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Resident cardiac stem cells

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Abstract. Regardless of erroneous claims by a minority of reports, adult cardiomyocytes are terminally differentiated cells which do not re-enter the cell-cycle under any known physiological or pathological circumstances. However, it has recently been shown that the adult heart has a robust myocardial regenerative potential, which challenges the accepted notions of cardiac cellular biology. The source of this regenerative potential is constituted by resident cardiac stem cells (CSCs). These CSCs, through both cell transplantation and *in situ* activation, have the capacity to regenerate significant segmental and diffuse myocyte losts, restoring anatomical integrity and ventricular function. Thus, CSC identification has started a brand new discipline of cardiac biology that could profoundly changed the outlook of cardiac physiology and the potential for treatment of cardiac failure. Nonetheless, the dawn of this new era should not be set back by premature attempts at clinical application before having accumulated the required scientifically reproducible data.

Keywords. Cardiac stem cells, myocardial regeneration, stem cell plasticity, stem cell niche, stem cell fate.

Introduction

The continuous increase in the average human lifespan and the progressive aging of the population in all developed countries has had the inevitable drawback of an increasingly severe epidemic of chronic diseases. Presently, there are >5 million patients with post-acute myocardial infarction (AMI) in chronic heart failure in the USA alone, and more than 550,000 patients per year are added to this group, which has an average mortality rate of ~18 % per year [1].
Early invasive treatment and optimization of medical therapy are increasingly successful in dealing with the acute manifestations of coronary artery diseases, i.e. acute coronary syndromes. This combined treatment strategy often extends the life of the patient, but may leave a chronic condition that represents a point of no return to a healthy life for the patient. Indeed, once congestive heart failure emerges, no therapies can improve long-term cardiac function [2]. The cellular basis of this downward spiral is a decrease in the number of viable cardiomyocytes, secondary to either acute ischemic injury or chronic cell apoptosis/necrosis, which is not balanced by the adaptive hypertrophy of the remaining cardiomyocytes.
It has been a long-standing goal of cardiology to find a source of cells capable of functionally replacing those lost as a consequence of AMI in order to prevent or reverse the pathological cardiac remodeling responsible for the development of heart failure.
Cardiac biology has for a long time rested on the dogma that the heart is a post-mitotic organ with no regenerative potential. Because of this, the only option to myocyte loss has been either palliative and/or aimed at improving or preserving the function of the remaining myocytes. This scenario left organ transplantation as the only means of improving cardiac function, with all

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the logistical and biological limitations associated with this type of intervention [3]. Moreover, the idea of generating human-compatible animal organs, such as the heart, through genetic manipulation has been elusive and full of pitfalls [4].

The recent popularity of the so-called field of regenerative medicine, based on the plasticity and potential of stem cells, has focused attention on the possibility of regenerating myocardial tissue through cell transplantation in order to treat cardiac diseases due to myocyte deficit [5]. Embryonic stem cells (ESCs), fetal myocytes, skeletal myoblasts, endothelial progenitor cells, bone marrow-derived mesenchymal and hematopoietic stem cells have all been transplanted into the post-infarcted myocardium of experimental animals in order to generate new myocytes and/or vascular structures [6]. ESCs represent the most fashionable and highly touted hypothetical source of cells to be used for tissue repair. However, their tendency to produce teratomas, the ethical issues surrounding their future use and the necessity of immunosuppression due to their heterologous nature will severely limit the potential use of these cells in regenerative medicine. The feasibility of nuclear transplantation and the 'therapeutic cloning' as a solution to obtain non-immunogenic, patient-matched ESCs remains in doubt after the recent scientific scandal and consequent setback for this interesting option [7].

Bone marrow-derived cells, along with circulating progenitors, have been used in small clinical trials with inconclusive but generally disappointing results [6, 8, 9]. Unfortunately, with very few exceptions [9, 10] most regenerative assays, including clinical trials, have been performed with non-cloned bone marrow-derived cell preparations, and it is thus impossible to unambiguously ascertain the nature of the regenerating cells, if any. Moreover, recent data have questioned the myogenic potential of bone marrow-derived stem cells [11–13], while this potential has been reaffirmed by others [10, 14–16]. These discordant results have left an unresolved controversy about the potential and biological bases of myocardial regeneration by bone marrow-derived cells, which threatens the future of this nascent field. It will require a broadly convincing and generally accepted experimental answer.

With very few exceptions, most experimental and clinical protocols for myocardial regeneration are based on transplantation into the damaged myocardium of exogenous cells with the capacity to differentiate into or perform the function of myocardial or vascular cells [6, 8, 9]. This has been so because, despite evidence to the contrary, the myocardium continues to be considered a tissue without regenerative potential. Indeed, Cardiac myocytes are terminally differentiated cells that permanently exit the cell cycle after a few rounds of cell division shortly after birth [17, 18]. During the past decade, it has been recognized that organs considered post-mitotic (e.g. brain) have, in reality, regenerative potential because they harbor a population of tissue-specific adult stem cells [19]. Since no such cells had been identified in the myocardium at the turn of the century, the heart was the only organ left as terminally post-mitotic and without intrinsic regenerative capacity. In this scenario, the only avenue open to replace the lost myocardial cells was by exogenous cell transplantation. Recently, however, it has been documented that the adult heart, too, contains a pool of cardiac stem-progenitor cells (CSCs) that not only can replenish the cardiomyocyte population [20–27] but are also able to regenerate coronary vessels [20, 28]. Therefore, the heart joins other organs, such as the central neurons system (CNS), in having regenerative potential yet being constituted mainly of post-mitotic cells.

