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## Life and Death of Cardiac Stem Cells A Paradigm Shift in Cardiac Biology

Piero Anversa, MD; Jan Kajstura, PhD; Annarosa Leri, MD; Roberto Bolli, MD

The recognition that myocyte mitosis occurs in the fetal, neonatal, adult, and hypertrophied heart and that a pool of primitive, undifferentiated cells is present in the myocardium has put forward a different view of the biology of the heart. The new paradigm suggests that myocyte formation is preserved during postnatal life, in adulthood or senescence, pointing to a remarkable growth reserve of the heart throughout the course of life of the organism. This article reviews a large body of novel information, which has been obtained in the last 2 decades, in favor of the notion that the mammalian heart has the inherent ability to continuously replace its parenchymal cells and that this unexpected characteristic has important implications in understanding myocardial homeostasis, cardiac aging, and tissue repair.

### The Heart Is a Self-Renewing Organ

The paradigm that the heart is a postmitotic organ incapable of regenerating parenchymal cells was established in the 1970s, and this dogma has profoundly conditioned basic and clinical research in cardiology for the last 3 decades. On the basis of this paradigm, cardiomyocytes undergo cellular hypertrophy<sup>1,2</sup> but cannot be replaced either by entry into the cell cycle of a subpopulation of nonterminally differentiated myocytes or by activation of a pool of primitive cells that become committed to the myocyte lineage. The only response of cardiomyocytes to stress is hypertrophy and/or death. Therefore, a tremendous effort was made to identify the molecular mechanisms of myocyte hypertrophy and their genetic control. A sophisticated knowledge of various signaling pathways has been achieved, and our understanding of the biology of hypertrophic myocyte growth has advanced markedly.<sup>3</sup> An array of new technologies has been introduced that has led to a scientific revolution in terms of questions, approaches, and interpretation of experimental results. Despite this enormous progress in our understanding of basic mechanisms of hypertrophy, however, very little has been translated into clinical interventions that interfere with the dramatic impact of ischemic myocardial injury and the devastating evolution of postinfarction heart failure.<sup>4</sup>

Recently, an established notion of cardiac biology has been questioned. The general belief that during prenatal life cardiac progenitor cells undergo progressive lineage commit-

ment and differentiation,<sup>5</sup> which results in an irreversible loss of developmental options in adulthood, may not be valid.<sup>6</sup> Similarly, the traditional view of the heart as an organ incapable of regeneration has been challenged. Myocyte replication, karyokinesis, cytokinesis, and foci of spontaneous myocardial regeneration have been documented in the human heart.<sup>7,8</sup> These observations, together with the recognition that cell division, telomerase activity, telomeric shortening, and apoptosis occur in myocytes with aging and cardiac failure,<sup>7,9,10</sup> suggest that in the adult heart, a class of parenchymal cells proliferates, undergoes telomere attrition, reaches terminal differentiation, and possibly experiences replicative senescence and ultimately, cell death.

If adult, fully differentiated myocytes ( $\approx 25\,000\ \mu\text{m}^3$  in volume) were to retain the ability to divide, cells in mitosis before cytokinesis would be expected to be  $\approx 50\,000\ \mu\text{m}^3$  to give rise to 2 daughter cells, each  $\approx 25\,000\ \mu\text{m}^3$ . Surprisingly, human myocytes in mitosis vary in size from  $<1000\ \mu\text{m}^3$  to at most  $5000\ \mu\text{m}^3$ , indicating that a different mechanism of myocyte formation is operative in the adult heart.<sup>8,10-12</sup> The replicating cardiomyocytes resemble small amplifying cells that have originated by activation of progenitor cells. These observations, together with the demonstration of male cells in female hearts transplanted in male recipients,<sup>13</sup> were the critical findings that led to the identification and characterization of stem cells in the adult myocardium. The presence of male cells in the female heart raised the possibility that these cells either were the progeny of primitive cells of the recipient's bone marrow, which homed to the heart through the systemic circulation, or represented the product of stem-like cells that had migrated to the allograft from cardiac remnants.<sup>14</sup> The discovery of cardiac stem cells (CSCs)<sup>6,15-20</sup> distinct from hematopoietic stem cells (HSCs) and minimal spontaneous engraftment of HSCs into the heart argue against a significant role of the bone marrow in the turnover of cardiac cells.<sup>21,22</sup>

The recognition that a pool of primitive cells resides in the myocardium and that these cells form myocytes, smooth muscle cells (SMCs), and endothelial cells (ECs)<sup>8,15,16,23</sup> has provided a different perspective concerning the biology of the heart and mechanisms of myocardial homeostasis and tissue repair. The new paradigm implies that myocytes are no longer

