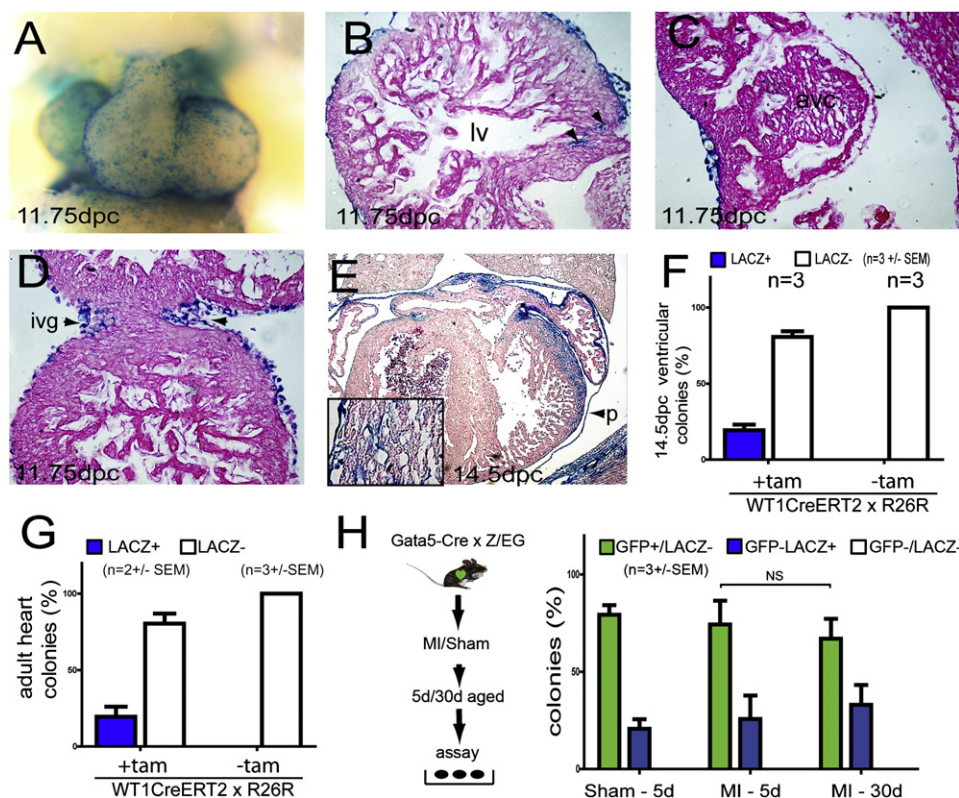
Figure 7. Conditional Lineage Tracing with *WT1*^{CreERT2}

(A–E) LacZ staining in 11.75 dpc embryonic hearts from *Wt1*^{CreERT2} × *R26R* crosses shows patchy labeling of epicardium, interstitial and interventricular groove (ivg) cells, and pericardium (p) (arrowheads).

(F and G) Percentage of LACZ$^+$ or LACZ$^-$ cCFU-F large colonies from *Wt1*^{CreERT2} × *R26R* progeny at 14.5 dpc (F) and adult whole hearts (G).

(H) Percentage of GFP$^+$/LACZ$^-$ cCFU-F large colonies from *Gata5Cre* × *Z/EG* adult mice was not significantly different 5 or 30 days post-MI compared to sham-operated controls.

Error bars = SEM between independent experiments. See also Figure S7.

MSCs can be mobilized in hypoxia (Rochefort et al., 2006) and pregnancy (O'Donoghue et al., 2004), or after treatment with G-CSF, albeit in the latter case, showing compromised growth potential (Lund et al., 2008). In this study, however, we have proven by two independent means that the vast majority of adult cCFU-Fs are not in flux with the BM. First, tagged whole BM transplantation studies found no contribution of BM to cCFU-Fs. While CFU-Fs from both BM and heart were radio-sensitive, nonirradiated cells from donor BM could substantially repopulate the marrow HSC and CFU-F compartments and rescue lethality, but neither population could supplement diminished cCFU-F numbers in lead-shielded hearts, even after aging, induction of MI, or mobilization of BM stem cells with G-CSF.