As well as playing an essential role in myocardial regeneration, it is increasingly evident that resident CSCs also play an important role in normal cardiac homeostasis and myocardial response to injury. Moreover, the CSCs likely represent the best candidate cell for long-term reconstitution of lost myocardium either by cell transplantation or through *in situ* activation [28]. Here we will review current progress in the identification and characterization of various types of CSCs reported to be present in the adult mammalian heart, including human. We will highlight the need to unravel the cellular and molecular events controlling the decision of adult CSCs to self-renew or to differentiate before we can develop an informed and rational approach to exploit their myocardial regeneration potential. Finally, we will put in perspective the prospects of CSC-based myocardial regeneration by *in situ* CSC activation.

The adult mammalian heart harbors a population of resident CSCs

Because the adult heart was considered a post-mitotic organ without regenerative capacity, it was axiomatic that it lacked a population of stem cells. Based on data obtained from sex mismatched cardiac transplants, we postulated the existence of such cells in the adult myocardium [29]. With Anversa's group, we first reported the identification and characterization of a distinct population of resident CSCs in rodents [20]. These cardiac c-kit^{pos} cells are self-renewing, clonogenic and multipotent, giving rise to a minimum of three different cardiogenic cell lineages: myocytes, smooth muscle cells and endothelial cells. When

grown in suspension, they form a ball of hundreds of cells similar to the pseudo-embryoid bodies formed by the neural stem cells (neurospheres), which by analogy we named cardiospheres and which represent a distinctive feature of multipotent cells. Out-growing cells from these spheres express biochemical markers of myocytes, smooth muscle cells and endothelial cells [20, 28]. When BrdU- or genetically tagged c-kit^{pos} cells were injected into the border zone of an experimentally produced infarction [20], a band of regenerating myocardium composed of tagged cells was observed replacing the infarcted zone. The labeled cells were either small cardiac myocytes expressing sarcomeric proteins or vascular cells forming new vascular structures [20].

Other cardiac stem/progenitor cells with similar characteristics were rapidly identified by other investigators (Table 1). Interestingly, each reporting group has placed emphasis on different markers which made their cells unique and different from those previously reported. With the exception of the Islet 1 cells, which seem to be remnants from the cardiac primordia [25], the different cardiac/stem progenitors identified by expression of other membrane markers are likely to be phenotypic variations of a unique cell type. It is highly unlikely that a tissue which until recently was believed to lack any self-renewal capability is indeed populated by several different types of tissue-specific stem cells.

A resident population of progenitor cells, confirmed by several investigators [21–23, 26, 27], has been isolated from the mouse heart based on expression of the stem cell antigen 1 (Sca-1^{pos}). These adult-heart derived Sca-1^{pos} cells were c-kit^{neg} but expressed Tie-2, Ang-1 and CD31, markers which might denote a primitive hemangioblast or its precursor [21]. These cells did not differentiate into cardiac lineages but expressed Nkx2.5 when treated with 5′-azacytidine *in vitro* [21], indicative of cardiomyogenic commitment. A similar enriched population of adult cardiac Sca-1^{pos} cells isolated from the hearts of 12-week-old mice formed beating cardiomyocytes with spontaneous calcium transients upon oxytocin treatment [22]. However, multipotency of these adult Sca-1^{pos} cells remains to be proven in cloned cell assays. A side population (SP) of cardiac cells within the Sca-1^{pos} fraction has also been described [23]. These cells, present in embryonic and fetal life and said to persist into the adulthood, are best characterized by expression of the transport protein Abcg2 [23]. Whether the cells present in prenatal and postnatal life represent the same or different cell populations remains unaddressed. Importantly, their ability to differentiate into contracting cardiac myocytes or to contribute to functional repair of damaged heart muscle has not yet been reported. Pfister et al. [26] have reported that among cardiac SP cells, the greatest potential for cardiomyogenic differentiation resides in the Sca1^{pos}CD31^{neg} populations, which are capable of both biochemical and functional cardiomyogenic differentiation into mature cardiomyocytes.

Tomita et al. [27] observed that rodent cardiac SP cell fractions from neonatal hearts (heterogeneously positive for c-kit, Sca-1 and CD34) formed cardiospheres in serum-free medium in the presence of epidermal growth factor and fibroblast growth factor 2 (FGF-2) [27]. These cells also expressed nestin and Musashi-1, markers of undifferentiated neural crest stem cells. Indirect evidence coming from Cre/lox transgenic mice also suggests that these SP-derived spheres were derived from neural crest-originated cells present in the myocardium [27]. However, direct and definitive proof that adult cardiac SP cells contain clonogenic, self-renewing and multipotent nestin^{pos}/Musashi-1^{pos} cells is still missing.

A population of undifferentiated cardiac cells expressing markers different from those described above has also been identified [30]. These cells are remnants of cells present since embryonic life in the heart fields and anterior pharynx [30]. These cells express the homeobox gene islet-1 (Isl1). They are found most commonly in the outflow tract, the atria and the right ventricle, in agreement with the embryonic contribution of the secondary heart field [30]. Interestingly, some Isl1^{pos} cells can be identified in the hearts of newborn rodents and humans and can differentiate into cardiac myocytes both *in vivo* and *in vitro;* the number of these cells falls dramatically in the first few weeks of post-natal life [25]. The multipotentiality of these progenitors and their capacity to engraft in the heart and to regenerate lost myocardium has not been tested [31].

CSCs and the human myocardium

The unambiguous identification of resident CSCs in the adult rodent heart raised the question of whether cells with similar characteristics and regenerative potential are also present in the human myocardium. Not surprisingly, Messina et al. [24] isolated c-kit^{pos} cells from biopsy samples of human myocardium. These cells cloned with efficiency similar to rodent CSCs, and when injected into immunodeficient animals after myocardial injury they regenerated functional myocardium.

We have recently confirmed and expanded these results. Indeed, we have obtained c-kit^{pos} human CSCs (hCSCs) from myocardial surgical or percutaneous biopsy samples from each of the four cardiac cham-

Table 1. Chronological order of discovery of resident putative stem cells and their characteristics in the adult heart by different research groups.

