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REVIEW

The Quest for the Adult Cardiac Stem Cell

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Over the past 2 decades, cardiac regeneration has evolved from an exotic fringe of cardiovascular biology to the forefront of molecular, genetic, epigenetic, translational, and clinical investigations. The unmet patient need is the paucity of self-repair following infarction. Robust regeneration seen in models such as zebrafish and newborn mice has inspired the field, along with encouragement from modern methods that make even low levels of restorative growth discernible, changing the scientific and technical landscape for effective counter-measures. Approaches under study to augment cardiac repair complement each other, and encompass grafting cells of diverse kinds, restarting the cell cycle in post-mitotic ventricular myocytes, reprogramming non-myocytes, and exploiting the dormant progenitor/stem cells that lurk within the adult heart. The latter are the emphasis of the present review. Cardiac-resident stem cells (CSC) can be harvested from heart tissue, expanded, and delivered to the myocardium as a therapeutic product, whose benefits may be hoped to surpass those achieved in human trials of bone marrow. However, important questions are prompted by such cells' discovery. How do they benefit recipient hearts? Do they contribute, measurably, as an endogenous population, to self-repair? Even if "no," might CSCs be targets for activation in situ by growth factors and other developmental catalysts? And, what combination of distinguishing markers best demarcates the cells with robust clonal growth and cardiogenic potential?

Key Words: Cardiac regeneration; Heart repair; Platelet-derived growth factor receptor-*α*; Stem cells

Cardiac Regeneration: Filling a Therapeutic Void

Heart disease has emerged as an auspicious target for regenerative medicine,[1–6] because of the sheer scope of the healthcare burden. Cardiovascular disorders are a paramount cause of death and disability, among which ischemic heart disease caused 13% of global mortality, or >7 million deaths worldwide in 2012.[7] Over the past 30 years, progress in treating ischemic heart disease has improved patient outcomes, reducing the early mortality from myocardial infarction. Yet, despite success in acutely restoring myocardial blood flow, the remaining cardiac damage predisposes to later heart failure.[8] This inexorable cause-effect relationship contributes to the mounting prevalence of heart failure, predicted to increase 50% within 15 years.[8–11] Hence, it may be imperative not merely to achieve timely reperfusion, the clinical state of the art, but also to reverse the existing tissue damage, to prevent the progressive dysfunction that follows.

In stark contrast, regeneration of the heart is robust in species such as the axolotl, newt, and zebrafish,[12–15] in which total limb regeneration even occurs. How the heart reconstitutes itself in these circumstances, at the cellular level, was long ambiguous, but recent evidence indicates that zebrafish generate new cardiomyocytes by proliferation of pre-existing ones as their basis for scarless healing. This Promethean ability is also found, albeit transiently, in mice after birth,[16,17] where it is contingent on neonatal macrophages[18] and the *Hippo* effector *Yap*.[19]

Whether substantial cardiac regeneration might also exist in adult mammals, and by what means if so, has been more contentious. A pulse-chase analysis of ^{14}C incorporated into cardiac DNA during the mid-century decades of atmospheric atom bomb testing confirmed that human cardiomyocytes renew themselves, albeit at the low rate of 1% per year in young adults and just 0.45% per year in the elderly.[20] Claims of much more extensive myocyte cycling have been retracted.[21] Analogous studies of adult mice, by stable isotope labeling and multi-isotope imaging mass spectrometry, confirmed cardiomyocyte renewal through the division of pre-existing cardiomyocytes.[22] Cardiomyocytes in young adults were labeled by [^{15}N]thymidine at an annual rate of 5.5%, most of which was polyploidization. Mononucleated [^{15}N]$^{+}$ myocytes also were detected (annual rate, 0.76%), more indicative of cell division. Conversely, studies using flow cytometry of isolated myocyte nuclei[23] and transgenic fluorescent anillin to mark cytokinesis[24] challenge the conclusions that measurable myocyte turnover occurs in the adult mouse heart and that repopulation occurs via pre-existing myocytes.

What is the alternative to this seeming impasse? Fate-mapping using the Cre/lox system provides a means to indelibly tag cells in model organisms, based on a gene's expression in a chosen cell type, at a chosen time, and suggests that the adult mammalian heart regenerates itself via the lineage decisions of resident progenitor/stem cells.[25–27] The constellation of

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relevant markers to demarcate the cells in question is introduced next, with details of these fate-mapping studies subsequently.