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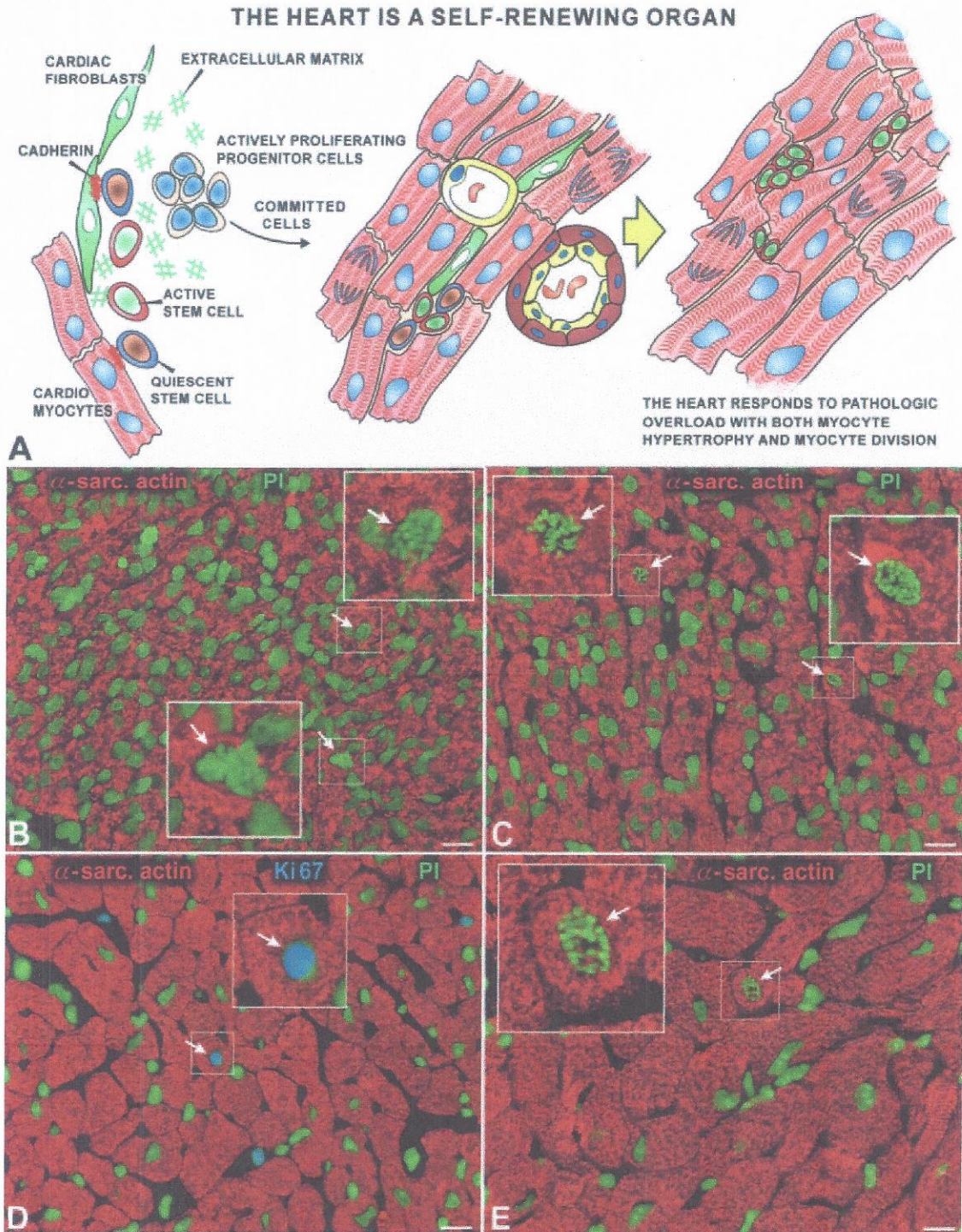
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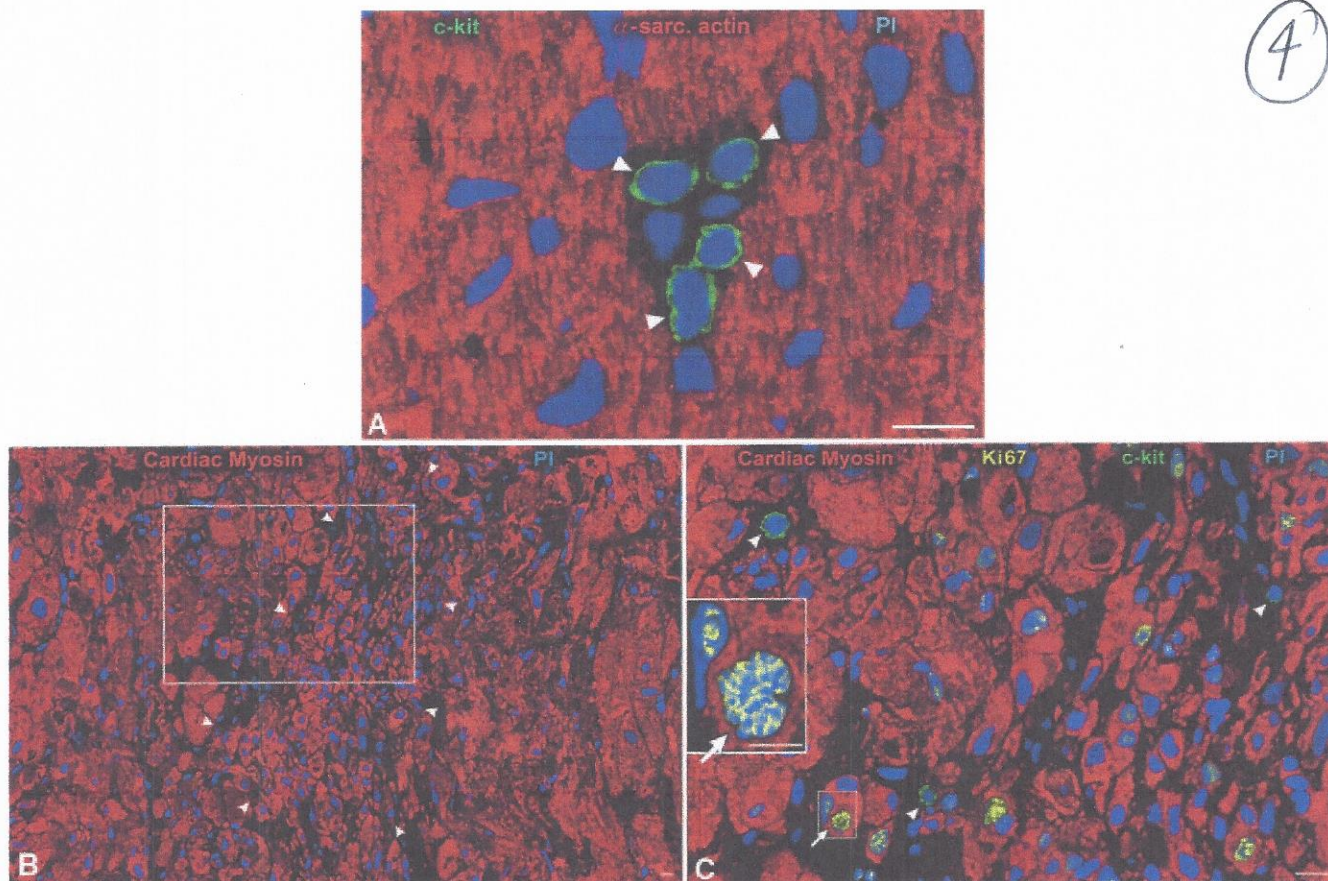
**Figure 1.** New paradigm of the heart. A, Cardiac niches contain stem cells, which, after activation, give rise to myocytes and vascular structures. B through E, Dividing myocytes ( $\alpha$ -sarcomeric actin, red) in fetal (B), neonatal (C), adult (D), and hypertrophied (E) rat heart. Mitotic myocytes (arrows) are shown in inserts. Bright blue denotes Ki67. Scale bars=10  $\mu$ m; for methods, see references.<sup>7,8,11,12</sup>

considered unusual cells that are created only during embryonic and fetal development so that their total number in the heart is established at birth and no further growth occurs postnatally, in adulthood, or during senescence.<sup>2,24</sup> This paradigm refutes the belief that all cardiomyocytes have the same age and that the age of these cells corresponds to the age of the organ and organism. Similarly, this paradigm suggests that the turnover and growth of vascular SMCs and ECs may

be regulated by differentiation of CSCs more than by the ability of these mature cells to reenter the cell cycle and divide.

Thus, a new conceptual framework of the heart has emerged. The heart is now viewed as a self-renewing organ in which myocyte regeneration occurs throughout the organism lifespan (Figure 1). Myocytes are the progeny of resident CSCs stored in niches. The niches control the turnover of





**Figure 2.** Primitive cells and myocardial regeneration. A, Cluster of c-kit–positive CSCs (green) in human heart. B and C, Arrowheads in B delineate new myocytes (myosin, red), and the rectangle delimits the area at higher magnification in C. Ki67 (yellow) labels developing myocytes, 1 of which is in mitosis (arrow). Arrowheads indicate c-kit–positive cells. Scale bars=10  $\mu$ m; for methods, see Urbanek et al.<sup>8</sup> B and C are reproduced with permission from Urbanek et al.<sup>7</sup> Abbreviations are as defined in text.

## IMPORTANT

myocardial cells and the growth, migration, and commitment of primitive cells that leave the niches to replace old, dying cells in the myocardium.<sup>25</sup> Regeneration implies that dead cells are replaced by newly formed cells that differentiate and organize in a complex pattern, restoring the original structure of the lost tissue. In adulthood, this process occurs in its completeness during physiological cell turnover, in the absence of injury. It is unknown why damage interferes with the recapitulation of cell turnover and restitutio ad integrum of the organ. A plausible hypothesis is that inflammation at the site of injury opposes the orderly reconstitution and spatial arrangement of myocytes and coronary vessels.<sup>26</sup> However, CSCs can be coaxed in vivo to home to the damaged area and promote the formation of parenchymal cells and vascular structures, resulting in recovery of ventricular performance.<sup>23,25</sup>

### Phenotype of CSCs

Investigators in several laboratories concur with the notion that the heart contains a compartment of undifferentiated cells with the characteristics of stem cells.<sup>6,8,15–20,23,25,27</sup> However, the actual number of CSCs remains controversial. Reports in mice,<sup>17,25</sup> rats,<sup>15</sup> dogs,<sup>23</sup> and humans<sup>8</sup> indicate that there is 1 stem cell per  $\approx$ 8000 to 20 000 myocytes, or  $\approx$ 32 000 to 80 000 cardiac cells. Some investigators have reported that primitive cells represent 2% of all cells, ie, 2 per  $\approx$ 25

myocytes, or  $\approx$ 100 cardiac cells.<sup>16,18</sup> This high value may reflect the contribution of endothelial progenitors.<sup>19,27</sup> Discrepancy also exists in the identification of the stem cell antigens c-kit and Sca-1 on cardiac primitive cells. The presence of Sca-1 has been reported,<sup>16–19,25,27</sup> whereas c-kit has been detected in 4 studies<sup>15,17,25,27</sup> and found to be absent in another.<sup>16</sup> c-kit–positive cells are an established component of the canine<sup>23</sup> and human<sup>7,8,13,20</sup> heart, and their activation leads to formation of new myocardium (Figure 2). The regenerative potential of c-kit–positive and Sca-1–positive cells after infarction<sup>15</sup> or ischemia/reperfusion injury<sup>16,28</sup> differs significantly; a robust regenerative response occurs with c-kit–positive cells,<sup>15,28</sup> whereas little engraftment and repair take place with Sca-1–positive cells.<sup>16</sup> Whether the modality of administration of cells, the animal model, and the properties of the injected cells are responsible for the different outcome is presently unclear.