	Frequency	Species	Phenotype	Clonogenic	Multipotent	Tested in *in vivo* regenerative assay
Beltrami et al. (2003)	1/10,000 myocytes	20–25 month old Fisher rats	Lin^{neg} $CD45^{neg}$ $CD34^{neg}$ $c\text{-}kit^{pos}$	Yes. Cardiosphere formation.	Gave rise to 3 different cardiac lineages (myocytes, endothelial, smooth muscle), *in vitro* and *in vivo*.	Yes, injected GFP^{+ve} $c\text{-}kit^{POS}$ cells gave rise to a band of regenerating myocardium after MI.
Oh et al. (2003)	14–17% of cardiac cells are $Sca1^{pos}$, and 0.03% of cardiac cells have SP profile	6–12 week C57BL/6 mice	$Sca1^{pos}$ Lin^{neg} $c\text{-}kit^{neg}$ $CD45^{neg}$ $CD34^{neg}$ $CD31^{pos}$	Not cloned	ND. Myocyte differentiation *in vivo* and *in vitro*.	Yes. Ischemia/reperfusion injury −1.5% myocyte differentiation in infarct border zone.
Matsuura et al. (2004)	Estimated ~0.3% of the total number of myocytes in the heart.	10–12 week C57Bl/6 mice	$Sca1^{pos}$ $CD45^{pos}$ $CD34^{pos}$ $c\text{-}kit^{pos}$	Not cloned	Myocyte, osteogenic and adipogenic differentiation *in vitro*.	ND
Martin et al. (2004)	Obtained a SP profile of 2% of total number of cells analyzed.	Embryonic and adult mice.	SP $Abcg2^{pos}$ $Sca1^{pos}$ $c\text{-}kit^{pos/low}$ $CD34^{pos/low}$ $CD45^{pos/low}$ $CD31^{neg}$	Not cloned	Myocyte differentiation (α-$actin^{pos}$) *in vitro*.	ND
Messina et al. (2004)	~10% of cardiac cells are positive for c-kit, CD34, Sca1 and CD31.	Transgenic mice. 1-month to 80 year old humans.	$c\text{-}kit^{pos}$ $Sca1^{pos}$ $CD34^{pos}$ $KDR/Flk\text{-}1^{pos}$ $CD31^{pos}$	Yes. Cardiosphere formation.	Endothelial and myocyte differentiation *in vitro* and *in vivo*.	Yes, gave rise to new myocytes and capillaries in infarcted myocardium.
Laugwitz et al. (2005)	137 ± 23 RA 198 ± 35 OT 67 ± 15 RV 25 ± 7 LV (taken from 1 day old rat heart).	Embryonic and Neonatal mice (1–5 days old) and rat. 2- and 8-day old humans.	$Isl1^{pos}$ $Sca1^{neg}$ $c\text{-}kit^{neg}$ $CD31^{neg}$ $Nkx2.5^{pos}$ $GATA4^{pos}$	Not cloned	Myocyte differentiation *in vitro* with sarcomeric structure.	ND
Pfister et al. (2005)	~500 to 1000/adult mouse heart	Adult mice (8–12 weeks)	SP $CD31^{neg}$ $Sca1^{pos}$ $CD45^{low/neg}$ $CD34^{low/neg}$ $CD44^{low/neg}$ $c\text{-}kit^{low/neg}$ $Nkx2.5^{pos}$ $GATA4^{pos}$ SMA^{pos} $Desmin^{pos}$ $Tie2^{pos}$	Not cloned. Colony-Forming Units	Myocyte differentiation with sarcomeric organization and spontaneous contractions at low frequency, *in vitro*.	ND
Tomita et al. (2005)	3.5% in 2 day heart 0.02% in 6 week heart	Neonatal (2 and 7 days) and adult (6 weeks) mice	SP $CD11b^{neg}$ $CD13^{neg}$ $CD45^{neg}$ $Ter119^{neg}$ $CD29^{pos}$ $CD44^{pos}$ $CD34^{pos}$ $c\text{-}Kit^{pos}$ $Flk\text{-}1^{pos}$ $Sca\text{-}1^{pos}$ $nestin^{pos}$ $Musashi\text{-}1^{pos}$ $Mdr1^{pos}$	Yes. Cardiosphere formation.	Differentiation of cardiosphere-derived cells into neurons, glia of CNS and PNS lineage (*in vitro and in vivo*) and beating cardiomyocytes (*in vitro*).	ND

SP denotes Side Population
ND denotes Not Determined

bers of patients with ischemic and non-ischemic heart disease [32] (Fig. 1). The c-kit^{pos} hCSCs can be cloned with similar efficiency to their rodent counterparts. All the hCSCs clones obtained so far express high levels of c-kit and MDR-1, and they score negative for the endothelial and hematopoietic markers CD34, CD45 and CD31. Many of these cloned hCSCs have undergone more than 60 passages so far without evidence of crisis or culture senescence, form cardiospheres and differentiate *in vitro* into cardiomyocytes, vascular smooth muscle cells and endothelial cells. Importantly, when injected into infarcted hearts of *nu/nu* rats, they form histological and functional human myocardium and microvasculature. At the level of analysis performed so far, there are no detectable differences among the isolated cells that can be attributed to the cardiac chamber of origin [32]. Thus, c-kit^{pos} hCSCs can be successfully and routinely isolated from small myocardial samples of the four cardiac chambers, expanded to large numbers and maintained undifferentiated and/or differentiated in culture as desired. From the progeny of a single cell, it is possible to obtain $>1\times10^{10}$ differentiation-competent hCSCs. Moreover, the density of Lin^{neg} c-kit^{pos} hCSCs in the adult human and rodent myocardium is similar: 1 cell per ~1000 myocytes or ~50,000 hCSCs per gram of tissue [33].