Signatures of the Cardiac Stem Cell (CSC)

A longish list of potential identifiers has accumulated, over the past 12 years, to describe, define, isolate, and purify the formerly unsuspected adult CSC.[1,5] What criteria are necessary and sufficient for "stemness", and what tests provide reliable proof? Stem cells reproduce themselves (are self-renewing) and give rise to differentiated cells. Growth potential is commonly assessed as the ability to form colonies derived from single cells (clones) and capable of long-term propagation. Surprisingly, few studies have assessed the cloning efficiency of freshly isolated cardiac cells, most relying on prior adaptation to cell culture to evoke clonability. Equally, little work has systematically compared the molecular profiles of cloned cells vs. the fresh starting cells, or investigated clonal variation and its basis. The value of clonal analysis is highlighted by the inherent inability of purified cells to prove multilineage potential: no matter how purified, the population might harbor an indeterminate variety of progenitor cell types. Ultimately, measuring cells' plasticity (1 type of differentiated progeny, 2, 3, many, or all?) can only be resolved using offspring expanded from a single starting cell.

Through the combination of single-cell expression profiling and rigorous clonal analysis, we unmasked several aspects of the complex inter-relationships among the reported cardiac progenitor cell (CPC)/CSC, suggesting a multiparameter strategy for their phenotypic and functional identification, not reliance on a single determinant alone[28] (**Figure**). Early on, we used the orphan receptor stem cell antigen 1 (Sca1) as a surface marker to identify adult cardiac-resident cells having the capacity to differentiate into cardiomyocytes from an immature undifferentiated state when delivered to the infarcted mouse heart.[29] The logic of testing Sca1 was its known essential role in both hematopoietic stem cells and mesenchymal stem cells (MSCs), revealed by gene ablation in mice.[30] Its ligand remains unknown. Independent studies confirmed the capacity of cardiac Sca1$^+$ cells to undergo cardiomyocyte differentiation, in the setting of cell grafting, and as an endogenous population tracked by lineage-tracing tools.[26,27,31–34] Fewer investigations have scrutinized the vascular contribution of Sca1$^+$ CPCs. Interestingly, their differentiation into endothelium is impaired by cardiomyocyte-specific deletion of STAT3 or the cardiotoxic anticancer drug doxorubicin, attributable to deficient erythropoietin production by heart failure-prone cardiomyocytes.[35]

Sca1 is reportedly co-expressed by the CPCs denoted by other markers, including the hematopoietic growth factor receptor c-kit,[33,36,37] and may even be essential for the function of c-kit$^+$ CSCs.[33] The Sca1$^+$ population comprises a large fraction of adult cardiac non-myocytes; hence, inherently, additional markers were likely needed to best pinpoint the clone-forming cardiogenic cells. The anticipated utility of other markers was also foreseen, given that the Sca1 gene, *Ly6a*, has no counterpart in species other than the mouse.

In bone marrow, a "side population (SP)" phenotype based on expelling Hoechst dye 33342 identifies hematopoietic stem cells with long-term repopulating capacity.[38,39] Analogous dye-extruding SP cells are enriched 100-fold in the cardiac Sca1$^+$ population relative to cardiac non-myocytes as a whole, and showed potential for differentiation in vitro and in vivo into the endothelial, smooth muscle and cardiomyocyte lineages.[28,29,31,40–42] Cardiac SP cells also differentiate into non-cardiac mesodermal derivatives (adipocytes, osteocytes), suggesting a MSC-like phenotype.[42] Only CD31$^-$ cardiac SP cells had cardiomyogenic potential.[41] However, the range of potency must be assessed at a clonal level, and these early studies did not determine whether a single cardiac SP cell has multilineage capacity.

Sca1 is also expressed in a subset of murine cardiosphere-derived cells, which are obtained via non-adherent growth, are intentionally heterogeneous, and have well-demonstrated heart repair potential.[43] Sca1$^+$ CSC/CPC, as well as the more diverse cardiospheres, express several diagnostic MSC markers.[28,31,44–46] Functional MSC-like cells (cardiac colony-forming units, fibroblast: cCFU-F) have been identified in the adult heart, with individual colonies showing cardiovascular tri-lineage differentiation capacity.[45] More rarely, colonies had trans-germ layer capacity, giving rise to endodermal (hepatocyte) and neurectodermal (neuron, glia, oligondrocyte) fates. cCFU-Fs are especially enriched within the platelet-derived growth factor receptor (PDGFR)α^+ subset of cardiac Sca1$^+$ cells.[45]

Mesoangioblasts are vessel-derived progenitor cells from the aorta and solid organs, including the heart, that typically express MSC markers, c-kit, and Sca1,[47,48] which drive heart repair in part via secreted proteins' promoting host myocyte survival: fibroblast growth factor (FGF)-2, hepatocyte growth factor (HGF), and insulin-like growth factor (IGF)-1.[49] Pericytes derive from the microvasculature, possess many overlapping properties (CD44, CD146, CD13, CD49b and Sca-1) but not the endothelial lineage marker Flk-1,[50] make direct and indirect contributions to angiogenesis, and exert paracrine effects on host myocyte proliferation and resident CSCs.[51]