Additional support for a non-BM origin for cCFU-Fs comes from comparative mapping of CRE lineage signatures in cardiac, aortic, and BM CFU-Fs. While the majority of cCFU-Fs were marked by *Gata5*-CRE and *Mesp1*-CRE, not a single bmCFU-F was tagged with these transgenes. Furthermore, the proportion of cCFU-Fs marked by *Gata5*-CRE was not diluted by unmarked CFU-Fs during the substantial influx of BM cells to the heart after MI. We conclude that BM and cCFU-Fs do not share a common postgastrulation lineage in the adult. While our aging protocol covers approximately one-quarter of the life of a

laboratory mouse, we cannot exclude a minor contribution from BM or other tissues over a longer time frame. A population of blood-borne human cells termed circulating mesoangioblasts appears to be MSC-like and increases in hearts of children undergoing heart surgery and cardiopulmonary bypass (Iwasaki et al., 2011). Like cCFU-Fs, they do not derive from BM, although they do express NG2 and CD31 and have a more limited potency than cCFU-Fs. BM does contain cells with cardiac potential (Xaymardan et al., 2009), and blood-borne cells are known to seed vessels and myocardium during fetal development in the chick (Zhang et al., 2006). However, whether such cells contribute to cardiac repair in the adult has been highly controversial, and much further work needs to be done on their nature, origins, and ultimate fate.

The diversity of CRE strains used in our lineage tracing studies allowed us to exclude a neuroectodermal, endodermal, and CM origin for the majority of cCFU-Fs. Furthermore, the *Gata5-Cre* and conditional *Wt1*^{CreERT2} strains, while not absolutely specific to the epicardial lineage, have only the epicardium and cell layers in immediate continuity with epicardium as their intersecting lineages. A proepicardial/epicardial origin for cCFU-Fs is strongly supported by our embryo data, which shows that colony-forming ability was restricted to the *Pdgfra*-GFP$^+$ cell

fraction of the proepicardium at 9.5 dpc and subsequent stages of ventricular development, during which *Pdgfra*-GFP marks the epicardium and its descendant lineages. Recent data suggest that the majority of mouse coronary ECs bud from the endothelial layer of the sinus venosus, which lies in close proximity to the proepicardium. However, we can exclude sinus venosus endothelium as a source of cCFU-Fs, since this layer is negative for *Pdgfra*-GFP (data not shown) and the S+P+ population is negative for the endothelial marker CD31.

The mammalian proepicardium and epicardium gives rise to the perivascular (SM) components of the coronary vessels, as well as interstitial fibroblasts (Carmona et al., 2010). The origin of cCFU-Fs in the epicardium and their likely contribution to SM layers of the coronary vessels and aorta in the fetus (divined via surrogate lineage tracing using *Pdgfra*-GFP mice) suggests that cCFU-Fs arise as a self-organizing component of the coronary vascular bed that persists throughout fetal and adult life. The distinct and indeed multi-germ layer origins of different CFU-F populations (heart, BM, aorta) correlates with the diverse and multi-germ layer origins of different vascular beds in the embryo, including their mural components that arise in the mesothelia that are associated with distinct organs (Armulik et al., 2011). We propose, therefore, that CFU-Fs have their origins in development in organ-cognate vascular progenitor beds, likely including mesothelia, which give rise to vascular mural cells of coelomic organs, and neural crest.

While the full range of lineage descendants of cCFU-Fs remain to be mapped, they likely give rise to SM and other mural components of the coronary vasculature, matrix-forming stromal cells such as fibroblasts and myofibroblasts, and other sorts of vascular and interstitial cells. cCFU-Fs may retain features of the embryonic or adult epicardium, the latter gaining attention as an active player in cardiac repair. Indeed, ~5% of total *Pdgfra*-GFP⁺ cells persist in the adult epicardium, although only small and micro colony-forming cells without any large colony-forming cells were detected, suggesting that the adult epicardium carries a population of committed cardiovascular progenitors. The rare epicardial-derived CMs detected by Smart et al. (2011) in β4-thymosin-primed hearts after MI may be derived from these progenitors, although the mechanism of their occasional diversion to CMs by β4-thymosin is not clear. Nor is it clear whether interstitial cCFU-Fs give rise to CMs normally—however, cCFU-Fs can form CMs in vitro and after transplantation. Formal lineage tracing strategies will be needed to test this in vivo.