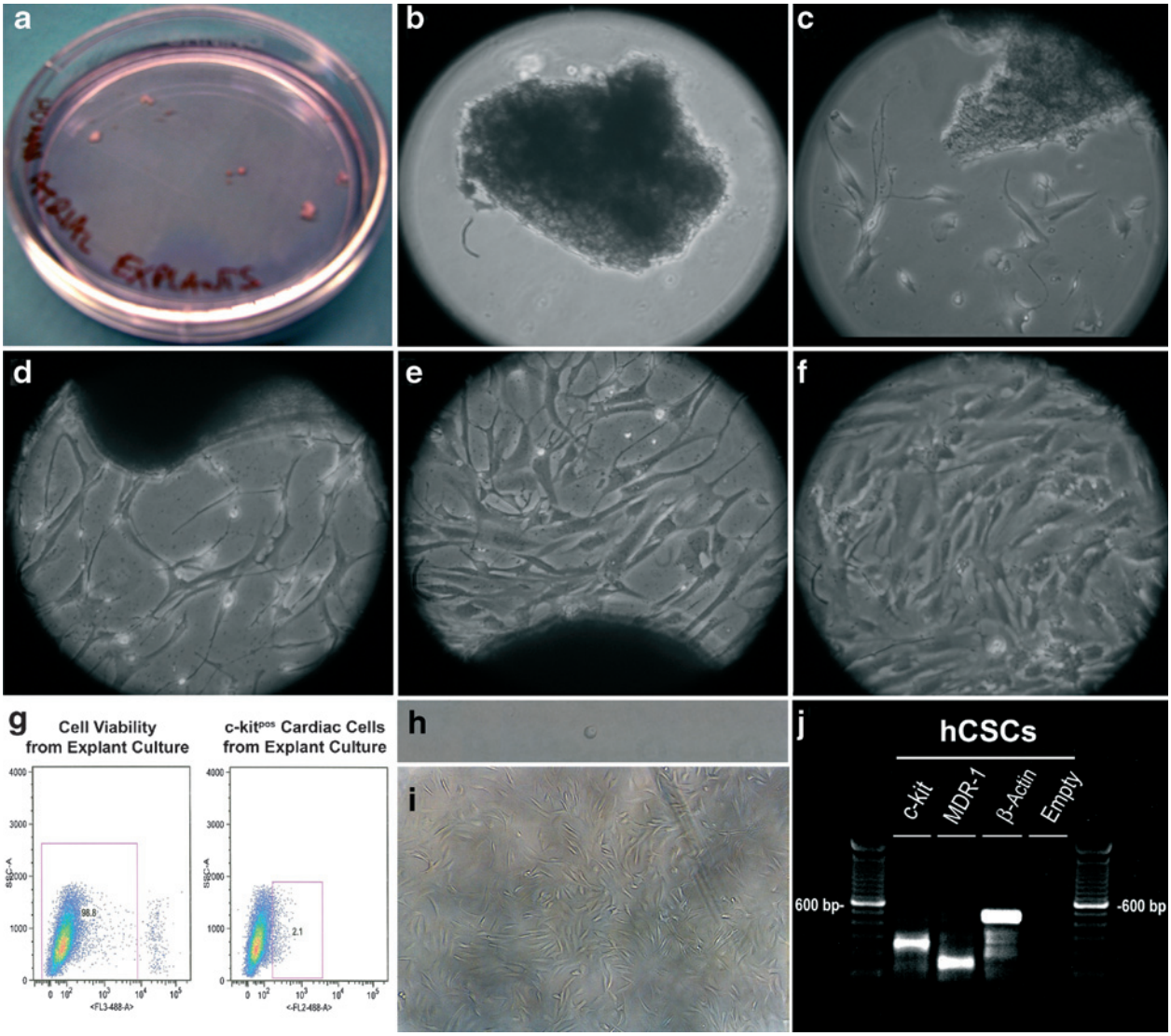

Figure 1. *Primary explant culture from human atrial explant and c-kit^{pos} cardiac stem cell (CSC) sorting.* Explant (*a*) and early stage outgrowth at 3, 7, and 14 days after explantation (*b–e*). Outgrowth confluent cells away from the explant at 28 days after explantation (*f*). c-kit^{pos} cells were sorted by flow-activated cell sorter (*g*). Single c-kit^{pos} cell suspension and formation of a clone of human c-kit^{pos} CSCs (*h–i*). hCSCs are positive for stemness genes, such as c-kit and multi-drug resistance 1 (MDR-1) shown by reverse transcriptase-polymerase chain reaction (*j*).

A new view of cardiac cellular homeostasis

As it was thought that the myocardium from early postnatal life until death was constituted by myocytes with the same chronological age as the individual, neither myocyte death nor new myocyte formation was understood to play any role in cardiac cell homeostasis. Yet over the past decade it has become increasingly evident from human and experimental animal data that the rate of myocyte loss under physiological and more so in pathological conditions is significant [5, 34]. This loss is in part compensated by hypertrophy of the remaining myocytes to preserve contractile muscle mass [5]. Despite this compensatory hypertrophy, normal myocyte death, in the absence of new myocyte formation, would inevitably result in the practical disappearance of the heart during the second half of the normal lifespan of most mammalian species, including human [5]. Since this does not happen, it naturally suggests that, concomitant with myocyte death, there must be compensatory new myocyte formation. Over the past decades a number of reports have revealed the presence of mitotic myocyte figures as well as incorporation of DNA labeling in myocytes of normal and pathological hearts [5]. Unfortunately, all these observations were misinterpreted by the authors who, based on these mitoses, reached the conclusion that adult myocytes were not terminally differentiated, post-mitotic cells but maintained the capacity to re-enter the cell cycle [35]. Identification of CSCs provided a satisfactory explanation for the ability of these immature myocytes to cycle once or twice before becoming terminally differentiated [5,28] and revealed a fundamental role for CSCs in the maintenance of normal cardiac cellular homeostasis.