By contrast to these stem cell-associated criteria (surface markers, dye expulsion, growth properties), others have identified CPCs based on reporter genes for embryonic heart transcription factors such as *Isl1* or *Wt1*. *Isl1* is a well-established marker of multipotent CSCs during embryogenesis and in differentiating embryonic stem cells (ESCs),[52] but its relevance to adult cardiac plasticity is unproven. *Wt1* is expressed in embryonic epicardium, including the pro-epicardial organ that generates the blood vessels and stroma of the heart. *Wt1* is reactivated after myocardial infarction and augmented by thymosin β4, a peptide that promotes heart repair by epicardium-derived cells through mechanisms including vascular and cardiomyocyte differentiation.[53,54] Activated adult Wt1$^+$ cells are c-kit$^-$ but 80% Sca1$^+$.[54]

Sca1 at the Crossroads of CSC Markers

These and other investigations suggest that Sca1 might overlap in mice with most if not all reported criteria that denote the adult CPC/CSC. Despite this, a finer and more exact definition was required to pinpoint the cells of greatest clonal growth capacity, ascertain their differentiation potential as single-cell progeny, compare the molecular signature of cloned populations with the native starting cells, and clarify the 2- and 3-way relationships among the seemingly diverse progenitors sharing Sca1 expression as a feature in common.

One attempt to compare the molecular signature of CPCs was performed on cardiac c-kit$^+$, Sca1$^+$ and SP cells by microarray-based transcriptome analysis.[55] Sca1$^+$ as well as SP cells were found to be enriched for 2 cardiogenic transcritpion factors[56] (*Mef2c* and *Hand2*), suggesting they might have a committed cardiac lineage potential, as previously found.[29,41] Cardiac Sca1$^+$ and SP cells are more closely related to cardiomyocytes by hierarchical clustering than are c-kit$^+$ cells, which correlate