The multipotency of pericytes has been recognized since the 1980s (da Silva Meirelles et al., 2008), and cells purified from BM and other organs on the basis of pericyte markers bear many of the properties of MSCs, including clonogenic and serial replating ability and multilineage differentiation (Méndez-Ferrer et al., 2010). BM can also seed pericytes or their progenitors to tumors and ischemic tissue (Lamagna and Bergers, 2006). However, in the adult heart, *Pdgfra*-GFP⁺ FACS fractions that contain all cCFU-Fs, including the S+P+ fraction, are negative or very low for NG2 and other pericyte markers. We propose, therefore, that cCFU-Fs are distinct from pericytes, although they may give rise to pericytes that retain some growth potential and lineage plasticity in vitro.

cCFU-Fs show broad and trans-germ layer potency in vitro and in vivo, although they do not form teratomas and are

therefore not pluripotent by this definition. Other stem cell populations, such as those recently described in lung, also possess trans-germ layer potency, as appropriate for the needs of this organ (Kajstura et al., 2011). We propose a distinction between the highly canalized and evolutionarily constrained pluripotent state in ESCs, as reflective of the inner cell mass of the early embryo, and the highly plastic state of adult stem cells that must retain a preparedness to respond to diverse stimuli encountered in stress and injury. Understanding the molecular underpinnings of this distinction is a major priority in stem cell biology.

The concept of MSCs as perivascular stem cells in many organs has important implications for the way we view tissue homeostasis and repair. In humans, heart failure incidence is rising exponentially and novel therapies are being sought using cell therapy or regenerative strategies. The distinct local vascular-bed origins of MSC-like cells may lay a foundation for enhancing tissue repair based, on the one hand, on exploring common signaling pathways regulating their activation, survival, and deployment, and on the other hand, their distinct organ-specific origins and differentiative biases. Deeper dissection of the role of these cells in homeostasis and repair may yield new options for enhancing tissue regeneration.

EXPERIMENTAL PROCEDURES

Mice

Mouse strains were as described in Table S1. All experiments were overseen by the St Vincent's Hospital/Garvan Institute of Medical Research Animal Ethics Committee.

Gene/Protein Expression

Probes and antibodies and methods are as described in Table S2.

Cell Isolation and Culture

Mononuclear cells were isolated from dissected hearts and aorta via mincing of tissue before digesting it in 0.1% Collagenase type II (Worthington) in PBS at 37°C for 30 min, and having it be triturated mechanically at 10 min intervals and with myocyte debris removed with a 40 μm filter. Dead cells were removed using MACS Dead Cell Removal kit (Miltenyibiotec) before incubation with fluorophore-conjugated antibodies (Table S1) and sorting using FACS Aria or FACS Canto (Becton and Dickinson) cytometers. For CFU-F assays, 5,000 S+P+ cells were plated on tissue culture plastic or Poly-L-lysine-coated coverslips and cultured in αMEM (GIBCO) plus 20% FCS for 12 days.

BM Transplantation and Irradiation

Mice were irradiated with 9 Gy using an X-RAD 320 (Precision Xray Inc, USA) irradiator. For BM reconstitution, 2.5×10^7 fresh GFP-tagged BM cells from Bag1 donors (Table S1) were injected in an aseptic manner into the tail vein. After 4–6 weeks, reconstitution was assessed. Lead shielding was 3 mm.

MI

Mice were anaesthetized by intraperitoneal injection of Ketamine (100 mg/kg) and Xylazine (20 mg/kg), intubated and ventilated with 30% O_2. Through a thoracotomy the left coronary artery was ligated. For G-CSF administration, 300 μg/kg rhG-CSF (Amgen, Australia) was injected subcutaneously for 6 days.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Figures S1–S7, Tables S1 and S2, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2011.10.002.

REFERENCES

Armulik, A., Genové, G., and Betsholtz, C. (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev. Cell 21, 193–215.

Beltrami, A.P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., et al. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114, 763–776.