If CSCs are involved in maintaining normal cardiac cellular homeostasis, what is their role in the compensatory responses of the myocardium to adapt to physiological and/or pathological demands that require an increase in cardiac work? It would be expected that if CSCs are an essential component of the cardiac reserve and adaptive mechanisms, they would become activated in response to a demand for increased myocardial mass. In contrast to the traditional idea that cardiac hypertrophy in the adult exclusively entails myocyte hypertrophy with no new myocyte formation (hyperplasia), we found that in human aortic stenosis, increased cardiac mass results from a combination of myocyte hypertrophy and hyperplasia. Intense new myocyte formation in the outflow tract of these patients is likely to be the result of the differentiation of hCSCs [36]. There are numerous clusters of stem cell progeny making the transition to cardiogenic and myocyte precursors, as well as very primitive myocytes that turn into terminally differentiated myocytes. These clusters provide a convincing link between resident hCSCs and new myocyte formation in the living human heart and strongly support the notion that they respond to an increased workload.

One of the arguments raised against the intrinsic regenerative capacity of the myocardium is that occlusion of a branch of coronary artery almost invariably results in a permanent myocardial scar instead of regenerated muscle. What this question overlooks is that the obstruction of a main parenchymal artery of any organ, no matter how abundant its resident stem cells (e.g. bone marrow, skin, intestine etc.), always evolves into a scar. This is so because during the evolution of long-lived organisms, the presence of adult stem cells is likely to have been selected as a mechanism to repair minor lesions and the normal wear and tear of the tissue, as it is required to support proper organ function for a long time and not to regenerate catastrophic acute segmental cell loss.

Myocardial regeneration occurs in humans following ischemic injury, but myocyte replacement appears to be restricted to the viable myocardium adjacent to and remote from the infarct [37]. Because hCSCs are distributed throughout the heart, it was of interest to determine whereas hCSCs become activated in response to ischemic insult to reconstitute the dead myocardium. To this end, hearts from patients who died acutely after infarction and explants from those undergoing transplantation for end-stage ischemic cardiomyopathy were examined [38].

The number of CSCs increased markedly in infarcted hearts, but striking differences were found between acute and chronic infarcts. Indeed, hCSC numbers were 7.5 and 3.5 times higher in acute and chronic infarcted left ventricles than in controls, respectively. Although most of these hearts exhibited small islands of active muscle regeneration in the infarcted area, they had the histological appearance of an abortive reparative phenomena. The level of regeneration definitely was not in the range expected from the dramatic increase in the number of hCSCs. This discrepancy is explained, at least in part, by the fact than in infarcted hearts many hCSCs acquire a senescent phenotype, as shown by expression of $p16^{INK4a}$, a reliable marker of the senescent state [38]. These senescent hCSCs, which can reach up to half of the total, are non-cycling and non-differentiating. Thus, after coronary occlusion, the magnitude of the infarct significantly exceeds the regenerative potential of hCSCs, which is further diminished by sequestration of a large fraction of them into the senescent non-functional pool. This regenerative cell

deficit fails to counteract the chronic loss of myocytes and vascular structures and might contribute to end-stage heart failure [38].

If human cardiac cell homeostasis throughout life is dependent on myocyte and vascular cell regeneration by hCSCs, it can be predicted that loss of hCSC function, either as a consequence of their death or because they become non-productive, should result in progressive net myocyte loss and impaired ventricular function, as has been shown in experimental animals. As ischemia induces the withdrawal of hCSCs from the functional pool, neutralizing their increase in absolute numbers, we inquired whether organismal aging affected hCSCs, as previously shown for murine CSCs [39]. Thus, endomyocardial biopsies from elderly patients with dilated cardiomyopathy, without any evidence of coronary or metabolic disease, were compared with age-matched individuals with normal ventricular function to determine whether there was evidence of hCSC senescence, leading to a deficit of myocardial cell regeneration over cell death, which might lead to cardiac failure [40]. Quantitatively, the density of hCSCs was ~2-fold higher in aged diseased as compared with control hearts. However, more than half of the hCSCs in the diseased heart were $p16^{INKa4\text{-}positive}$. Apoptosis and necrosis of hCSCs was detected in all diseased hearts. In all cases the dying cells were $p16^{INKa4pos}$. Myocyte death in both groups was detected only in $p16^{INKa4pos}$ cells but was higher in diseased hearts than in the controls. Evidently, like experimental animal models, older humans can develop a regenerative cell deficit despite an increase in the total number of hCSCs due to the accumulation of functionally impaired hCSCs (senescent), which results in a lower myocyte regeneration rate and increased myocyte death rate [40].

We conclude that the adult myocardium has a robust intrinsic regenerative capacity that resides in the CSCs. Myocyte death and new myocyte formation by the CSCs are thus the two sides of the coin that allow this organ to maintain proper and uninterrupted cardiac output from the cradle to the grave.