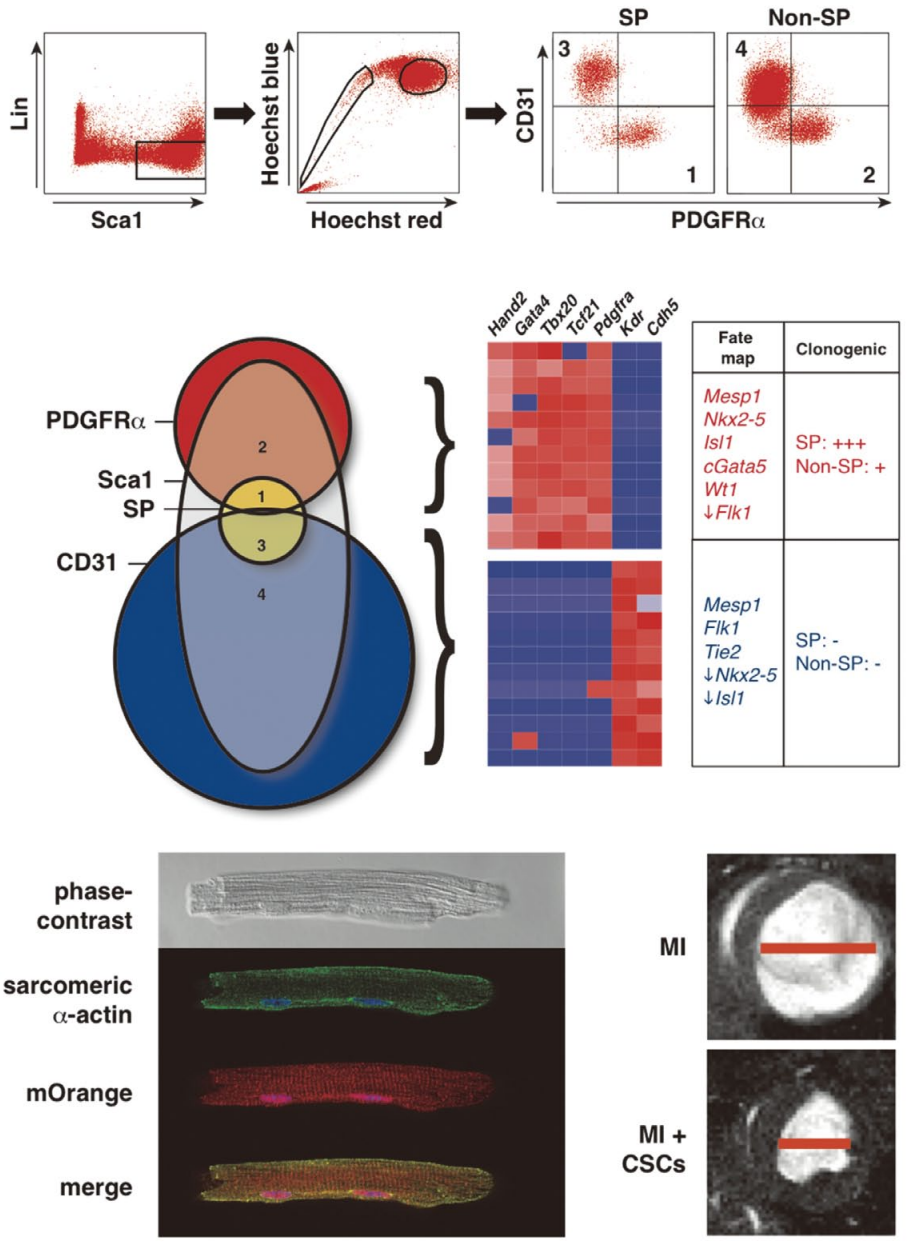

Figure. Platelet-derived growth factor receptor (PDGFR)*α* demarcates the clonogenic cardiogenic Sca1+ cell. (**Upper**) Hematopoietic lineage (Lin) negative non-myocytes from dissociated adult mouse myocardium purified by flow-sorting based on Sca1 expression, Hoechst dye efflux, and reciprocal expression of PDGFR*α* vs. CD31. (**Middle**) Regardless of their side population (SP) phenotype, PDGFR*α*+ cells expressed a cardiogenic gene signature (representative single-cell qRT-PCR results are shown), arose from ancestors expressing cardiac transcription factors, and were highly clonogenic. Sectors 1–4 from the flow cytometry results are mapped to the corresponding regions of the Venn diagram. (**Lower left**) Donor-derived cardiomyocyte 12 weeks after stem cell grafting. Single-cell progeny from the illustrated clone also demonstrated vascular smooth muscle and endothelial differentiation and incorporation into vascular structures. (**Lower right**) Grafted cardiac stem cells reduce adverse remodeling. End-systolic images are shown by 9.4 Tesla magnetic resonance tomography. MI, myocardial infarction. Adapted from Noseda M, et al.[28]

least with cardiomyocytes and other CSCs, perhaps indicating a more primitive population.[55] These findings are consistent with the presence by qRT-PCR of *Hand2*, *Mef2c*, and most other essential cardiogenic transcription factors in the Sca1+ population.[29] In contrast, relative to bone marrow cells, cCFU-Fs lack enrichment for cardiogenic transcription factors excepting *Mef2c*.[45,46] Differences between studies may be related to the cell isolation methods, which might either select or provoke distinguishable phenoptypes, the techniques used to profile gene and protein expression, or dilutional effects in mixed populations.

The intricate relationship among CPCs having Sca1 in

Table. Clinical Trials of Cardiac Repair With Heart-Derived Progenitor/Stem Cells

Study	CADUCEUS (CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction)	SCIPIO (Cardiac Stem Cell Infusion in Patients With Ischemic CardiOmyopathy)	TICAP (Transcoronary Infusion of Cardiac Progenitor Cells in Patients With Single Ventricle Physiology)	ALCADIA (AutoLogous Human CArdiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy)
Clinicaltrials.gov identifier	NCT00893360	NCT00474461	NCT01273857	NCT00981006
Country	USA	USA	Japan	Japan
Principal investigator	Eduardo Marban, Cedars-Sinai Medical Center	Roberto Bolli, University of Louisville	Hidemasa Oh, Okayama University	Hiroaki Matsubara, Kyoto Prefectural University of Medicine
Type	Phase I	Phase I	Phase I	Phase I
Status	Completed, results reported	Completed, results reported	Completed, results reported	Completed, preliminary results reported
References	Makkar et al[91] Malliaris et al[92]	Bolli et al[93] Chugh et al[94]	Ishigami et al[95]	Takahara et al[96] (abstract)
Diagnosis	Ischemic LV dysfunction/ recent MI	Coronary artery disease/ CHF/ICM	HLHS, single ventricle, heart failure	CHF, ICM, ventricular dysfunction
Enrollment	31	33	14	6
Age, sex	18–80 years, both sexes	18–75 years, both sexes	≤6 years, both sexes	20–80 years, both sexes
Cell type	Autologous cardiosphere-derived cells from RV endocardial biopsy	Autologous c-kit$^+$ cardiac stem cells from RAA (magnetic sorting)	Autologous atrial cardiosphere-derived stem cells	Autologous human CD105$^+$/CD90$^+$ cardiosphere-derived cells, enriched for ESC markers and mesenchymal features
Intervention route	Intracoronary	Intracoronary	Intracoronary	Transepicardial, in and around scar at time of CABG
Dose	12.5 or 25×10^6 cells within 36 days of sampling; 3 injections lasting 15 min	1×10^6 cells 4 months after harvest; 4 injections lasting 3 min	0.3×10^6 cells/kg; up to 3 vessels; 1 month after Norwood-Glenn, Glenn, or Fontan procedure	0.5×10^6 cells/kg (20 sites), overlaid by gelatin hydrogel sheet containing 200 μg bFGF
Inclusion criteria				
NYHA class	1–2	2.1	NYUPHI 10	3.8 (mean)
LVEF	25–45%	<40%	Not applicable	15–35%
Results				
NYHA class improvement	No	Yes	Yes (NYUPHI 6.1 at 18 months)	Yes
LVEF improvement	Improved regional function but not LVEF at 1 year	13.7% at 1 year	9% at 18 months (RVEF)	12.1% at 6 months
Scar reduction	11% at 1 year	30% at 1 year	Not applicable	4% at 6 months