Bergmann, O., Bhardwaj, R.D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B.A., Druid, H., et al. (2009). Evidence for cardiomyocyte renewal in humans. Science 324, 98–102.

Bersell, K., Arab, S., Haring, B., and Kühn, B. (2009). Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 138, 257–270.

Bonyadi, M., Waldman, S.D., Liu, D., Aubin, J.E., Grynpas, M.D., and Stanford, W.L. (2003). Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. Proc. Natl. Acad. Sci. USA 100, 5840–5845.

Caplan, A.I. (2007). Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J. Cell. Physiol. 213, 341–347.

Carmona, R., Guadix, J.A., Cano, E., Ruiz-Villalba, A., Portillo-Sánchez, V., Pérez-Pomares, J.M., and Muñoz-Chápuli, R. (2010). The embryonic epicardium: an essential element of cardiac development. J. Cell. Mol. Med. 14, 2066–2072.

Christoffels, V.M., Mommersteeg, M.T.M., Trowe, M.-O., Prall, O.W.J., de Gier-de Vries, C., Soufan, A.T., Bussen, M., Schuster-Gossler, K., Harvey, R.P., Moorman, A.F.M., and Kispert, A. (2006). Formation of the venous pole of the heart from an Nkx2-5-negative precursor population requires Tbx18. Circ. Res. 98, 1555–1563.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3, 301–313.

da Silva Meirelles, L., Caplan, A.I., and Nardi, N.B. (2008). In search of the in vivo identity of mesenchymal stem cells. Stem Cells 26, 2287–2299.

Ebihara, Y., Masuya, M., Larue, A.C., Fleming, P.A., Visconti, R.P., Minamiguchi, H., Drake, C.J., and Ogawa, M. (2006). Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes. Exp. Hematol. 34, 219–229.

Fazel, S., Cimini, M., Chen, L., Li, S., Angoulvant, D., Fedak, P., Verma, S., Weisel, R.D., Keating, A., and Li, R.K. (2006). Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. J. Clin. Invest. 116, 1865–1877.

Fazel, S.S., Chen, L., Angoulvant, D., Li, S.-H., Weisel, R.D., Keating, A., and Li, R.K. (2008). Activation of c-kit is necessary for mobilization of reparative bone marrow progenitor cells in response to cardiac injury. FASEB J. 22, 930–940.

Friedenstein, A.J., Chailakhjan, R.K., and Lalykina, K.S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 3, 393–403.

Galvez, B.G., Sampaolesi, M., Barbuti, A., Crespi, A., Covarello, D., Brunelli, S., Dellavalle, A., Crippa, S., Balconi, G., Cuccovillo, I., et al. (2008). Cardiac mesoangioblasts are committed, self-renewable progenitors, associated with small vessels of juvenile mouse ventricle. Cell Death Differ. 15, 1417–1428.

Hsieh, P.C., Segers, V.F., Davis, M.E., MacGillivray, C., Gannon, J., Molkentin, J.D., Robbins, J., and Lee, R.T. (2007). Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat. Med. 13, 970–974.

Iwasaki, M., Koyanagi, M., Kossmann, H., Monsefi, N., Rupp, S., Trauth, J., Paulus, P., Goetz, R., Momma, S., Tjwa, M., et al. (2011). Hepatocyte growth factor mobilizes non-bone marrow-dividing circulating mesoangioblasts. Eur. Heart J. 32, 627–636.

Kajstura, J., Urbanek, K., Perl, S., Hosoda, T., Zheng, H., Ogórek, B., Ferreira-Martins, J., Goichberg, P., Rondon-Clavo, C., Sanada, F., et al. (2010). Cardiomyogenesis in the adult human heart. Circ. Res. 107, 305–315.

Kajstura, J., Rota, M., Hall, S.R., Hosoda, T., D'Amario, D., Sanada, F., Zheng, H., Ogórek, B., Rondon-Clavo, C., Ferreira-Martins, J., et al. (2011). Evidence for human lung stem cells. N. Engl. J. Med. 364, 1795–1806.

Kern, S., Eichler, H., Stoeve, J., Klüter, H., and Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24, 1294–1301.

Kikuchi, K., Holdway, J.E., Major, R.J., Blum, N., Dahn, R.D., Begemann, G., and Poss, K.D. (2011). Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. Dev. Cell 20, 397–404.

