Regenerating new heart with stem cells

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Introduction
Our understanding of the process of myocardial regeneration is currently under debate. Although the adult human heart is no longer considered a postmitotic organ, but is viewed as a self-renewing organ characterized by a resident stem cell compartment responsible for tissue homeostasis and cardiac repair following injury. Additionally, HSCs possess the ability to transdifferentiate and acquire the cardiomyocyte, vascular endothelial, and smooth muscle cell lineages. Both cardiac and hematopoietic stem cells may be used therapeutically in an attempt to reverse the devastating consequences of chronic heart failure of ischemic and nonischemic origin.

CSCs
The shift in paradigm dictated by the discovery of c-kit–positive CSCs has been difficult for the field to accept. The recognition that myocyte regeneration, mediated by activation and lineage specification of endogenous CSCs, is an important determinant of cardiac homeostasis and tissue repair was received with skepticism. Studies suggesting a limited role of myocyte renewal in animals and humans (2, 36) were highly publicized with accompanying editorials neglecting CSC function and reiterating the limited nature of myocyte replacement in the adult heart (37, 38). The simple concept of a requisite equilibrium between myocyte death and renewal physiologically has often been ignored.

Myocyte apoptosis in the normal human heart involves at least 1 in 100,000 cells (39). Considering that apoptosis lasts at most 4 hours, 0.006% of myocytes are lost per day, which accounts for a decrease of 2.2% of myocytes per year. Moreover, myocyte apoptosis increases linearly with age, so that over a period of 30 years approximately 95% of the original ventricular myocytes are lost as a result of wear and tear of the organ (12). This magnitude of cell death does not include cell necrosis, which has recently been documented independently by the presence of cardiac troponin in the circulation of apparently healthy individuals (40, 41). Therefore, a level of myocyte regeneration significantly higher than that predicted by the extent of cell apoptosis is required to preserve cardiac mass and function.

Following the discovery of c-kit–positive CSCs (15), several additional, distinct CSC classes have been described, including ISL1 progenitors, epicardial progenitors, side population progenitors, Sca1 progenitors, progenitors generating cardiospheres, and mesenchymal stem cells (24–27, 29). This unusual number of CSC categories is in sharp contrast to the properties of all other self-renewing organs in the organism, in which a single tissue-specific adult stem cell has been found. By definition, stem cells possess well-defined growth properties, suggesting that it is unrealistic that the heart contains such a variety of primitive cells all performing the same biologic function.

Among the different progenitor cell subsets, the c-kit–positive CSC has been well characterized in the mouse (42), rat (15), dog (16), and human (17, 21) heart. The expression of the stem cell antigen c-kit is associated with a pool of undifferentiated cells that have essentially identical properties in vitro and in vivo and are indistinguishable among species. Typically, stem cells reside in niches (30), where they are structurally connected to the supporting cells by gap and adherens junctions made by connexins and cadherins.
respectively (Figure 1A). The niche constitutes the microenvironment within which stem cells retain their undifferentiated state and receive growth signals from the supporting cells (43, 44). Following growth activation, stem cells divide symmetrically or asymmetrically, generating new stem cells and cells destined to acquire specialized functions; c-kit–positive CSCs self-renew, form multicellular clones, and give rise to a differentiated progeny in vitro and in vivo (Figure 1B). By employing viral transduction for clonal tracking of individual mouse and human CSCs, their fate in vivo has been determined (42). The clonality and multipotentiality of c-kit–positive CSCs (Figure 1C) was demonstrated in physiological conditions and following myocardial injury.

This extensive work has provided strong evidence in support of the notion that the adult heart possesses a class of c-kit–positive CSCs that modulates organ homeostasis and conditions tissue repair. During physiological myocardial aging in humans, from 20 to 100 years, the growth and differentiation of c-kit–positive CSCs replaces the entire myocyte compartment approximately 12 times (12). Similarly, activation and myocyte specification of c-kit–positive CSCs occurs in response to myocardial infarction (MI) and chronic aortic stenosis in humans (7, 9, 10). The crucial role of resident c-kit–positive CSCs in the turnover of cardiomyocytes in the adult rat and mouse heart has also been shown (30, 42, 45).