*Hidemasa Oh, personal communication. bFGF, basic fibroblast growth factor (FGF-2); CABG, coronary artery bypass graft; CHF, congestive heart failure; DCM, dilated cardiomyopathy; EF, ejection fraction; HLHS, hypoplastic left heart syndrome; ICM, ischemic cardiomyopathy; LV, left ventricle; MI, myocardial infarction; NYHA, New York Heart Association; NYUPHI, New York University Pediatric Heart Failure Index; RAA, right atrial appendage; RV, right ventricle.

(Table continued the next page.)

common was clarified using multiparametric flow cytometry, single-cell qRT-PCR, cell cloning by single-cell deposition, and a detailed analysis of clonal progeny, revealing subsets of cells and microheterogeneities that were previously obscured.[28] We undertook a multistep approach to enrich for defined subsets of interest (Figure). In this way, it was possible to identify cardiac Lin$^-$ Sca1$^+$ SP cells that co-express PDGFRα as unprecedentedly clonogenic (efficiency, 30%). Their cloned progeny faithfully retain the molecular signature of fresh cells and have tri-lineage potential for the major differentiated cell types of the heart after grafting. Our findings reconcile many prior reports on the adult CSC and are summarized as an explanatory Venn diagram (Figure).

Under our current conditions, Sca1 is detected in nearly one-third of the hematopoietic lineage-negative, cardiomyocyte-depleted, stromal fraction of the adult mouse heart. Size alone suggests that Sca1 encompasses a heterogeneous pool of cardiac cells, as further shown by flow cytometry even in the earliest reports.[29] Indeed, Lin$^-$ Sca1$^+$ cells can be fractionated into 2 subpopulations that largely are non-overlapping and mutually exclusive: PDGFRα^+/CD31$^-$ and PDGFRα^-/CD31$^+$ cells, the latter being roughly 4-fold more numerous. At the other extreme of abundance, SP cells when gated stringently represent 1–3% of the overall Sca1$^+$ cells, likewise existing as dichotomous PDGFRα^+/CD31$^-$ and PDGFRα^-/CD31$^+$ forms.[28,29,41,42] Compared with the "main" population (Non-SP) cells, SP cells are enriched 4-fold for PDGFRα^+.[28] Notably, PDGFRα is a key marker of cardiac specification in ESCs and flow-sorted cells from E7–7.5 embryos.[57,58]

Single-Cell Gene Expression Profiles

By qRT-PCR for a panel of 45 genes in freshly isolated single cells, the phenotypic dichotomy defined by PDGFRα and CD31 in the broad Sca1$^+$ population and among Sca1$^+$ SP cells correlates precisely with a sharp partition in the molecular

Study	PERSEUS (Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease)	ALLSTAR (Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration)	DYNAMIC (Dilated cardiomYopathy iNtervention With Allogeneic Myocardlally-regenerative Cells)
Clinicaltrials.gov identifier	NCT01829750	NCT01458405	NCT02293603
Country	Japan	USA	USA
Principal investigator	Hidemasa Oh, Okayama University	Andrew Hamer, Capricor	Rajendra Makkar, Cedars-Sinai Heart Institute; Andrew Hamer, Capricor
Type	Phase II	Phase I and II	Phase I
Status	On-going	On-going	On-going
References	Not applicable	Not applicable	Not applicable
Diagnosis	HLHS, single RV, single LV	MI	DCM, ICM, non-ischemic cardiomyopathy, CHF
Enrollment	34	274	42
Age, sex	≤20 years, both sexes	≥18 years, both sexes	≥18 years, both sexes
Cell type	Autologous atrial cardiosphere-derived stem cells	Allogeneic cardiosphere-derived cells CAP-1002 (from whole hearts)	Allogeneic cardiosphere-derived cells CAP-1002
Intervention route	Intracoronary	Intracoronary	Sequential intracoronary infusion in up to 3 coronary arteries supplying 3 major cardiac territories
Dose	0.3×10^6 cells/kg; 1 month after Norwood-Glenn, Glenn, or Fontan procedure	25×10^6 cells; single dose by intracoronary infusion	Phase Ia: stepwise dose escalation; up to 3 coronary arteries supplying 3 major heart territories
Inclusion criteria			
NYHA class	NYUPHFI 13–15*	≤3	3 or ambulatory 4
LVEF	<60%	≤45%	<35%
Results			
NYHA class improvement	Not available	Not available	Not available
LVEF improvement	Not available	Not available	Not available
Scar reduction	Not available	Not available	Not available