Lamagna, C., and Bergers, G. (2006). The bone marrow constitutes a reservoir of pericyte progenitors. J. Leukoc. Biol. 80, 677–681.

Laugwitz, K.L., Moretti, A., Lam, J., Gruber, P., Chen, Y., Woodard, S., Lin, L.Z., Cai, C.L., Lu, M.M., Reth, M., et al. (2005). Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433, 647–653.

Li, M., Naqvi, N., Yahiro, E., Liu, K., Powell, P.C., Bradley, W.E., Martin, D.I., Graham, R.M., Dell'Italia, L.J., and Husain, A. (2008). c-kit is required for cardiomyocyte terminal differentiation. Circ. Res. 102, 677–685.

Lie-Venema, H., Eralp, I., Markwald, R.R., van den Akker, N.M., Wijffels, M.C.E.F., Kolditz, D.P., van der Laarse, A., Schalij, M.J., Poelmann, R.E., Bogers, A.J.J.C., and Gittenberger-de Groot, A.C. (2008). Periostin expression by epicardium-derived cells is involved in the development of the atrioventricular valves and fibrous heart skeleton. Differentiation 76, 809–819.

Linke, A., Müller, P., Nurzynska, D., Casarsa, C., Torella, D., Nascimbene, A., Castaldo, C., Cascapera, S., Böhm, M., Quaini, F., et al. (2005). Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc. Natl. Acad. Sci. USA 102, 8966–8971.

Louis, S.A., Rietze, R.L., Deleyrolle, L., Wagey, R.E., Thomas, T.E., Eaves, A.C., and Reynolds, B.A. (2008). Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay. Stem Cells 26, 988–996.

Lund, T.C., Tolar, J., and Orchard, P.J. (2008). Granulocyte colony-stimulating factor mobilized CFU-F can be found in the peripheral blood but have limited expansion potential. Haematologica 93, 908–912.

Ma, Q., Zhou, B., and Pu, W.T. (2008). Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity. Dev. Biol. 323, 98–104.

Martínez-Estrada, O.M., Lettice, L.A., Essafi, A., Guadix, J.A., Slight, J., Velecela, V., Hall, E., Reichmann, J., Devenney, P.S., Hohenstein, P., et al. (2010). Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. Nat. Genet. 42, 89–93.

Mellgren, A.M., Smith, C.L., Olsen, G.S., Eskiocak, B., Zhou, B., Kazi, M.N., Ruiz, F.R., Pu, W.T., and Tallquist, M.D. (2008). Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. Circ. Res. 103, 1393–1401.

Méndez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466, 829–834.

Merki, E., Zamora, M., Raya, A., Kawakami, Y., Wang, J., Zhang, X., Burch, J., Kubalak, S.W., Kaliman, P., Belmonte, J.C., et al. (2005). Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation. Proc. Natl. Acad. Sci. USA 102, 18455–18460.

Messina, E., De Angelis, L., Frati, G., Morrone, S., Chimenti, S., Fiordaliso, F., Salio, M., Battaglia, M., Latronico, M.V., Coletta, M., et al. (2004). Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ. Res. 95, 911–921.

Mouquet, F., Pfister, O., Jain, M., Oikonomopoulos, A., Ngoy, S., Summer, R., Fine, A., and Liao, R. (2005). Restoration of cardiac progenitor cells after myocardial infarction by self-proliferation and selective homing of bone marrow-derived stem cells. Circ. Res. 97, 1090–1092.

O'Donoghue, K., Chan, J., de la Fuente, J., Kennea, N., Sandison, A., Anderson, J.R., Roberts, I.A., and Fisk, N.M. (2004). Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. Lancet 364, 179–182.

Oh, H., Bradfute, S.B., Gallardo, T.D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L.H., Behringer, R.R., Garry, D.J., et al. (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc. Natl. Acad. Sci. USA 100, 12313–12318.

Pelekanos, R., Li, J., Gongora, M., Chandrakanthan, V., Scown, J., Suhaimi, M., Grooke, G., Christensen, M.E., Doan, T., Rice, A.M., et al. (2011). Comprehensive transcriptome and immunophenotype analysis of renal and cardiac MSC-like populations supports strong congruence with bone marrow MSC despite maintenance of distinct identities. Stem Cell Research 8, 58–73.