The recurrent statement made against the presence of resident CSCs and the significant growth reserve of the human heart is that spontaneous cardiac repair does not occur after infarction and that necrotic tissue is not restored by intact myocardium (14); instead, the healing process results in a thick scar. There is validity in this comment because it describes correctly the progression of MI and the need for cell therapy with appropriate cells that repair a non-contracting scar with functionally competent cardiomyocytes and coronary vessels. However, a lack of endogenous regeneration after infarction is present in organs including the skin, liver, intestine, kidney, and bone marrow. In all cases, occlusion of a supplying artery leads to scar formation, mimicking cardiac pathology (46–50). In the case of polyarteritis nodosa and vasculitis, microinfarcts develop in the intestine, and skin and resident stem cells do not repair the damaged tissue (51). Infarcts of the bone and bone marrow occur with sickle cell anemia, but HSCs and mesenchymal stromal cells do not reconstitute the structural integrity of the organ (46, 49). The simplest example is provided by the occlusion of a branch of a mesenteric artery; a segmental infarct of the intestine develops rapidly, but viable stem cells of the crypts fail to regenerate necrotic tissue (52). The stem cell compartment appears to be properly equipped to regulate tissue homeostasis but does not respond effectively to ischemic injury or, late in life, to aging and senescence of the organ (Figure 2).

Some comments must be made concerning the ISL1-positive cardiac progenitor that has been argued to represent the master human heart stem cell (27, 53). The cardiomyocyte specification dictated by the expression of ISL1 defeats the inclusion of ISL1-positive cells into the category of stem cells. As recently stated, adult stem cells are undifferentiated, long-lived cells that have the capacity to self-renew and give rise to a differentiated

Figure 1
Human CSCs. (A) Cluster of c-kit–positive CSCs (green) surrounded by fibronectin (fib; yellow). Myocytes are labeled by α-sarcomeric actin (α-SA) (white). White rectangle indicates the area shown at higher magnification at right. Scale bar: 5 μm. Connexin 43 (Cnx43; red) and N-cadherin (N-Cadh; magenta) are expressed between c-kit–positive CSCs and between CSCs and cardiomyocytes (arrows). Scale bar: 10 μm. (B) Clone derived from deposition of a single c-kit–positive CSC in a well of a Terasaki plate. Cells in the clone are all c-kit positive (green). Scale bar: 200 μm. (C) In differentiating medium, clonal c-kit–positive CSCs differentiate into myocytes (α-SA, red), smooth muscle cells (α-smooth muscle actin [α-SMA]; magenta), endothelial cells (von Willebrand factor [vWf], yellow), and fibroblasts (procollagen [procoll], blue). Some undifferentiated c-kit–positive cells (green) are also present. Scale bar: 50 μm.
The notion that HSCs transdifferentiate and form cardiomyocytes and coronary vessels has been sharply attacked, and criticisms have been made, regarding the implementation of this form of cell therapy in human beings (79–82). However, based on positive results obtained in phase 1 and phase 2 clinical trials (83, 84), the possibility that HSCs are involved in the restoration of damaged myocardium and contribute to the cardiac repair process suggests that bone marrow–derived cells are capable of forming cardiomyocytes and coronary vessels in spite of their predetermined lineage specification. During prenatal life, stem cells undergo a hierarchical progressive restriction of developmental options, and this mechanism of embryonic specification was thought to be irreversible and inviolable in adulthood (63, 66). However, this notion has been challenged by several examples of transition from one cell type to another or, more unexpectedly, from one cell lineage to another lineage (67–70). The unanticipated plasticity of adult HSCs to generate cells beyond their own tissue boundary became the driving force of a series of studies in which HSCs were implemented experimentally to restore the necrotic myocardium after infarction (71–78).

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Figure 2
Infarcts in stem cell–regulated organs. (A) Infarcted mouse heart characterized by collagen accumulation (white, arrowheads) in the region of healing. Myocytes are labeled by cardiac myosin heavy chain (MHC, red). Reproduced with permission from Proceedings of the National Academy of Sciences of the United States of America (ref. 72; copyright 2001, National Academy of Sciences, USA). (B) Schematic representation of infarcted tissue in various organs. Regeneration of the infarct is absent in all cases.
different classes of bone marrow cells are currently being used in patients with acute and chronic ischemic cardiomyopathy. A phase 3 trial supported by the European Union is ongoing (BAMI; http://www.euram.ltd.uk/BAMI/). But whether the apparent benefit of bone marrow–derived cells is dictated by the ability of these cells to regenerate damaged myocardium is the subject of intense debate. The predominant view is that these cells secrete a variety of cytokines that activate endogenous progenitors, which are actually responsible for the repair process and the improvement in ventricular function (82). Unfortunately, the documentation of the paracrine effects mediated by bone marrow cells in animals has been elusive at best, and data in humans have not helped to resolve this conundrum. Findings have been reported that support the notion that HSCs acquire the myocyte lineage and differentiate into functionally competent cardiomyocytes (75, 85), strengthening the view that HSCs may have therapeutic import for the human disease by acquiring the myocyte and endothelial cell lineage (Figure 4).

Despite animal and human results, myocardial repair continues to be viewed with suspicion and trepidation. The traditional view of the ability of the heart for proliferative growth was defined as “on shadings between none and almost none” (86), whether it derives from activation of resident CSCs or HSCs.