In search of an identity: origin of resident cardiac stem cells

The existence of CSCs raises a key question as to their origin. With the exception of Isl^{pos} cells (which seem to be remnants of primitive cardioblasts derived from the second heart field [25, 31]), the data available do not distinguish between intrinsic cardiac cells present in the myocardium from fetal life and cells of extracardiac origin which have colonized the myocardium in postnatal life. The strongest argument in favor of postnatal origin, for at least a subset of adult CSCs, has been provided by cases of sex-mismatched cardiac and bone marrow transplants [29, 41, 42]. In all the cases, after transplant, it has been possible to identify in the myocardium cells with the characteristics of stem-progenitor cells with sex chromosomes and/or genetic markers of the recipient in the case of heart transplants or of the donor for bone marrow transplantation. The main counterpoint to this compelling argument comes from the believers of a heart-restricted origin of CSCs [43]. It is considered that new myocyte formation in sex-mismatched cardiac transplants is actually the product of resident CSCs migrating from the surgical stumps of the host atria and not from circulating cells [43]. However, this hypothesis is unfortunately internally inconsistent because it poses a different origin of CSCs for transplanted hearts and for bone marrow transplantation. Indeed, selective cardiac homing of bone marrow-derived stem cells restores SP cardiac stem-progenitor cells following myocardial infarction [44]. Moreover, a parabiosis experiment performed in our group, which joins a green fluorescent protein (GFP) transgenic male mouse to a wild-type female mouse, provided evidence of a GFP-positive myocyte in the female heart after diffuse myocardial damage [D. Torella et al., unpublished observations] (Fig. 2). It should, however, be pointed out that CSCs isolated from the adult heart have a phenotype not present in the bone marrow. Therefore, if CSCs originate from the bone marrow, they either represent a specific and rare bone marrow cell population not yet identified or they have resided in the myocardium long enough to have lost the epitopes of bone marrow-derived stem cells. The latter possibility is supported by the fact that bone marrow-derived stem cells repopulating the CSC pool after MI rapidly lose CD45 expression after engrafting into the heart [44].

Other pieces of evidence argue in favor of an embryonic origin of CSCs. In addition to the presence of a small number of Isl^{pos} cells in the adult, SP cardiac stem cells and $c\text{-}kit^{pos}$ CSCs have also been identified in embryonic and fetal life [23, 24]. However, because of the lack of specific prospective markers for the CSCs, there is no test to determine whether cells isolated from embryonic life are actually the same as those obtained from the adult heart.

Arguing against the hypothesis that adult CSCs are remnants of the heart fields is the different developmental potential of the two cell types. Indeed, the main cardiac lineages of the developing heart, myocytes, vascular cells and connective tissue cells, clearly derive from spatially and temporally different precursors [28, 45]. This is at odds with the findings that, aside from the $Isl\text{-}1^{pos}$ cardiac cells, some of the resident adult CSCs so far characterized are able to

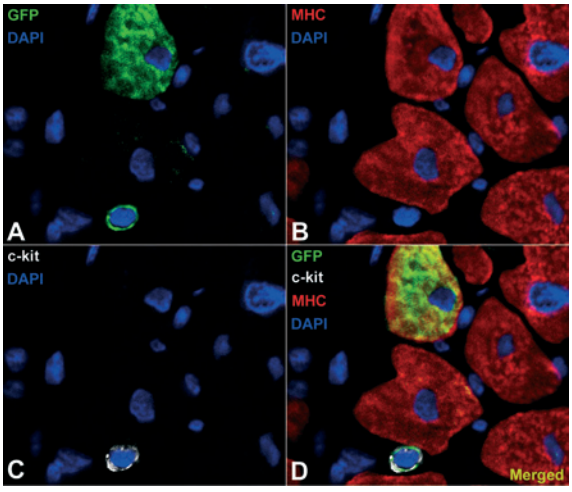

Figure 2. Parabiotic mouse model: circulating cell-derived new myocyte formation. Histological detection of a EGFP^{pos} cardiomyocyte (yellow, merged image, panel *d*) at the border of a serverely damaged myocardial area of a wild-type mouse 'parabiotically' joined with an EGFP transgenic mouse. Immunostaining shows myosin heavy chain (MHC, panel *b*, TRITC detection, red) and EGFP (panel *a*, green). In the same area, a c-kit^{pos} (Cy5 detection, white, panel *c*)-EGFP^{pos} (derived from joint circulation, green, panel *a*) cell is observed. DAPI staining (blue) detects cell nuclei.

give rise to new myocytes, endothelial cells and smooth muscle vascular cells.

The origin of CSCs is not a biological curiosity or a negligible detail without practical import, as some researchers in the field believe. Indeed, the origin of CSCs is of paramount importance if clinical protocols for true myocardial regeneration based on stem cell transplantation are going to be developed. In fact, if bone marrow does harbor the precursors of CSCs, it would provide an excellent and accessible source of those cells. Moreover, it would open new avenues to stimulate their mobilization and homing to the myocardium. Importantly, human bone marrow-derived multipotent stem cells with cardiogenic potential have recently been described. These cells can be generated from a single cell, exhibit unlimited self-renewal and are capable of triple-lineage differentiation: cardiomyocytes, endothelial cells and smooth muscle cells [10].

As discussed above, independent of the origin of CSCs it is important to determine whether phenotypically distinct CSCs described by different groups (20–27] (Table 1) represent different physiological states and/or developmental stages of a generic cardiac stem cell. Could it be that there are different stem cell populations within the adult heart? In the latter case it would be important to determine whether they are chamber specific. Clearly, c-kit^{pos} CSCs are different from endothelial progenitor cells (EPCs) since the former are negative for CD34; they are also different from hematopoietic stem cells and do not express CD45 [20] (Table 1). Cardiac Sca-1^{+} cells were initially reported as c-kit, CD34 and CD45 negative [21]; however, other reports described the enriched population of cardiac Sca-1^{+} cells as being c-kit^{pos} [22] or being c-kit^{low} [23, 26] (Table 1). Low expression of c-kit in SP cardiac cells was recently reported as the artificial consequence of enzymatic cleavage during the digestion process of the adult murine heart [26]. Curiously, in other hands the same procedure does not appear to decrease the efficiency of isolation of c-kit^{pos} CSCs from adult rodent hearts [20, 24].