signature.[28] Cells expressing PDGFRα are enriched for *Pdgfra* mRNA, pluripotency markers (*Nanog*, *Oct4*, *Klf4*), and genes encoding essential cardiogenic transcription factors (*Gata4/6*, *Tbx5/20*, *Hand2*, *Mef2a/c*) but not sarcomeric genes. Because several other cardiac transcription factors are silent (*Isl1*, *Nkx2–5*), the cells resemble an incomplete form (*forme fruste*) of the multipotent CSC during embryogenesis[56] or pluripotent cells' differentiation to a cardiac fate.[59] These PDGFRα^+ cells are largely negative for endothelial lineage markers (*Flk1*, *Cdh5*, *Vwf*). Conversely, Sca1$^+$ cells lacking *Pdgfra*/PDGFRα, but expressing CD31, do not express *Gata*, *Tbx* and *Hand* genes, being enriched for endothelial lineage markers instead.[28]

This dichotomous signature immediately suggests a mechanistic basis for the divergent cardiogenic potential reported for CD31$^-$ and CD31$^+$ SP cells.[41] Indeed, dissecting the Sca1$^+$ cells into 4 populations (combining Hoechst 33342, PDGFRα, and CD31), the expression profile of incomplete cardiac mesoderm segregates precisely with PDGFRα, independently of SP status.[28]

As mentioned, this Sca1$^+$ PDGFRα^+ consensus signature encompasses many cardiac transcription factors that are essential for cardiogenesis during development.[56] However, single-cell profiling revealed that individual fresh cells express, chiefly, just 2 or 3 of the core factors *Gata4*, *Hand2*, *Mef2c*, and *Tbx5*, in various combinations.[28] This level of microheterogeneity was previously unknown, and the mosaic phenotype of fresh cells was recapitulated in their clonal progeny. For a discussion of molecular mechanisms that might underlie mosaic gene expression see Suter et al[60] and Chang et al.[61] When forcibly expressed, none of the single factors *GATA4*, *MEF2C*, or *TBX5* was sufficient to trigger the cells' differentiation program, whereas cardiac gene and protein expression was provoked by *TBX5* in concert with *MYOCD* – a co-activator missing from the cells at baseline.[62]

Stratifying Clonal Growth

The initially reported clonogenic potential of cardiac Sca1$^+$ cells was as 0.1%, and long-term expandable cells just 0.03%,[63] consistent with several explanations, including innately poor growth potential, inadequate culture conditions, and heterogeneity of the cells tested. Most prior attempts to show clonal growth of CSCs with other markers, including c-kit and the SP phenotype, relied on pre-adaptation of cells in culture, feeder

layers, limiting dilution, or colony formation,[31,45,64] rather than cloning fresh single cells deposited by flow-sorting. Under the latter conditions, each droplet is individually and unambiguously interrogated, to ensure it does not contain a doublet or aggregate.

Based on preparative cell sorting with various combinations of the discussed identifiers, single cells were deposited in 96-well plates to assess the subpopulations' clonal growth, self-renewal, and multilineage potential.[28] SP status was a potent predictor of clonal growth under these exacting conditions, as seen earlier by colony formation in methylcellulose.[41] However, the SP phenotype was dispensible: PDGFRα^+ Non-SP cells were clonogenic (2% efficiency in physiological hypoxia), yet the combined PDGFRα^+ SP phenotype was highly synergistic (cloning efficiency >25%).[28] Cells lacking PDGFRα but expressing CD31 were rarely if ever clonable under our conditions, even if positive for the SP phenotype. Cloned Sca1$^+$ SP cells could be propagated for over 1 year, retained the cognate features (Sca1, SP status), maintained the cardiogenic gene signature, and were enriched for generating secondary clones, features together indicating self-renewal.[28]

ATP-binding cassette transporters that mediate the SP dye-efflux phenotype include Abcg2 and Mdr1. Both were expressed at equal levels in SP and Non-SP cells, suggesting that differences in pump activity, not expression, might distinguish the 2 states.[28]

Lineage Potential and Cell Therapy

We emphasize the importance of our clonogenicity data, not merely as a metric of "stemness", but more importantly as a tool to assess multilineage differentiation in the progeny of single cells. All the clones of Sca1$^+$ SP cells tested expressed *Pdgfra* mRNA and PDGFRα.[28] Within 2 weeks of cardiac grafting, molecular markers of tri-lineage potential emerged (cardiac troponin I, myosin light chain 2v, sarcomeric α-actin, sarcomeric myosin heavy chain, CD31, von Willebrand factor, smooth muscle myosin heavy chain). At 12 weeks, donor-derived differentiating cells were incorporated into the endothelial and smooth muscle layers of blood vessels, and, if cardiac, were typically rod-shaped, bi-nucleated, and striated (**Figure**). Thus, single-cell derivatives have tri-lineage capacity and can override the "developmental arrest" of a mosaic, incomplete cardiogenic program.