**Myocyte dedifferentiation**

Myocytes with decreased myofibrils and expansion of the undifferentiated cytoplast have been found in pathological cardiac hypertrophy (9), idiopathic dilated cardiomyopathy (IDC), and acute MI (7, 35) and in the presence of hibernating myocardium (87). Partial loss in the normal distribution pattern of titin, desmin, and cardiotin has been reported, together with the reexpression of fetal genes, including α-smooth muscle actin, atrial natriuretic peptide, α-skeletal actin, and α-smooth muscle actin (35, 87). Cardiomyocytes with these structural characteristics have been interpreted as the product of cell dedifferentiation, resulting in the acquisition of an immature proliferative cell phenotype. Dedifferentiation of adult cardiomyocytes has also been claimed following inhibition of p38 MAPK (32), myocardial injection of the extracellular matrix component peristin (33), or the growth factor neuregulin (34), although the effect of peristin on myocyte replication in vivo has been challenged (88).

Myocytes essentially identical to those commonly involved in the prenatal and early postnatal development of the myocardium (89, 90) reappear in the diseased human heart, and this process may be mediated by the release from macrophages of a member of the IL-6 inflammatory cytokines, oncostatin M (35). Surprisingly, divid-

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**Figure 3**

Lineage tracing of CSCs. (A) Representation of fate mapping involving the use of a fluorescent reporter gene (EGFP, green) driven by an inducible promoter coding for a stem cell–specific protein. Following differentiation and loss of stem cell antigen, myocytes, ECs, and SMCs continue to express EGFP, indicating a lineage relationship between ancestors and descendants. However, the labeled progeny may derive from activation of one or several stem cells, failing to document the multipotency of the parental stem cell. (B) Representation of viral gene tagging. Infection of CSCs with EGFP lentivirus results in the semirandom insertion of the proviral integrant in the genome of the recipient cell. Transcription and translation of the viral DNA result in expression of EGFP and fluorescent labeling of the infected CSCs. The unique insertion site of the viral genome is inherited by the entire population derived from the parental cell and can be amplified by PCR. CSCs nested in atrial and apical niches were labeled in situ to identify their progeny in vivo. (C) Four distinct clones were identified in EGFP-tagged CSCs, ECs, fibroblasts (Fbl), and cardiomyocytes (Myo) isolated from the ventricle of one mouse heart. Multiple PCR products (bands in agarose gel) were identified. Bands of the same molecular weight correspond to identical sites of integration of the proviral sequence in the host genome of CSCs, myocytes, ECs and fibroblasts, documenting the multipotency of CSCs in vivo. Reproduced with permission from *Proceedings of the National Academy of Sciences of the United States of America* (ref. 42; copyright 2009, National Academy of Sciences, USA).
ing myocytes repeatedly observed in IDC and acute and chronic MI in humans (6, 7, 10) have not been detected (35). Additionally, enhanced delivery of oncostatin M by macrophages in an in vivo model of dilated cardiomyopathy or following MI recapitulates the fetal-neonatal cell program but fails to promote myocyte regeneration (35), questioning the mechanisms involved in the negative (dilated myopathy) and positive (MI) effects of myocyte dedifferentiation on ventricular remodeling and cardiac hemodynamics. Similarly, the dedifferentiated cardiomyocytes identified in the human heart with IDC or acute MI never reached karyokinesis or cytokinesis. The expression of the fetal gene program with hemodynamic overload may simply reflect regenerated myocytes derived from commitment of resident CSCs rather than dedifferentiation of terminally differentiated postmitotic myocytes, which acquire an unwanted, mechanically inefficient cell phenotype (13).

Oncostatin M has also been argued to reverse in vitro the structural organization of postmitotic myocytes, resulting in the upregulation of several stem-related genes, including c-kit (35). Although the c-kit protein was never identified and the potential contamination of culture preparations from resident c-kit–positive progenitor cells was not excluded, a reinterpretation was immediately offered for the data on c-kit–positive CSCs (91). In the absence of in vivo findings, and despite the disclaimer from the authors, the validity of multiple demonstrations of resident c-kit–positive CSCs (15–23) was challenged (91). The rationale for this scientific view is obscure at best.

Recently we tested whether fetal myocytes, obtained from mice at E16–E18 expressing EGFP under the control of the c-kit promoter, were able to dedifferentiate and reacquire a degree of stemness, including the expression of c-kit (61). A total of 102 myocytes in mitosis were examined and none showed EGFP labeling. Moreover, non-dividing fetal myocytes expressing different levels