The developing and adult heart typically contain a CSC pool that has the ability to efflux the Hoechst 33342 dye.<sup>6,16,18,19,29</sup> Similar cells, which express an ATP-binding cassette transporter, have been identified in other organs and termed side-population (SP) cells.<sup>19,30</sup> The classic member of this family is a P-glycoprotein that confers to the cells multidrug resistance by extruding anticancer drugs and rhodamine 123. These putative CSCs are 93% Sca-1–positive



and appear to represent a subset, 1 per  $\approx 10\,000$  cells, of the 2% Sca-1–positive cells in the mouse heart.<sup>16</sup> According to a different study, however, SP cells comprise 2% of all cells (2 per  $\approx 100$  cardiac cells).<sup>18</sup> Cardiac SP cells appear to express CD31 and form hematopoietic colonies in vitro.<sup>18</sup> The presence of CD31, common to bone marrow SP cells,<sup>21,30</sup> together with the peculiar growth behavior of these cells in vitro, raises questions concerning their actual origin and suggests possible colonization to the heart from the hematopoietic system.

Recently, a novel Sca-1–positive, Hoechst 33342 dye–low, and CD31–negative cardiac SP cell has been identified.<sup>19,29</sup> The modest expression of c-kit in these cells was attributed to methodological limitations inherent in the enzymatic cleavage of this receptor during digestion of the myocardium and cell isolation.<sup>19</sup> There is 1 SP cell per  $\approx 30\,000$  cardiac cells in the mouse heart. CD31–negative cardiac SP cells acquire the myocyte lineage, reach the adult phenotype through a process mediated by cellular coupling with differentiated cardiomyocytes, and contract in vitro.<sup>19</sup> These results strengthen the notion of a functional role for resident SP cells in the heart.

The *Isl1* transcription factor is associated with the commitment to the myocyte lineage of cardiac cells that have lost their undifferentiated stem cell state. *Isl1* and *GATA4* are transcriptional coactivators of the myocyte transcription factor *MEF2C*.<sup>31</sup> Homozygous deletion of *Isl1* alters development of the mouse heart, affecting the atria, right ventricle, and outflow tract,<sup>32,33</sup> and *Isl1*–positive cells may have implications for myocyte replacement in the newborn.<sup>34</sup>

Together, these observations are consistent with the notion that the heart possesses an intrinsic capacity for regeneration, but whether differences in the expression of surface antigens reflect CSC subclasses, which are functionally distinct, is difficult to ascertain. A direct comparison among stem cell phenotypes would require transplantation studies in which single CSCs are delivered to the chemically ablated or irradiated organ. To date, no information is available concerning the repopulating ability of individual CSCs in the depleted heart. Stem cell depletion is complex in solid organs, and currently, there are no protocols available to eliminate resident CSCs in the absence of extensive parenchymal damage and myocardial scarring.<sup>26</sup> So far, only limited characterization of the in vitro properties of CSCs has been performed. However, these data do not answer the question whether the expression of 1 stem cell antigen versus another carries a greater or reduced regeneration potential for the diseased heart.

### Classification of CSCs and Their Progeny

Information obtained so far on the phenotype of adult CSCs is incomplete. The nomenclature is imprecise, and frequently, cells with distinct growth behaviors are defined by the same terminology. Some comments may be helpful. CSCs are undifferentiated, lineage–negative cells that possess a high growth potential.<sup>6,15–20,23,25</sup> At the single–cell level, CSCs are self-renewing, clonogenic,<sup>15,19,23</sup> and capable of differentiating in mature progenies in vitro<sup>15–17,19,23</sup> and in vivo.<sup>8,15,16,23,25</sup> Stem cells divide rarely, whereas the committed, amplifying

cells are the actual group of replicating cells. Stem cells in the bone marrow, some areas of the brain, skeletal muscle, and heart share in different proportions the stem cell–related antigens c-kit, *MDR1*, and *Sca-1*.<sup>8,23,25</sup>

We propose a classification of cardiac immature cells into 4 classes: CSCs, progenitors, precursors, and amplifying cells. These cell types can be viewed as subsequent steps in the progressive evolution from a more primitive to a more differentiated phenotype (Figure 3). The first 3 cell types express c-kit, *MDR1*, and *Sca-1*, whereas the last type no longer expresses these antigens. This relatively simple terminology defines the fundamental characteristics of CSCs and the classes of cells generated by their lineage commitment.

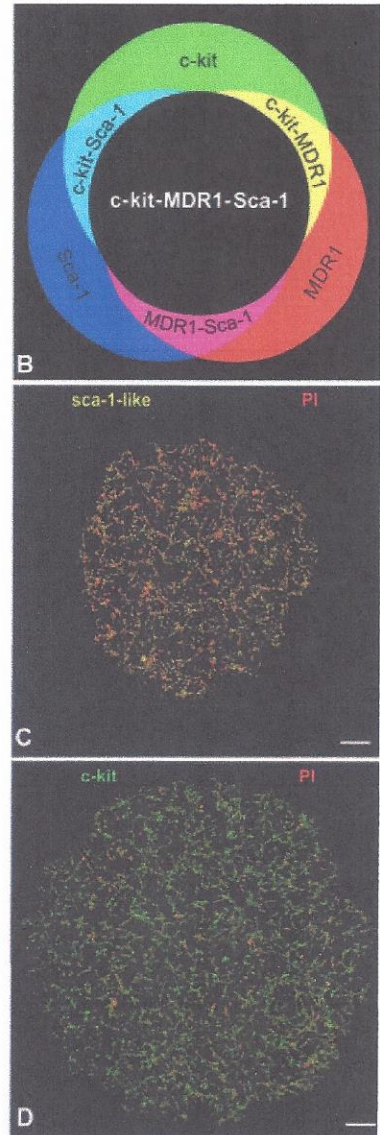
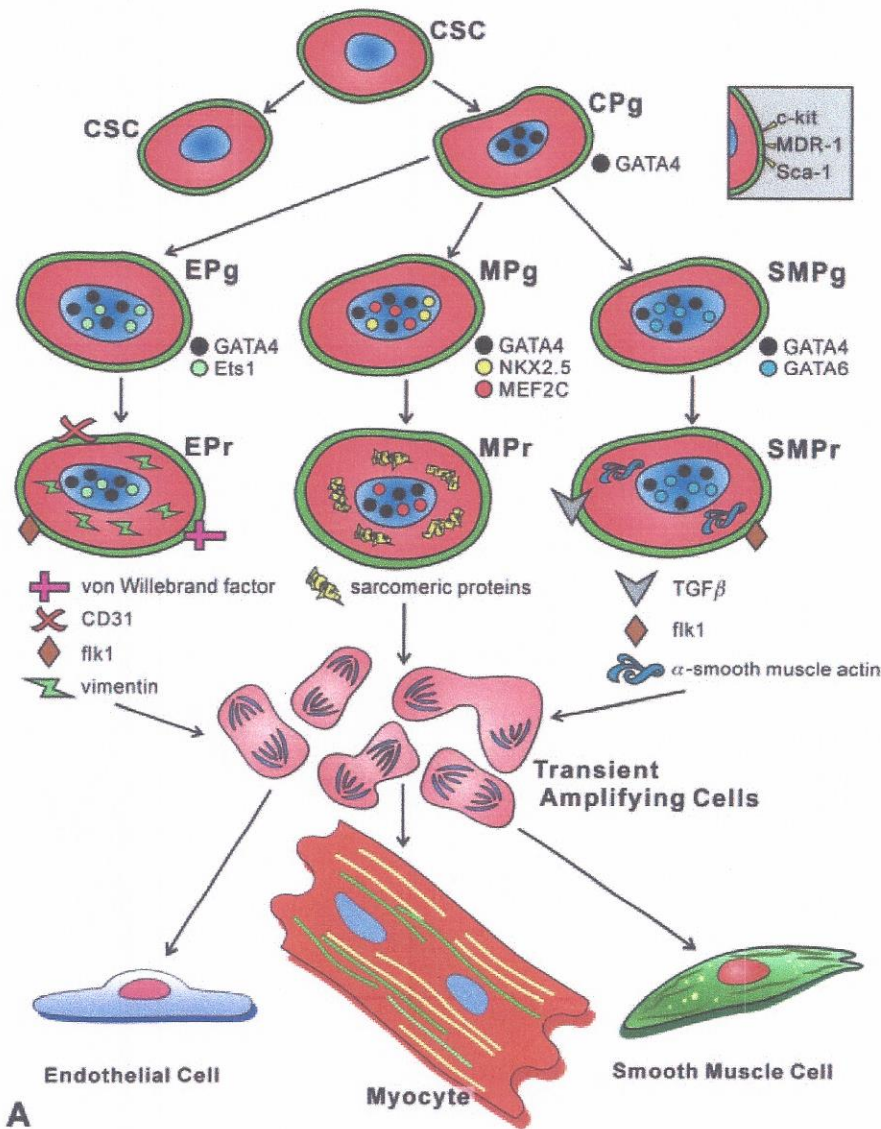
The recognition of lineage–negative CSCs and their progenies is technically demanding and complex in the rat,<sup>15</sup> mouse,<sup>6,16–19,25</sup> dog,<sup>23</sup> and human<sup>8</sup> heart. These cell categories can be identified in isolated cardiac cell preparations or in sections of atrial and ventricular myocardium. In both cases, this is accomplished by utilizing several mixtures of antibodies directly labeled by fluorochromes to avoid cross-reactivity and nonspecific staining.<sup>8,23</sup> With histological approaches, major screening with cocktails of fluorescein–conjugated antibodies may be followed by immunostaining with indirect labeling; this approach requires the use of secondary antibodies. Additionally, it is necessary to achieve the level of resolution afforded by confocal microscopy.<sup>35</sup> Contrary to common belief,<sup>2,36–38</sup> confocal microscopy is the only method that permits quantitative measurement of the frequency and distribution of CSC classes in the myocardium.<sup>26,39</sup> Fluorescence–activated cell sorting analysis is extremely efficient in separating cells in specific categories according to the expression of 1 or more surface epitopes,<sup>40,41</sup> but the distinction between lineage–negative and committed cells requires the analysis of nuclear and cytoplasmic proteins. This can only be obtained by fixation of cells to make them permeable for the detection of intracellular components.<sup>42,43</sup> Similar problems exist with immunolabeling. With either protocol, cell viability is lost, precluding further study.