It is important to note that cardiac c-kit^{pos} cells are the only stem-progenitor cells proven to fulfill the requirement to define a bona fide stem cell. Indeed, they are clonogenic, self-renewing and multipotent, giving rise to three different cardiac lineages [20]. This remains to be demonstrated for the other cardiac progenitors described above (Table 1). Remarkably, cardiac c-kit^{pos} cells express MDR1, which is a P-glycoprotein of the same family of membrane transporters as Abcg2, and Sca-1 [46]. This would suggest that c-kit^{pos} CSCs represent a more primitive cell population with the potential to generate SP and Sca-1^{pos} cells. Lending support to this hypothesis, CD31^{pos}/Sca1^{pos} compose the majority of total SP cardiac cells (~80 %), and only the CD31^{neg} fraction of Sca-1^{pos} cells are able to undergo myocyte differentiation *in vitro*. Cardiac Sca-1^{pos} are also enriched for Ang2, which would suggest an early angioblast phenotype of these cardiac cells [21]. This would suggest Sca-1 as a marker for cardiac-derived vascular precursors. Indeed, Sca1^{pos} CSCs are highly proficient in endothelial differentiation [47]. Only experimental data will determine the validity of this putative developmental hierarchy of CSCs.

Finally, the resident population of isl1^{+} cardiac progenitors described by Laugwitz and colleagues [25] differs fundamentally from c-kit^{pos} CSCs, Sca-1^{+} progenitors and cardiac SP cells (see Table 1). Indeed, they probably represent specified cardiac progenitors or cardioblasts, as suggested [31]. Their relationship, if any, to the other CSCs remains to be determined.

Cell fusion vs. developmental plasticy of CSCs

An issue that has muddled the interpretation of some of the results with stem cells for myocardial regeneration has been the process of *in vivo* stem-cell fusion with differentiated host cells [48]. For some reason, this issue has caught the interest of a large number of investigators, with the result that there are almost as many papers dealing with cell fusion of transplanted

cells in the myocardium as there are publications dealing with the physiological effect and potential of this putative therapeutic approach.

Cell fusion has been described in different settings, involving both direct transplantation of non-cardiac cells into the myocardium and through the general circulation. Following the initial demonstration of occasional cell fusion between bone marrow cells and cardiomyocytes *in vitro* [49] and *in vivo* [50], phenotypes consistent with cell fusion events were subsequently demonstrated in several others studies, including transfusion of cardiac progenitor cells [21] and bone marrow-derived hematopoietic stem cells injected into the myocardium [51, 52], transplantation of skeletal myoblasts into normal hearts [52–54], transfusion of human peripheral blood $CD34^{pos}$ cells into the mouse heart [55] and co-culture of human bone marrow stem cells with rat cardiomyocytes [10]. Cardiomyocytes apparently derived from cell fusion were also recently observed in a xenogeneic context in which human blood haematopoietic stem cells were transplanted into neonatal immunodeficient mice [56]. Cell fusion has also been claimed to occur in transplanted hearts [57].

Although there is little doubt that cell fusion as a biological phenomenon occurs in the myocardium *in vivo* and with myocardial cells *in vitro,* it is also clear that it occurs at a very low frequency and that its physiological significance remains to be identified.

Moreover, in the context of myocardial regeneration, it is an incontrovertible fact that the fusion of transplanted cells with host myocytes does not increase the number of myocytes in the treated heart. Thus, as pointed out elsewhere [5], when cell therapy results in an increase in myocyte number, it cannot be explained by cell fusion.

When cardiac Sca-1^{pos} progenitor cells were injected into mice after ischemia-reperfusion injury using the cre/lox technology system, they generated new myocytes by both direct differentiation and fusion [21]. However, the study could not ascertain whether the 'fused' host cell (with the Sca-1^{pos} cell) was a resident myocyte or of another cardiac lineage.

Furthermore, the methods used to detect and quantify cell fusion, adapted from other systems, might not be as reliable as expected when used for the myocardium. Indeed, the most widely used method *in vitro* and in the mouse has been the so-called cre-lox system, which has proven very reliable in many cell types. Unfortunately, this system might not be as foolproof in the heart because the cre protein is of low molecular weight, and a defining characteristic of myocytes is to generate gap junctions that allow the transit of large molecules. Moreover, the phenomenon of metabolic complementation [58] between donor cre^{pos} cells and the recipient *floxed* mice cannot be dismissed as a potential explanation for the origin of the fused cells [28] in any of the published cases. In this respect it is relevant to notice that different cell types, among them EPCs and myocytes, form intercellular bridges that allow for the interchange of all sorts of macromolecules and even organelles such as mitochondria [59]. Such bridges, so far documented only *in vitro* and whose functionality *in vivo* has not yet been determined, could also lead to transfer of cre between cells, which would result in erroneously scoring the floxed cell as the product of cell fusion. Thus, the so-called genetic gold standard test to rule out cell fusion in the heart [13] needs to be further evaluated to determine its specificity in this organ. On the other hand, in the study of Beltrami et al. [20], c-kit^{pos} cell fusion was excluded by a set of stringent criteria [20], including the small size of the new myocytes, which were far too small to be the product of fusion, the high amplification of the transplanted cells and number of new myocytes produced, and the absence of 'fusing partners' in the dead infarct zone. Furthermore, islet-1^{+} cells [25] and c-kit^{pos} cells [G. Ellison et al., unpublished observations] undergo myocyte differentiation in the absence of cell fusion. In addition, most publications on the myogenic potential of CSCs have excluded cell fusion as an explanation for their developmental plasticity [25, 26].

Self-renewal vs. Differentiation: the molecular unknown

The new holy grail of stem cell biology is to unravel the molecular mechanisms which regulate the binary decision between self-renewal (and expansion) of the stem cell and uncommitted precursors versus their commitment to the differentiation pathway leading to the formation of one of the progeny cell types. Recently, progress has been made in understanding the self-renewal divisions of some adult stem cells [60–62]. However, little, if anything, is known about the molecular mechanisms regulating the choice between self-renewal and commitment to differentiation of CSCs.