As in related studies using other defined cells or cardiospheres,[31,65–69] grafted cells retained for 3 months were vanishingly rare. Nonetheless, serial magnetic resonance imaging (MRI) demonstrated that cloned Sca1$^+$ SP cells delivered to myocardium at the time of infarction successfully reduced infarct size, increased left ventricular ejection fraction, and reduced adverse remodeling.[28] This recurring combination of marked functional improvement despite meager cell retention plausibly suggests that, under the present conditions of cell delivery, a paracrine mechanism of benefit predominates.[31,65–69] Paracrine effects of clonally expanded Sca1$^+$ cells on cardiomyocyte survival and angiogenesis are attributable in part to soluble VCAM-1 and soluble junctional adhesion molecule-A.[31,70] Related effector mechanisms of grafted heart-derived cells include enhancing cardiomyocyte proliferation and activating host CSCs, via HGF, IGF-I, stromal cell-derived factor-1, angiopoietin-2, vascular endothelial growth factor,[66,68,71,72] and exosomes,[73–75] secreted extracellular vesicles containing microRNAs that regulate the recipient tissue. Suppressing Sca1 (*Ly6a*) by RNA interference downregulates HGF and the benefits of cell transplantation on angiogenesis and myocyte survival.[63] The functional superiority of expanded cardiac Sca1$^+$ cells and cardiosphere-derived cells over bone marrow and non-cardiac MSCs in side-by-side comparisons of heart repair is at present ascribed to their paracrine potency.[66,70] Such findings are of heightened interest, given the failure of promising bone marrow trials in the most authoritative meta-analysis.[76] The benefits of c-kit$^+$ GATA^{high} CSCs on cardiomyocyte survival are negated by blocking IGF-I;[72] however, depleting c-kit$^+$ cells from cardiospheres has no effect on the observed improvements.[44]

In light of this emerging consensus, paracrine effects plausibly dominate not just in experimental studies delivering CSCs, but also in the emerging human trials (**Table**). Whereas an empirical case exists that mixed cell populations might be better therapeutically than homogeneous ones,[77–79] defining each component and its precise contribution may lead best to therapies that are stem cell-inspired yet ultimately cell-free. The greater extent of cardiomyocyte formation by heart-derived cells than other adult stem cells could matter more, once engraftment is durable. This prediction may require a tissue engineering solution,[80,81] not just the right cell, and remains to be tested directly.

Insights From Cell Origins

The developmental origin of adult CPCs has only recently received close study, confounding factors being the multitude of cells described and lack of an agreed marker. Among the a priori possibilities, cardiac-resident stem cells might arise as a dedifferentiated product of "lapsed" cardiomyocytes, a trans-differentiated product of bone marrow cells in the cardiac milieu, a derivative of migratory neural crest cells, offspring of the hemangioblast, or an undifferentiated remnant of the first or second heart field during embryogenesis. In our dissection of the cardiac Sca1$^+$ population, nearly all cells were fated by the primitive mesodermal transcription factor *Mesp1*, regardless of their SP status or PDGFRα Expression.[28] Little or no contribution was seen from committed hematopoietic cells (*Vav-Cre*), neural crest (*Wnt1-Cre*), or preformed cardiomyocytes (*Myh6-Cre*).

Intermediate levels of recombination were seen with tracers of mesoderm patterning and commitment to a cardiovascular fate.[28] Regardless of SP status, PDGFRα^+ and PDGFRα^- cells were partially derived from ancestors expressing *Nkx2–5* and *Isl1*, cardiogenic genes that are silent in these adult cells but demarcate bipotent and multipotent embryonic populations that form the heart and are indispensable for normal cardiogenesis.[82,83] Large differences between PDGFRα^+ and PDGFRα^- cells were found using markers of epicardial and vessel-associated origins: PDGFRα^+/CD31$^-$ cells were preferentially fated by the embryonic epicardial genes *Wt1* and *cGATA5*, whereas PDGFRα^-/CD31$^+$ cells arose chiefly from precursors expressing *Flk1* and *Tie2*.[28]

Does this possible signify a dual origin for Sca1$^+$ cells? With cardiospheres, using *Myh6-MerCreMer*×Z/EG mice, dedifferentiation of cardiomyocytes was noted as a source for a subset of the cells.[84] But, cardiospheres by design are heterogeneous, and more than 1 source is expected in this instance. A more parsimonious explanation for the fate map of PDGFRα^+ cells may be the contribution of *Nkx2–5* and *Isl1* to the pro-epicardial organ, source of the epicardium, coronary vessels, and most cardiac interstitial cells, epicardially-derived cells being a potential reservoir of cardiac progenitors.[54,80] A largely congruent fate map was proposed for the cCFU-F, which as mentioned expresses PDGFRα and Sca1, but not

most cardiogenic genes, and may be a precursor or more primitive form of the cells we studied.[45] It is unknown whether *Nkx2–5*- and *Wt1*-fated cells differ in molecular or functional terms.