Although stem cells have been known for quite some time, the importance of stem cell antigens in the growth and differentiation potential of these cells remains poorly understood.<sup>44–46</sup> These limitations are not restricted to the heart and CSCs but also apply to stem cells in other organs, including the bone marrow. As indicated earlier, c-kit, *MDR1*, and *Sca-1* are present not only on the surface of primitive cells but also in cells undergoing lineage differentiation, complicating recognition of the actual primitive cells in the population. Similarly, the detection of these antigens in cells collected by enzymatic digestion of the myocardium is highly dependent on the isolation protocol, which inevitably affects the yield, properties, and purity of the preparation, as well as the integrity of surface antigens. For this reason, some of the differences encountered in the characterization of CSCs and various subsets may be accounted for by technical difficulties inherent in the separation and collection of these cells from a solid organ.<sup>19</sup>

To gain insights into the functional implications that different CSC surface antigens may have in myocardial growth in large mammals, CSCs were isolated and charac-



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**Figure 3.** Hierarchy of CSC growth and differentiation. A, Asymmetrical division of a CSC into a daughter CSC and a daughter cardiac progenitor (CPg). CPg gives rise to myocyte progenitor (MPg) and precursor (MPr), EC progenitor (EPg) and precursor (EPr), and SMC progenitor (SMPg) and precursor (SMPp). Precursors become transient amplifying cells, which divide and differentiate into mature myocytes, ECs, and SMCs. CSCs are lineage-negative cells that express only c-kit, MDR1, or Sca-1. Progenitors express stem cell antigens and transcription factors of cardiac cells but do not exhibit specific cytoplasmic proteins. Precursors possess stem cell antigens, transcription factors, and membrane and cytoplasmic proteins typical of myocytes, ECs, and SMCs. Amplifying cells have nuclear, cytoplasmic, and membrane proteins of cardiac cell lineages but are negative for stem cell antigens. TGFβ indicates transforming growth factor β receptor; other abbreviations are as defined in text. B, Colocalization of c-kit, MDR1, and Sca-1 in CSCs. Areas corresponding to cells positive for 1 antigen only were enlarged 3 times. C and D, Clones derived from a single Sca-1-like-positive cell (C, yellow) and a single c-kit-positive cell (D, green). Scale bars=100 μm. Reproduced with permission from Linke et al.<sup>23</sup>

terized in the dog heart.<sup>23</sup> Consistent with results in the mouse,<sup>25</sup> rat,<sup>15</sup> and human<sup>8</sup> heart, canine CSCs expressing 3 surface antigens constitute the prevailing population, (~60%); those expressing 2 antigens, the second most frequent fraction (~25%); and those expressing 1 antigen, the least represented component (~15%). CSCs with 1 epitope were equally distributed, nearly 5% for each surface antigen. The size of clones and subclones varies when CSCs express different epitopes (Figure 3).

Clones from c-kit-positive CSCs are 2.3-fold, 5.9-fold, and 7.6-fold larger than MDR1-, Sca-1-like-, and c-kit/MDR1/Sca-1-like-positive clones, respectively.<sup>23</sup> When the same

number of clonogenic cells, ≈100, from each of these clones is driven to growth and differentiation, the magnitude of the population of committed cells differs, depending on whether it develops from c-kit-, MDR1-, Sca-1-like-, or c-kit/MDR1/Sca-1-like-positive clonogenic cells. Over a period of 4 weeks, 1.05×10<sup>6</sup>, 3.9×10<sup>5</sup>, 2.4×10<sup>5</sup>, and 1.6×10<sup>5</sup> differentiated cardiac cells are formed from c-kit-, MDR1-, Sca-1-like-, and c-kit/MDR1/Sca-1-like-positive clonogenic cells, respectively.<sup>23</sup> Therefore, the ability of c-kit-positive cells to generate cardiac cell lineages is greater than that of MDR1-, Sca-1-like-, and c-kit/MDR1/Sca-1-like-positive cells. Clonogenic MDR1-positive cells produce more myocardial cells



than do Sca-1-like- and c-kit/MDR1/Sca-1-like-positive cells. Although there is no appreciable difference in the relative proportion of myocytes formed by each clonogenic CSC class (myocytes,  $\approx 50\%$ ; SMCs,  $\approx 40\%$ ; and ECs,  $\approx 10\%$ ), the aggregate number of myocytes, SMCs, and ECs created by the c-kit-positive cells is several-fold larger than that obtained from other CSC categories.<sup>23</sup>

Understanding the relation between stem cell antigens and growth characteristics of CSCs has important implications in the future implementation of CSC therapy in patients. A critical question that needs to be addressed is whether human c-kit-positive CSCs, followed by MDR1-positive cells, constitute the more powerful CSC classes for acute regeneration of myocytes and coronary vessels in the damaged heart. Sca-1-like- and c-kit/MDR1/Sca-1-like-positive CSCs may be less relevant for emergency need and may be implicated in the formation of coronary arterioles, capillary structures, and myocytes to compensate for a defective growth in the other stem cell populations. Alternatively, these latter 2 classes may be prevalently involved in the low physiological turnover of cardiac cells. These possibilities are not mutually exclusive.