Rochefort, G.Y., Delorme, B., Lopez, A., Hérault, O., Bonnet, P., Charbord, P., Eder, V., and Domenech, J. (2006). Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. Stem Cells 24, 2202–2208.

Sarugaser, R., Hanoun, L., Keating, A., Stanford, W.L., and Davies, J.E. (2009). Human mesenchymal stem cells self-renew and differentiate according to a deterministic hierarchy. PLoS ONE 4, e6498.

Shenje, L.T., Field, L.J., Pritchard, C.A., Guerin, C.J., Rubart, M., Soonpaa, M.H., Ang, K.L., and Galinanes, M. (2008). Lineage tracing of cardiac explant derived cells. PLoS One 3, e1929.

Simmons, P.J., Przepiorka, D., Thomas, E.D., and Torok-Storb, B. (1987). Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature 328, 429–432.

Smart, N., Bollini, S., Dubé, K.N., Vieira, J.M., Zhou, B., Davidson, S., Yellon, D., Riegler, J., Price, A.N., Lythgoe, M.F., et al. (2011). De novo cardiomyocytes from within the activated adult heart after injury. Nature 474, 640–644.

Stottmann, R.W., Choi, M., Mishina, Y., Meyers, E.N., and Klingensmith, J. (2004). BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. Development 131, 2205–2218.

Takashima, Y., Era, T., Nakao, K., Kondo, S., Kasuga, M., Smith, A.G., and Nishikawa, S. (2007). Neuroepithelial cells supply an initial transient wave of MSC differentiation. Cell 129, 1377–1388.

Tomita, Y., Matsumura, K., Wakamatsu, Y., Matsuzaki, Y., Shibuya, I., Kawaguchi, H., Ieda, M., Kanakubo, S., Shimazaki, T., Ogawa, S., et al. (2005). Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J. Cell Biol. 170, 1135–1146.

Wu, S.M., Fujiwara, Y., Cibulsky, S.M., Clapham, D.E., Lien, C.-L., Schultheiss, T.M., and Orkin, S.H. (2006). Developmental Origins of a Bipotential Myocardial and Smooth Muscle Cell Precursor in the Mammalian Heart. Cell 127, 1137–1150.

Xaymardan, M., Cimini, M., Fazel, S., Weisel, R.D., Lu, W.-Y., Martin, U., Harvey, R.P., and Li, R.K. (2009). c-Kit function is necessary for in vitro myogenic differentiation of bone marrow hematopoietic cells. Stem Cells 27, 1911–1920.

Zamora, M., Männer, J., and Ruiz-Lozano, P. (2007). Epicardium-derived progenitor cells require beta-catenin for coronary artery formation. Proc. Natl. Acad. Sci. USA 104, 18109–18114.

Zhang, N., Mustin, D., Reardon, W., Almeida, A.D., Mozdziak, P., Mrug, M., Eisenberg, L.M., and Sedmera, D. (2006). Blood-borne stem cells differentiate into vascular and cardiac lineages during normal development. Stem Cells Dev. 15, 17–28.

Zhang, Y., Li, T.-S., Lee, S.-T., Wawrowsky, K.A., Cheng, K., Galang, G., Malliaras, K., Abraham, M.R., Wang, C., and Marbán, E. (2010). Dedifferentiation and proliferation of mammalian cardiomyocytes. PLoS ONE 5, e12559.

Zhou, B., Ma, Q., Rajagopal, S., Wu, S.M., Domian, I., Rivera-Feliciano, J., Jiang, D., von Gise, A., Ikeda, S., Chien, K.R., and Pu, W.T. (2008a). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454, 109–113.

Zhou, B., von Gise, A., Ma, Q., Rivera-Feliciano, J., and Pu, W.T. (2008b). Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. Biochem. Biophys. Res. Commun. 375, 450–453.

Zhou, B., von Gise, A., Ma, Q., Hu, Y.W., and Pu, W.T. (2009). Genetic fate mapping demonstrates contribution of epicardium-derived cells to the annulus fibrosis of the mammalian heart. Dev Biol. 338, 251–261.