In summary, given the heterogeneity of cardiac Sca1 cells and the complex potential origins, we sought to study the inception of specific subsets, based on combinatorial use of multiple identifiers. Partitioning 4 Sca1$^+$ populations (ie, SP/PDGFRα^+, SP/CD31$^+$, Non-SP/PDGFRα^+ and Non-SP/CD31$^+$) allowed us to identify a shared developmental pathway that bifurcates to form PDGFRα^+ cells vs. CD31$^+$ ones, associated, respectively, with cardiogenic vs. vasculogenic genes.

PDGFRα^+ cells exist in human hearts, whose developmental and reparative potential is beginning to be investigated.[84,85] No significant cardiogenic differentiation was observed in PDGFRα^+ human fetal heart cells treated as monolayers with 5'-azacytidine or as embryoid body-like aggregates with the cardiogenic growth factors activin A and bone morphogenetic protein-4.[84] Conversely, cardiac differentiation and angiogenic responses were demonstrated in PDGFRα^+ human myocardial pericytes, purified using CD146 and expressing other pericyte markers (NG2, PDGFRβ).[85] Thus, the potential utility of PDGFRα^+ human cardiac cells warrants further study.

Endogenous CSCs and Self-Repair

What about differentiation by native cells, in situ? Lineage-tracing experiments mapped the progeny of cardiac Sca1$^+$ cells by multi-color Cre tagging (*Sca1-tTA/LC1-Cre/R26R-Confetti*),[26] in which single cells stochastically undergo distinct recombination events and express 1 of 4 distinct fluorescent colors.[86] Sca1-fated cells infrequently displayed multilineage cardiovascular potential, suggesting that any multipotent Sca1 population is rare.[26] Roughly 4% of Sca1-fated cells were cardiomyocytes at 2–12 months of age, one-third of which were generated after birth.[26] Myocardial infarction elicited no change, but Sca1-derived cardiomyocytes increased 3-fold during pressure-overload hypertrophy.[26]

Analogously, cardiomyocytes were reportedly regenerated from existing cardiac-resident c-kit$^+$ cells using lentiviral delivery of Cre driven by a *c-kit* promoter and high-dose isoproterenol, a model of diffuse myocardial damage resembling takotsubo cardiomyopathy.[37] Those findings have been emphatically challenged by discordant results using tamoxiphen-dependent Cre targeted to the native *c-kit* locus: generating endothelial cells was the predominant effect.[87] Ordinarily, a "knock-in" offers the greatest fidelity of gene expression and would logically be given the benefit of doubt here. Countervailing arguments that have been raised include theoretical concerns that *c-kit-Cre* knock-in mice were defective for regeneration (because of *c-kit* haploinsufficiency), or might fail to capture as many c-kit-fated cells in the heart (because of lesser expression than with viral delivery).[88]

Rather than emphasize this ensuing technical debate, we wish to emphasize a conceptual difference among the various fate-mapping studies. Even if little or no myocyte creation occurs via a given cell type, under unassisted conditions, we view the relevant translational question as whether myocyte creation by that route can be enhanced, experimentally. (In much the same way, transdifferentiation of fibroblasts to cardiomyocytes is rightfully pursued, without being known to occur spontaneously.) At least 4 interventions enhance cardiomyocyte formation from a non-myocyte precursor. Grafting bone marrow or cardiospheres augments new myocyte creation by non-myocytes within the heart.[69,89] Treatment with thymosin β4 is essential for cardiac myogenesis by Wt1$^+$ cells, following infarction.[54] Of most direct relevance to our laboratory's studies, cardiomyocyte formation by the native Sca1$^+$ population appears responsible for the regenerative effect of prostaglandin E2 (PGE2) in mice.[27] In those experiments, cardiomyocyte replacement in the border zone by progenitor cells was blocked by cyclooxygenase (COX) inhibitors (indomethacin, celecoxib), increased by PGE2, and required the EP2 receptor.[27] Although it is uncertain if this action is entirely cell-autonomous, or involves known effects of PGE2 on the inflammatory milieu, direct effects were demonstrated in culture, with induction of Nkx2–5 and cardiac troponin T.[27]

In short, despite differences in the cells and models used, current data point to the existence of a cardiac regenerative capacity, albeit meager if unassisted. Thus, the possibility of activating endogenous progenitor cells in situ to regenerate viable and functional myocardium is an attractive therapeutic option, distinct from CSCs' utility as a cell therapy product. There are precedents for more than 1 population being led to a given cell fate; indeed, fate-mapping studies of brown adipogenesis implicate both Sca1$^+$ PDGFRα^+ stromal cells and pre-existing white adipocytes.[90] Hence, whether endogenous PDGFRα^+ precursors generate new cardiomyocytes merits investigation, and it may be more straightforward to interpret than the Sca1 fate map, given PDGFRα^+ cells' greater homogeneity.

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