### CSCs and Cell Turnover

Researchers from 8 laboratories have shown that stem cells are present in the adult heart of small and large mammals, including humans.<sup>6-8,13,15-20,23,25,27,29</sup> Despite these findings, the shift in paradigm from the heart as a postmitotic organ to a self-renewing organ has not yet occurred. At best, the heart is now viewed as an organ capable of some but severely limited regeneration.<sup>1,2,18,47</sup> On the basis of this premise, strategies aimed at reversing the terminally differentiated state of myocytes have been proposed as an alternative intervention for the cellular repopulation of the diseased heart.<sup>48</sup> In this section, we discuss the reasons for the unwillingness to lay to rest the old dogma and adopt a novel biological perspective of the heart. The new paradigm sees the heart as a highly dynamic organ in which old, poorly functioning myocytes and vascular SMCs and ECs are continuously replaced by activation and commitment of resident CSCs. The recognition of the mechanisms that regulate the physiological turnover of cardiac cells may offer a unique, unprecedented approach for cardiac repair. Potential of this naturally occurring process may become the most efficient and successful form of tissue reconstitution for the failing heart. In this scenario, exogenous expansion of autologous CSCs,<sup>15,16,20</sup> bone marrow progenitor cells,<sup>35,49-53</sup> and skeletal myoblasts<sup>54</sup> for subsequent myocardial implantation<sup>55,56</sup> would be seen as a secondary model of cell therapy and not as the primary method of choice. Similarly, the use of genetically manipulated, nonmalignant, nonimmunogenic embryonic stem cells<sup>57,58</sup> would be envisioned only in extreme cases. Currently, embryonic stem cells are immunogenic<sup>59</sup> and tumorigenic,<sup>60-62</sup> precluding their clinical relevance in the near future.<sup>26</sup>

The reluctance to accept a reassessment of cardiac biology and pathology has its foundation on 4 major questions: (1) Are CSCs responsible for myocardial homeostasis and therefore, for an effective physiological replacement of dying

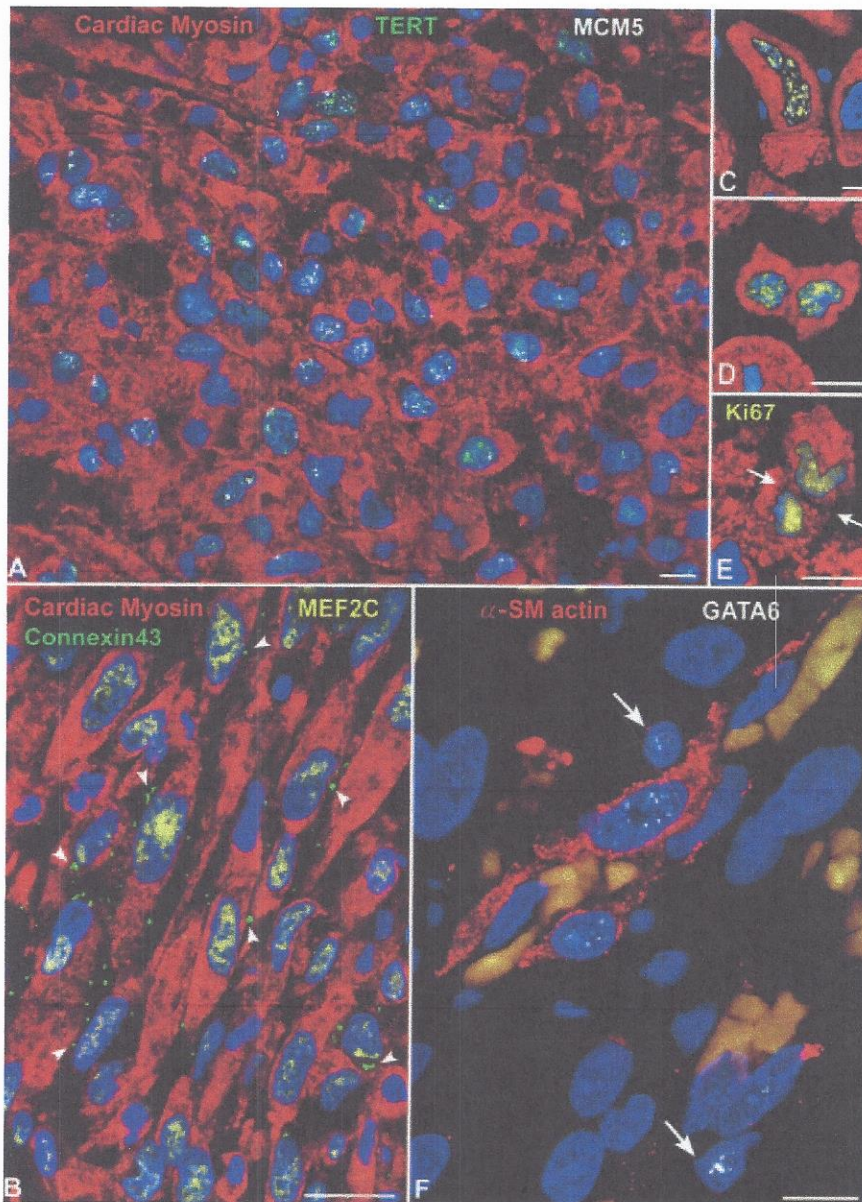
cardiac cells, including cardiomyocytes? (2) Is the CSC pool activated after injury, and if so, does this lead to a relevant increase in the number of myocytes and coronary vessels? (3) If properly activated, are CSCs able to translocate to areas of damage, grow, and differentiate, thereby restoring the lost myocardium? (4) Assuming that the heart has a powerful growth reserve dictated by the CSC compartment, do aging and chronic heart failure reduce the pool of functionally competent CSCs, and if so, is this defect important in the development of end-stage failure? The answers to these questions will provide a framework for a cardiac paradigm that does not ignore the discovery of CSCs and their role in the life and death of the heart.

Cardiac homeostasis is regulated by the balance between old, senescent cardiac cells undergoing apoptosis/necrosis and the formation of new cells through the commitment of CSCs. CSCs are clustered in the atria and apex but are also present throughout the ventricular myocardium.<sup>25</sup> An equivalent loss and generation of cardiomyocytes, vascular SMCs, and ECs would be expected to occur in the entire heart to preserve its equilibrium, before aging effects become apparent.<sup>10,63,64</sup> Because of the conviction that myocytes cannot be replaced<sup>1,2</sup> and that "adult" SMCs and ECs reenter the cell cycle and divide,<sup>65,66</sup> independently from lineage specification of primitive cells, the possible function of CSCs in myocardial cell turnover has only been recently explored.

Information obtained so far is incomplete but strongly supports the notion that the heart contains a pool of replicating, lineage-negative CSCs, together with myocyte, SMC, and EC progenitors and precursors, and highly proliferating amplifying cells. The retroviral tagging of undifferentiated cells in vivo or in vitro before their intramyocardial injection has demonstrated that developing and mature cells are the progeny of activated CSCs.<sup>15,25,28</sup> A similar analysis could not be obtained in the human heart. All of these cell classes have been identified in the ventricular myocardium, however, suggesting that a similar growth behavior occurs in the human heart (Figure 4) and that it involves CSC multilineage commitment and differentiation.<sup>7,8,10-13</sup> Myocytes, and SMCs and ECs in coronary vessels, die continuously, and the constant replacement of cells preserves the integrity of the tissue and the function of the organ. Whether cell death triggers regeneration or whether regeneration activates cell death is not known, but cellular aging with loss of replicating cells is invariably associated with initiation of the death pathway.<sup>67-69</sup>

In recent work,<sup>7,8,10-13</sup> the numbers of cycling and noncycling lineage-negative CSCs, myocyte progenitors/precursors, cycling amplifying myocytes, terminally differentiated non-aged myocytes, and senescent myocytes have been measured in the human heart. Similarly, the mitotic index and Ki67 and MCM5 labeling of myocyte nuclei have been obtained in humans.<sup>7,8,11,12</sup> On the basis of the fact that mitosis is a rapid process,<sup>70</sup> the duration of the cell cycle in forming myocytes has been estimated previously to be  $\approx 25$  hours.<sup>7,11,12</sup> Additionally, the ratio of progenitors to precursors to amplifying cells to lineage-negative CSCs was determined<sup>8</sup> to compute the number of transit generations from the uncommitted state to terminal differentiation, ie,  $\approx 7$  rounds of the cell cycle.<sup>71</sup>





**Figure 4.** Regeneration of human myocardium. A and B, Small, developing myocytes ( $\alpha$ -sarcomeric actin, red) are positive for telomerase (A, green) and MCM5 (A, white). MEF2C is also present in the nuclei (B, yellow), and connexin 43 is detected (B, green, arrowheads). C through E, Metaphase chromosomes (C), karyokinesis (D), and cytokinesis (E, arrows) are present in dividing myocytes. Ki67 in the chromosomes is apparent (C through E, yellow). Developing arteriole with SMCs ( $\alpha$ -smooth muscle actin, red) contains erythrocytes (yellow). Dispersed SMCs are observed (F, arrows); GATA6 is present in SMC nuclei (white dots). Scale bars=10  $\mu$ m. Reproduced with permission from Urbanek et al<sup>7</sup> (C-E) and Urbanek et al<sup>8</sup> (A, B, F).