It is clear that the niche plays a fundamental role in the regulation of quiescence, self-renewal and commitment to differentiation of stem cells in general [61–62]. This opens the treasure hunt searching for cardiac stem cell niches. Unfortunately, despite claims to the contrary [63], nothing is presently known about the cellular and cytokine/growth factor environment of the CSC niche. This is so because at this time there is not even consensus about which cell represents the true cardiac stem cell instead of its amplifying progeny. Since one of the characteristics of the

better-known stem cells is that they leave their niche upon activation, claims about identification of the CSC niche based on the study of amplifying cells [63] is likely to be irrelevant for understanding CSC biology. More evidence is available about the role of some gene families in the regulation of growth and/or differentiation of adult mammalian stem cells in general and the CSCs in particular. Recent evidence from a variety of stem cells, both embryonic and adult, indicates that stem cell fate is at least in part regulated by the Polycomb Group (PcG) family of genes [64]. Bmi-1, a member of the PcG family, promotes cell proliferation because it is a potent negative regulator of the Ink4a/Arf locus, which encodes cell cycle regulators and tumor suppressors $p16^{Ink4a}$ and $p19^{ARF}$ and, therefore, inactivates and/or downregulates the two main cell cycle regulators, p53 and pRB [65], thereby favoring cell growth. In recent years, direct evidence of the essential role of bmi-1 on the self-renewal of several adult stem cells, including HSCs [66] and neural stem cells [67], has been obtained. In both cell types, bmi-1 loss suppresses the self-renewal of the stem cell, while its overexpression rescues the self-renewal capacity. Very recently, the Bmi-1/$p16^{Ink4a}$ axis was also shown to finely regulate stem - cell replicative senescence and aging in HSCs [68] and neural stem cells [69] *in vivo*.

At least in the cerebellum, Bmi-1 is induced by the secreted morphogen Sonic hedgehog (Shh) [70], which also modulates the Wnt and Notch signaling networks [71]. This provides a connection between the PcG gene family and the main regulatory pathways of cell fate and differentiation represented by the Wnt and Notch gene families [72,73]. Therefore, based on these data, it is fair to conclude that the regulatory mechanisms for stem cell self-renewal on the one hand and commitment to a particular differentiation pathway on the othe, are likely to be closely intertwined [71].

Similar mechanisms appear to be involved in regulating CSC self-renewal and commitment. Wnt-conditioned media as well as Bmi-1 upregulation increase CSC proliferation, clonal efficiency and cardiosphere formation. The opposite effects were noticed when Bmi-1 and the Wnt canonical pathway were inhibited [74]. These preliminary findings point towards a key role for Bmi-1 and Wnt proteins in adult CSC biology, modulating their clongenesis and self-renewal.

Identification of the signaling mechanisms regulating CSC self-renewal and differentiation will be a major step towards the goal of regulating the CSC population both *in vivo* and *ex vivo*, thereby enhancing its clinical potential in cell replacement therapeutic strategies.

A Preview of the future: CSC-Based myocardial Regeneration without cell transplantation

From the evidence presented so far, it is clear that the future of myocardial regeneration rests on the success or failure in unraveling the genetic regulatory pathways controlling the self-renewal, differentiation and aging of CSCs.

Recent work from our group shows that cardiac myocytes exhibit a stereotypical response to different forms of stress: they activate the expression and secretion of a large number of growth factors and cytokines, among them IGF-1, HGF, SCF and SDF-1 [75, 76]. Not surprisingly, CSCs have receptors for these factors, which upon ligand binding signal to downstream effector pathways involved in cell survival, cell replication and differentiation [9]. Through an auto/paracrine feedback loop at least some of these growth factors trigger the production of the activating ligands by the CSCs themselves. This auto/paracrine loop is responsible for maintenance of the activated state of the CSCs for some time after the disappearance of the primary stimulus and supports their growth and differentiation [9].

Based on the data summarized above, it was logical to test whether in situ activation of CSCs by some of the growth factors produced by the stressed myocytes could be as effective as CSC transplantation in regenerating post-ischemic myocardium. Not surprisingly, a combination of IGF-1 and HGF proved very effective in mice [9]. Because the significance of murine data for human pathology is debatable, we have tested the effectiveness of local growth factor administration on a porcine model of acute MI with closed chest and open coronary arteries [77]. The results show that this therapeutic modality can produce extensive myocardial regeneration in an animal heart of size and anatomy similar to human [77]. Therefore, it should be possible to produce myocardial regeneration in humans exploiting the intrinsic regenerative capacity of the myocardium, without having to resort to cell transplantation. It should be stressed that, despite this promising outlook, before planning clinical trials using these approaches we need to obtain answers to a set of important questions such as identification of optimal compounds and doses, route and schedule of administration, duration of therapeutic effects and secondary effects, similar to those applicable to experimental clinical protocols for cell therapy in general [78].

Conclusions

Until very recently the accepted paradigm considered the mammalian heart a post-mitotic organ, without regenerative capacity and with a relatively constant but diminishing number of myocytes from shortly after birth to adulthood and senescence. The identification of CSCs that can divide and mature into heart muscle as well as vascular structures suggests that the heart has a previously unrecognized plastic and regenerative potential.

Despite the enticing therapeutic potential of cell-based cardiac regeneration, a host of important questions remain to be answered before rational approaches will allow for optimal clinical utility of CSCs. Elucidation of the regulatory mechanisms determining the self-renewal and differentiation of these cells will improve our understanding of cardiac biology and, hopefully, will foster the development of novel non-invasive stem cell therapies. Such therapies are needed if cardiac regeneration intervention is to become available to a significant fraction of the patients who can benefit from it.

Learning from the past is one of the major advantages for modern scientists, and repeating the mistakes of the past would be inexcusable. Indeed, given the long time and effort required to dismantle an old dogma of cardiac biology and open the door to the brave new world, of regenerative therapies directed to the myocardium, we must resist the temptation both to rush to their applications to humans and/or to foster the development of new dogmatic points of view. What we need is to accumulate a solid body of reproducible data about the new biology of the myocardium which, in turn, is likely to point us in the best direction to implement its practical application to the human condition.

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