**Figure 4**

Transdifferentiation of c-kit–positive HSCs. (A) Schematic representation of transgene constructs used in the generation of donor mice for the acquisition of HSCs to be delivered after infarction. In each case, the promoter that controls the ubiquitous (β-actin) or myocyte-restricted (α-MHC) expression of the transgene (EGFP or c-myc–tagged nuclear-targeted Akt) is shown. c-kit–positive HSCs from donor males were injected intramyocardially in wild-type female infarcted mice. (B, C) Examples of myocytes isolated from the regenerated myocardium of mice injected with HSCs collected from β-actin EGFP, α-MHC EGFP, or α-MHC c-myc–tagged nuclear-targeted Akt mice. Top panels illustrate the localization of EGFP (left and middle, green; arrowheads) and c-myc (right, yellow nuclei; arrows) in newly formed cardiomyocytes. Bottom panels show the colocalization of α-SA and EGFP (yellow, arrowheads) and of α-SA and c-myc (yellow nuclei, arrows). Large spared myocytes negative for EGFP or c-myc are also present (ref. 75). Scale bar: 50 μm. (D) Myocyte-specific ionic currents and action potentials of a cell isolated from the regenerated infarcted myocardium. EGFP-positive myocytes show contractile activity (bottom). Original magnification, ×200. Reproduced with permission from *Proceedings of the National Academy of Sciences of the United States of America* (ref. 75; copyright 2007, National Academy of Sciences, USA).
of sarcomeric proteins were all EGFP negative, excluding cell dedifferentiation. Importantly, the generation of myocytes by dedifferentiation of mature cells, if it occurs, would have a minor role in physiological cell renewal, being restricted to cardiomyocytes (Figure 5). Similarly, dedifferentiated myocytes would be severely limited in their ability to sustain or improve ventricular performance following injury. Myocytes in the absence of adequate vascular supply and tissue oxygenation would be mechanically inefficient. Conversely, CSCs are multipotent and form in a coordinated manner cardiomyocytes and coronary vessels, fundamental components of myocardial repair.

Future directions
Endothelial progenitor cells, mononuclear bone marrow cells, mesenchymal stromal cells, and CD34-positive cells have been administered to patients suffering from acute MI or chronic ischemic cardiomyopathy (83, 84). Based on meta-regression analyses, these interventions have had positive outcomes, documenting the feasibility and safety of this therapeutic strategy and, in most cases, the beneficial effects on cardiac function. An average significant increase in ejection fraction of 3 percentage points has been reported (83, 84), although studies have also been published that indicate no changes in ejection fraction (92–96). Although enroll-
The documentation that a local RAS is present in human CSCs and that formation of Ang II and expression of Ang II receptors increase with age provides evidence in favor of the role that this octapeptide has in CSC senescence and death. Ang II generates reactive oxygen species, resulting in DNA damage; 8-OH-deoxyguanosine (8-OH-dG) accumulates at the GGG triplets of telomere, resulting in telomeric shortening and uncapping (103). Conversely, IGF1 decreases oxidative stress (60) and repairs DNA damage by homologous recombination (104). Therefore, changes in the proportion of these growth factor receptor systems in CSCs influence their growth reserve, a critical determinant of stem cell–based therapy.

Importantly, CSCs cannot be implemented in acute events in view of the time required for their preparation, whereas mononuclear bone marrow cells constitute an appealing form of cell intervention. Mononuclear bone marrow cells can be easily collected from bone marrow aspirates or the peripheral blood upon their mobilization from the bone marrow with cytokines. However, mononuclear bone marrow cells should not be confused with c-kit–positive HSCs that constitute a minute fraction of the mononuclear bone marrow cell pool (105). Experimentally, remarkable levels of myocardial regeneration after infarction have been obtained with c-kit–positive HSCs (71, 72, 75, 85) but not with mononuclear bone marrow cells (106). At present, it is unknown whether CSCs and HSCs are similarly effective in reconstituting the necrotic and scarred tissue, or whether limitations exist in CSC growth and HSC transdifferentiation, resulting in inadequate restoration of the damaged myocardium. A fundamental question to be answered is whether CSCs are superior, equal, or inferior to HSCs for the regeneration of myocytes and coronary vessels in animal models of CHF.

The therapeutic efficacy of these two stem cell classes depends on their ability to survive in the hostile milieu of the damaged heart, to engraft within the myocardium, and to grow and differentiate. HSCs may have a growth potential that is superior to CSCs, but transdifferentiation could affect this characteristic, and CSCs may constitute a more powerful form of therapy for cardiac repair. The process of transdifferentiation may alter the growth behavior of HSCs, which may lose in part their capability of dividing through alterations of the telomere–telomerase system, premature cellular senescence, and apoptosis. The opposite may also be true, and HSCs may retain after transdifferentiation a stronger regenerative capacity than CSCs. However, with HSCs, the newly formed myocytes have fetal–neonatal properties and may not reach the adult phenotype, a problem that may not affect the commitment of CSCs. Understanding the biology of HSC transdifferentiation and the molecular control of CSC growth and lineage commitment is a challenging and exciting endeavor due to the extraordinary clinical importance of myocardial regeneration for patients affected by acute MI and CHF.

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