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Together, these data define the rate of turnover (death/formation) of the myocyte population. In the normal human heart, there are  $\approx 5 \times 10^9$  parenchymal cells in the left ventricle inclusive of the interventricular septum,<sup>72,73</sup> and  $\approx 3 \times 10^6$  terminally differentiated myocytes are generated per day (Table) by commitment and growth of CSCs.<sup>7,8,11,12</sup> This degree of myocyte formation ensures that the entire cell population of the heart is replaced approximately every 4.5 years. Thus, parenchymal cells cannot live, as is generally believed, as long as the organism,  $\approx 80$  years.<sup>74</sup> The human heart replaces completely its myocyte population  $\approx 18$  times during the course of life, independently from cardiac diseases.

There is a remarkable balance between the formation and loss of myocytes physiologically (Table). It has been shown that in the normal adult human heart, cell death by apoptosis/necrosis<sup>75</sup> accounts for the loss of  $\approx 3 \times 10^6$  myocytes per day, a value essentially identical to that of regenerated

myocytes. The rate of cell death is calculated from the measurement of apoptosis and necrosis of myocytes,<sup>75,76</sup> together with the duration of apoptosis,  $\approx 3$  hours,<sup>77</sup> and necrosis,  $\approx 24$  hours.<sup>78</sup> If the heart were postmitotic and dying myocytes were not constantly replaced, the entire organ would disappear in  $\approx 4.5$  years. These conservative estimates project a remarkably different image of the human heart, in sharp contrast to the contention that this organ has a poor ability to regenerate myocytes, inadequate for significant myocardial reconstitution.<sup>1,2,79</sup>

Whether SMCs and ECs organized in vascular structures experience higher or lower turnover rates than do myocytes in mammals is difficult to predict. At present, only scattered information is available on the growth and death of these cell populations, precluding a meaningful analysis of their short- and long-term fate in the control and pathological heart. However, EC and SMC progenitors and precursors are easily detectable in the human,<sup>7,8</sup> dog,<sup>23</sup> rat,<sup>15,28</sup> and mouse<sup>25</sup> myo-



**Generation and Loss of Myocytes in the Normal and Infarcted Human Heart**

	Control		Acute Infarcts		Chronic Infarcts	
	Mean±SD	References	Mean±SD	References	Mean±SD	References
CSCs, cells/mm <sup>3</sup>	3.7±0.5	7, 8	41±7*	8	11±2*†	8
Cycling CSCs, %	3.5±1.8	7, 8	33±6*	7, 8	15±3*†	7, 8
Myocytes generated, cells · d <sup>-1</sup> · mm <sup>-3</sup>	18±7	7, 8	1160±290*	7, 8	130±31*†	7, 8
Viable LV mass, g	164±27	7, 8, 11, 12	190±31	8, 12	260±38*†	8, 11
Myocytes generated in LV, cells/d	2.9±1.0×10 <sup>6</sup> §	...	220±42×10 <sup>6</sup> §	...	34±6×10 <sup>6</sup> †§	...
Myocytes in LV	4.9±1.0×10 <sup>9</sup>	7, 8, 11, 12	4.6±0.7×10 <sup>9</sup>	8, 12	4.3±0.7×10 <sup>9</sup>	8, 11
Time to replace LV myocytes, d	1668±740	...	21±6*	...	125±37*†	...
Myocytes lost in LV, cells/d	2.9±0.9×10 <sup>6</sup>	75, 76	179±49×10 <sup>6</sup> *	75, 76	97±21×10 <sup>6</sup> *†	75, 76
Myocytes generated-to-lost ratio	1.0±0.47‡¶	...	1.22±0.41‡¶	...	0.35±0.10¶	...

\*P<0.05 vs control hearts.  
 †P<0.05 vs acute infarcts.  
 ‡Not significantly different from 1.00.  
 §Product of line 3 and line 4.  
 ||Quotient of line 7 and line 6.  
 ¶Quotient of line 5 and line 8.

*because the macrophages must be activated*

cardium. These observations support the notion that lineage specification of CSCs contributes to the formation of coronary vessels and the replacement of ECs and SMCs within the vascular wall. However, functionally competent ECs and SMCs can reenter the cell cycle and divide, and this growth reserve represents a relevant component of angiogenesis and/or cell turnover.<sup>65,66</sup> Importantly, circulating endothelial progenitor cells are a powerful source of vasculogenesis.<sup>50,51,80</sup> How CSCs, replicating vascular SMCs, and endothelial progenitor cells interplay in the homeostasis and growth of coronary vessels remains a critical, unanswered question.

In summary, the adult heart is not a static but a very dynamic organ whose homeostasis is governed by a compartment of multipotent CSCs, which continuously repopulate the myocardium, replacing parenchymal cells and vascular SMCs and ECs that die by apoptosis/necrosis. CSCs provide the adult heart with a growth reserve that can restore a large number of myocytes and replace the entire myocyte compartment several times during the course of life in animals and humans. The notion that the heart retains the capacity to regenerate cardiomyocytes, coronary resistance arterioles, and capillary structures throughout life has far-reaching implications.

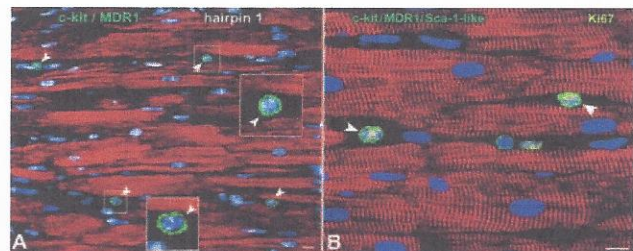
**Stem Cells and Cardiac Repair**

The adult heart is well equipped to sustain organ homeostasis, which efficiently preserves the integrity of the tissue during physiological cell turnover. The equilibrium between the rates of dying and forming myocytes suggests that the CSC compartment senses signals from cells undergoing apoptosis or necrosis, which in turn activate the growth and commitment of primitive cells replacing dying myocytes. More complex is the issue of whether the heart has the intrinsic ability to regenerate large quantities of myocytes after myocardial infarction. The setting of myocardial infarction has been cited as an indisputable argument against myocyte regeneration and significant growth reserve of the adult

*Very important*

heart.<sup>81</sup> Although this logic is deeply ingrained in the clinical and scientific community, it falls short for several reasons.

The outcome of infarction is essentially identical in all organs of the organism, regardless of whether the parenchymal and nonparenchymal cells are able to enter the cell cycle and divide. The presence of stem cells does not prevent the inevitable evolution of the infarct with scar formation. Occlusion of a major conductive artery or large branch results in loss of tissue in the skin, kidney, intestine, brain, liver, and reproductive organs,<sup>82-85</sup> in a manner identical to the heart.<sup>86</sup> This general outcome of ischemic injury is dictated by 2 crucial factors: (1) Stem cells within the infarct die, as do all other cells deprived of oxygen supply, and (2) resident stem cells cannot migrate from the viable tissue to the damaged area, home, grow, and differentiate to replace the dead cell lineages. CSC death within the infarct has been well documented,<sup>23,25</sup> but viable, cycling CSCs as well as replicating myocyte progenitors and precursors, in combination with highly dividing amplifying myocytes, are commonly found<sup>7,8,12,23,25</sup> throughout the surviving myocardium (Figure 5). These observations provide an explanation for the lack of myocardial regeneration within the infarct and the intense



**Figure 5.** Cardiac progenitor cells in the infarcted heart. A, Infarcted myocardium contains c-kit/MDR1-positive progenitor cells (green, arrowheads) and myocytes undergoing apoptosis (hairpin 1, white). B, c-kit/MDR1/Sca-1-like-positive progenitor cells (green) in the surviving myocardium are cycling (Ki67, yellow, arrowheads). Scale bars=10 μm. Reproduced with permission from Dawn et al.<sup>28</sup>



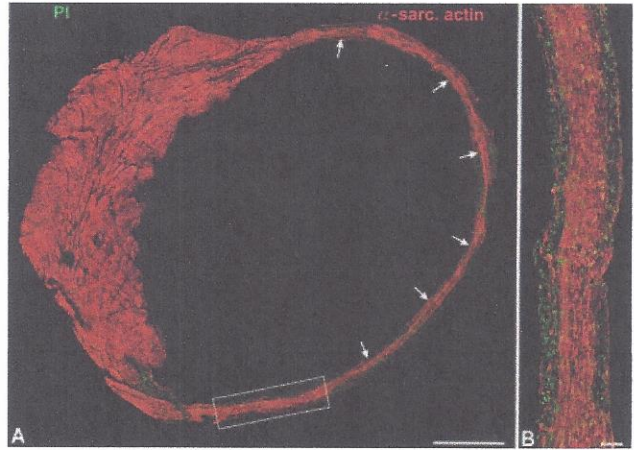
YOU CAN cross reference organs!!

myocyte formation in the nonischemic region of the ventricular wall.<sup>8,11,12</sup> The important point is that the behavior of the heart is not different from that of other self-renewing organs, including the bone marrow, skin, intestine, brain, kidney, and liver.

Although myocardial infarcts do not regenerate spontaneously, the endogenous repair mechanisms of the heart can be manipulated and exploited to accomplish this objective. Thus, the critical question is whether the growth reserve of the adult heart is sufficient to reconstitute acute and massive losses of myocardium. To resolve this issue, the ability to form parenchymal cells needs to be examined in 2 distinct regions of the diseased heart, the nondamaged and damaged myocardium.<sup>7,8,10-13,15,16,23,25</sup> The latter can only be measured by interventions that promote the translocation of CSCs from the surviving to the dead tissue and, by necessity, can only be determined experimentally, because similar strategies have not been developed in humans.

In humans, myocyte formation within the noninjured tissue is dramatically potentiated in acute and chronic heart failure, and this increased generation of myocytes is sustained by enhanced replication and lineage commitment of CSCs. Patients who die shortly after infarction<sup>8,12</sup> give us a good indication of the maximal regeneration potential of the human heart. An infarcted ventricle with  $\approx 190$  g of viable myocardium<sup>8</sup> and a myocyte mitotic index<sup>12</sup> of  $\approx 500/10^6$  can create  $\approx 2.20 \times 10^8$  myocytes per day (Table). This value is  $\approx 70$ -fold higher than the baseline turnover rate (see earlier sections). If this magnitude of myocyte growth were to persist, a 40% infarct that reflects a loss of  $\approx 2 \times 10^9$  myocytes would be repaired in 10 days. In end-stage postinfarction cardiomyopathy, there is an  $\approx 70\%$  reduction in myocyte mitotic index,<sup>11</sup> to a value of  $\approx 140/10^6$ . However, the viable ventricular muscle mass is increased to  $\approx 260$  g.<sup>8</sup> These factors lead to the generation of  $\approx 3.4 \times 10^7$  myocytes per day, which is  $\approx 11$ -fold higher than physiological turnover.

Therefore, the severely decompensated human heart possesses a relevant growth reserve, which is, however, restricted to the noninfarcted region of the wall. Additionally, ongoing myocyte death in the surviving myocardium comprises  $\approx 1.80 \times 10^8$  myocytes acutely after infarction (authors' unpublished data, 2006) and  $\approx 10^8$  myocytes in end-stage postinfarction heart failure.<sup>75</sup> On the basis of these observations, myocyte regeneration modestly exceeds myocyte death acutely after infarction, but myocyte replacement is markedly less than myocyte death in the long term. Thus, myocyte death significantly interferes with the recovery of structure and function after an acute infarct and results in loss of myocardium in long-term ischemic heart disease (Table). In the early phases of postinfarct myopathy, growth of the spared myocardium is not associated with invasion and repair of the infarcted area that conditions the chronic alterations in size, shape, and performance of the heart. The time-dependent worsening of cardiac anatomy and hemodynamics can only be prevented and reversed if the dead tissue is restored with new, functionally competent myocardium. In its absence, myocyte hypertrophy occurs and cellular enlargement depresses ventricular function further. Regeneration attenuates the load on the surviving myocardium and atten-



**Figure 6.** Myocardial infarction and cardiac repair. A, Regeneration of the infarcted myocardium by mobilization of resident cardiac progenitor cells. The area delineated by the rectangle is shown at higher magnification in B. Myocytes,  $\alpha$ -sarcomeric actin (red)+ nuclei, propidium iodide (green). A, Scale bar=1 mm; B, scale bar=100  $\mu$ m. Reproduced with permission from Urbanek et al.<sup>25</sup>

uates or corrects cavitory dilation and wall thinning. Successful strategies in this direction have been developed in the mouse<sup>25</sup> and dog<sup>23</sup> heart. Local growth factor activation of CSCs within the myocardium can rescue mice with large infarcts (Figure 6), in the long term leading to reconstitution of the infarcted ventricular wall, normalization of the ventricular mass-to-chamber volume ratio, and acquisition of the adult phenotype by a fraction of newly formed myocytes.<sup>25</sup>

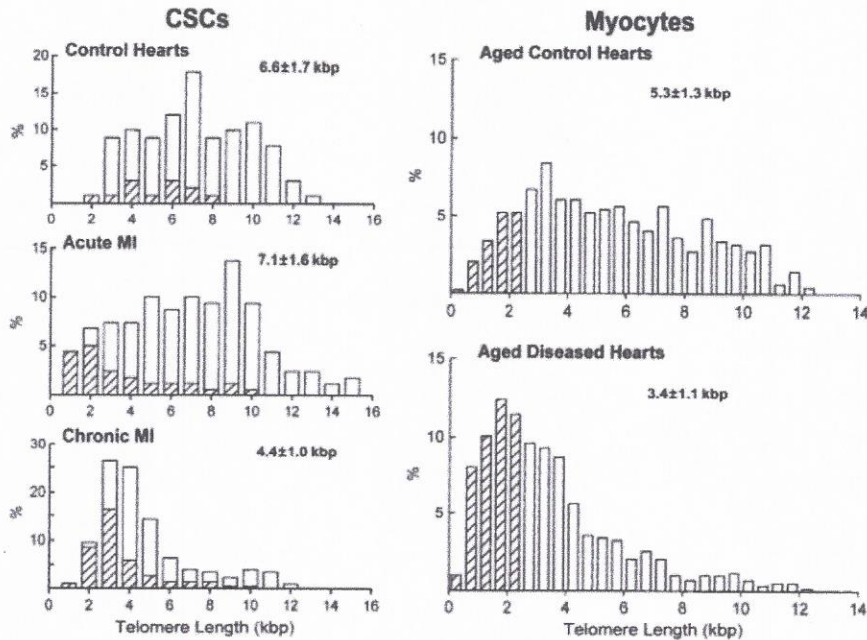
### CSC Aging and Death

The evidence reviewed earlier indicates that the heart is a self-renewing organ and that the growth of its parenchymal cells is regulated by the CSC compartment and by the ability of CSCs to self-renew and differentiate. Telomeres are chromatin structures composed of tandem G-rich repeats bound to an array of proteins that cap the ends of chromosomes and are critical for chromosomal stability and cell viability.<sup>67</sup> Proliferation of human stem cells is dependent on a functional telomere.<sup>87</sup> Telomere shortening coupled to cell division in the absence of telomerase activity is 1 of the major causes of telomere dysfunction in human cells. Severe telomeric shortening to a critical length triggers chromosome end-to-end fusion, which affects cell replication and survival.<sup>67,87</sup> Telomerase prevents telomere erosion and chromosomal instability by protecting against the loss of DNA at the chromosomal ends during cell division, which, with time and repeated doublings, results in irreversible growth arrest, cellular senescence, and apoptosis.<sup>9,10</sup> Typically, stem cells undergo replicative senescence with loss of self-renewing capacity and commitment and differentiation.<sup>87</sup> Telomeric shortening affects CSCs and myocytes in the aged and diseased heart (Figure 7).

The length of telomeres is a good predictor of the regenerative potential of a cell,<sup>87,88</sup> and cycling human CSCs express telomerase. This morphological characteristic has its molecular equivalent in telomerase activity.<sup>7-9</sup> These results are consistent with the notion that control of telomere length



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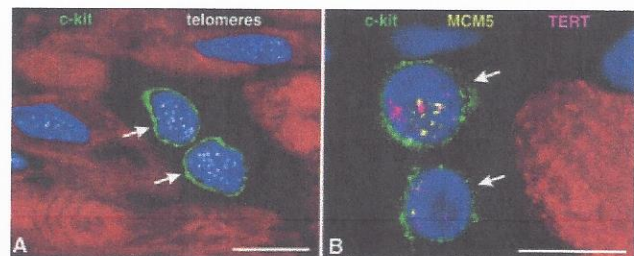


**Figure 7.** Telomeric shortening in CSCs and myocytes of the failing human heart. Frequency distribution of telomere length in CSCs of control and infarcted (MI) hearts (left) and myocytes of normal and failing aged hearts (right). The distribution of telomere length in the decompensated heart is shifted to the left. Average values are indicated in each panel. The hatched areas within the bars correspond to nuclei expressing p16<sup>INK4a</sup>. kbp indicates kilobase pairs. Modified and reproduced with permission from Urbanek et al<sup>8</sup> and Chimenti et al.<sup>10</sup>

in CSCs is regulated by telomerase and not by alternative telomere-lengthening mechanisms.<sup>89</sup> However, other factors are crucial for telomere maintenance. The loss of integrity of telomere structure, which is composed of repetitive sequences of DNA and proteins, significantly conditions telomeric shortening. Telomeres are protected by a complex of proteins, including TRF1 and its partners tankyrase and TIN-2, TRF2, poly(ADP-ribose) polymerase, DNA-dependent protein kinase, Ku80, Ku70, and Pot1.<sup>8,90</sup> Therefore, replicative senescence of CSCs can be induced by changes in the protected status of telomeres rather than by a pure loss of telomeric DNA. In myocardial aging and chronic heart failure, CSCs are characterized by telomere dysfunction with alterations in telomeric binding proteins.<sup>8,10</sup> These molecular modifications are accompanied by increased expression of p14<sup>ARF</sup>, p16<sup>INK4a</sup>, phospho-Ser-15-p53, and p53; p14<sup>ARF</sup> inhibits p53 degradation, and p16<sup>INK4a</sup> inhibits cdk4 and cdk6, blocking cells at the G<sub>0</sub>-G<sub>1</sub> boundary. Moreover, p53 inhibits the cell cycle at the G<sub>1</sub>-S or G<sub>2</sub>-M checkpoint. Bmi-1, which is a negative modulator of the age-associated genes p14<sup>ARF</sup> and p16<sup>INK4a</sup>, decreases in CSCs in chronic heart failure.<sup>91</sup> Telomere dysfunction, cellular senescence, and the expression of genes that interfere with cell replication and that activate cell death are enhanced in CSCs of hearts suffering from aging and end-stage failure. Together, these factors result in the loss of functionally competent CSCs, which can no longer reenter the cell cycle and divide because they have reached replicative senescence.<sup>8,10,63,64</sup> Telomere dysregulation, in combination with the expression of p53 and p16<sup>INK4a</sup>, activates the death program, further decreasing the size of the CSC pool in the heart. Despite cellular senescence and death, however, the old heart<sup>10</sup> and the chronically decompensated heart<sup>8</sup> possess a number of CSCs that retain the ability to divide and differentiate. These CSCs have long telomeres, express telomerase and cycling proteins such as Ki67 and MCM5, and are p53- and p16<sup>INK4a</sup>-negative (Figure 8). Whether these CSCs maintain their youth and self-renewing property or

whether they represent a residual pool of defective old cells is currently unknown.

The rate of organ aging depends on the pace of cell-autonomous processes resulting in the accumulation of older cells with impaired function.<sup>92</sup> This accumulation is in turn linked to a decline in the capacity of the organ to replace old cells with better-functioning, younger cells. Therefore, aging of the heart is defined at the level of the controlling cell, ie, the CSC. CSC aging decreases the pool size of functionally competent stem cells, impairing the compensatory growth mechanisms of the heart and its ability to sustain sudden increases in workload and ventricular performance.<sup>8,10</sup> These limitations are apparent in aging human hearts in which ischemic injury has much more dramatic effects in terms of morbidity and mortality than in younger individuals.<sup>93</sup> In the elderly, premature cardiac aging manifests itself with loss of CSC growth, CSC apoptosis, and heart failure.<sup>10</sup> Similarly, chronic cardiac failure of ischemic and nonischemic origin is characterized by a remarkable increase in the number of senescent CSCs and senescent, poorly contracting, hypertrophied myocytes.<sup>7,8</sup> Regeneration is activated, but the long-term outcome of CSC division is telomeric shortening,



**Figure 8.** Cycling CSCs in the human heart. A, Two c-kit-positive CSCs (green) possess long telomeres (white dots in nuclei). B, Two c-kit-positive CSCs (green) express the cell cycle protein MCM5 (yellow dots) and the catalytic subunit of telomerase (TERT, magenta dots). Scale bars=10  $\mu$ m. Reproduced with permission from Urbanek et al.<sup>8</sup>



replicative senescence, cell death, reduction of the stem cell pool, and ultimately, exhaustion of their growth reserve.

### Conclusions

In self-renewing organs, cell number is controlled by the stem cell compartment. A good example of this paradigm is found in the hematopoietic system. Despite the fact that proliferation and self-renewal of HSCs are limited and dependent on telomere length and telomerase activity, these cells may not reach their intrinsic limits during the organism's lifespan.<sup>94</sup> The turnover of mature blood cells is maintained in old age, although alterations in gene expression have been reported.<sup>95</sup> Conversely, the turnover of myocytes and vascular SMCs and ECs is not preserved in the aging heart of mammals, including humans.<sup>72,73</sup> Rarefaction of the coronary vasculature and myocyte loss characteristically occur in men, whereas the number of parenchymal cells is not reduced in age-matched women.<sup>96</sup> This growth defect occurs in women nearly a decade later than in men. When telomere attrition and replicative senescence of CSCs become apparent, myocardial aging and heart failure develop in humans.<sup>10</sup> Similarly, CSCs cannot supply the acutely and chronically decompensated human heart with the necessary number of cardiomyocytes and vascular structures, so the diseased heart is composed of a reduced number of parenchymal cells and coronary vessels. Hypertrophied, senescent myocytes accumulate, and the ratio of old to young cells severely increases; this condition is present in CSCs and in other cardiac lineages. Equivalent conditions to the aging and failing heart are found HSC disorders, such as aplastic anemia and myelodysplastic syndromes.<sup>97,98</sup> These pathological conditions cause bone marrow failure, which manifests itself with a drastic decline in HSCs and in the bone marrow's ability to produce mature blood cells. This is mimicked in the aged and decompensated heart in which the balance between cell loss and cell formation is altered in favor of the former, indicating CSC failure.<sup>8,10</sup>

Thus, heart failure and the myopathy of aging share a common underlying cellular alteration that involves the stem cell compartment. Telomeric dysregulation and shortening lead to growth arrest and apoptosis of CSCs, and these processes negatively affect the creation of parenchymal cells and coronary vessels in the myocardium. Additionally, CSCs are not equipped to sense signals from areas of damage that could promote their recruitment and regeneration potential, although they can be coaxed to home to sites of injury, where they can restore dead myocardium. However, their growth and survival, together with their translocation and speed of locomotion, are impaired with aging and most likely with chronic heart failure, and these defects are the consequence of downregulation of the local c-Met–hepatocyte growth factor and insulin-like growth factor-1/insulin-like growth factor receptor systems.<sup>23,64</sup> Although CSCs are efficient in preserving organ homeostasis and cell turnover, the decompensated heart is characterized by a loss of myocytes and vascular structures that cannot be counteracted by the activation and differentiation of CSCs, which undergo progressive replicative senescence, leading to a dramatic reduction of the stem cell compartment. In this sense, cardiac aging and chronic

heart failure can be viewed as stem cell disorders. It is hoped that the new paradigm of cardiac biology and an improved understanding of the fundamental role of CSCs in cardiac physiology and pathophysiology will promote the development of novel therapeutic strategies for alleviating heart failure and perhaps even for preventing cardiac senescence.

### Disclosures

None.

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