Chapter 2
Understanding Tissue Repair Through the Activation of Endogenous Resident Stem Cells

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1 The Clinical Need of Stem Cells for Cardiovascular Diseases

Heart failure (HF) has been singled out as an epidemic and is a staggering clinical and public health problem, associated with significant mortality, morbidity, and healthcare expenditures, particularly among those aged ≥65 years (Roger 2013). In particular, HF has a prevalence of roughly six million in the United States (a similar number is reached in Europe) and more than 23 million worldwide. After the diagnosis of HF, survival remains quite poor with estimates of 50 % and 10 % at 5 and 10 years, respectively. Despite modest progress in reducing HF-related mortality, hospitalizations for HF remain frequent and rates of readmissions continue to rise (Roger 2013). It is predicted that the constant progress in the primary prevention of

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HF will eventually lead to decreasing incidence of the disease. Accordingly, the constant improvement in modern medical care of the acute cardiac diseases will result in improved survival. However, the latter in turn will anyway increase the prevalence of HF. This is so because modern management for HF is mainly a symptomatic treatment that does not fight against its cause, leave organ transplantation as the only alternative available to restore function, with all the logistic, economic and biological limitations associated with this intervention (Kahan 2011). Indeed, the root problem responsible for the poor outcome of the CHF is a deficit of functional myocardial contractile cells (cardiomyocytes) and adequate coronary circulation to nurture them resulting in pathological cardiac remodelling, which, in turn, triggers the late development of cardiac failure in these patients (Jessup and Brozena 2003). For this reason, it has been a goal of cardiovascular research for the past decade to find methods to replace the cardiomyocytes lost as a consequence of the MI in order to prevent or reverse the pathological cardiac remodelling. Overall, the need to identify new therapies has become a key research area in regenerative cardiovascular medicine and stem cell-based therapies are fast becoming an attractive and highly promising experimental treatment for heart disease and failure (Terzic and Nelson 2010).

2 Adult Heart Self-Renewal and Tissue-Specific Endogenous Cardiac Stem/Progenitor Cells

Out of the limelight and apart from the cultural and philosophical wars, over the past 15 years there has been a slow but steady re-evaluation of the prevalent paradigm about adult mammalian—including human—tissue cellular homeostasis. It has been slowly appreciated that the parenchymal cell population of most, if not all, adult tissues is in a continuous process of self-renewal with cells continuously dying and new ones being born. Once cell turnover was accepted as a widespread phenomenon in the adult organs, it was rapidly surmised that in order to preserve tissue mass, each organ constituted mainly of terminally differentiated cells needed to have a population of tissue-specific regenerating cells. Not surprisingly, this realization was rapidly followed by the progressive identification of stem cells in each of the adult body tissues (Yamanaka 2007; Robinton and Daley 2012; Rountree et al. 2012; Reule and Gupta 2011; Kopp et al. 2011; Kotton 2012; Buckingham and Montarras 2008; Suh et al. 2009).

For a long time, the cardiovascular research community has treated the adult mammalian heart as a post-mitotic organ without intrinsic regenerative capacity. The prevalent notion was that the >20-fold increase in cardiac mass from birth to adulthood and in response to different stimuli in the adult heart, results exclusively from the enlargement of pre-existing myocytes (Hunter and Chien 1999; Soonpaa and Field 1998; Laflamme and Murry 2011). It was accepted that this myocyte hypertrophy, in turn, was uniquely responsible for the initial physiological adaptation and subsequent deterioration of the overloaded heart. This belief was based on two generally accepted notions: (a) all myocytes in the adult heart were formed during fetal life or shortly thereafter, were terminally

differentiated and could not be recalled into the cell cycle (Nadal-Ginard 1978; Chien and Olson 2002); therefore, all cardiac myocytes have to be of the same chronological age as the individual (Oh et al. 2001); (b) the heart has no intrinsic parenchymal regenerative capacity because it lacks a stem/progenitor cell population able to generate new myocytes. Despite published evidence that this prevalent view was incorrect (Beltrami et al. 2001; Quaini et al. 2002; Urbanek et al. 2003, 2005; Anversa and Nadal-Ginard 2002; Nadal-Ginard et al. 2003), it took the publication of Bergmann et al. in 2009, based on ^{14}C dating in human hearts showing that during a lifetime the human heart renews ~50 % of its myocytes (Bergmann et al. 2009), to produce a significant switch in the prevalent opinion. After this, intensive research and still much controversy on the adult mammalian heart's capacity for self-renewal has finally brought a consensus that new cardiomyocytes are indeed formed throughout adult mammalian life (Bergmann et al. 2009; Hsieh et al. 2007). However, the physiological significance of this myocyte renewal, the origin of the new myocytes as well as the rate of adult myocyte turnover are still highly debated. Indeed, while Bergmann et al. (2009) have calculated a yearly cardiomyocyte turnover of about 1 % (Bergmann et al. 2009) others calculated 4–10 % (Senyo et al. 2013) and some as high as 40%/year (Kajstura et al. 2012). This very high spread on the "measured" values of such an important phenomenon raises questions about the conceptual and methodological approaches used in these studies. If Bergmann's calculations were the one more close to the reality, because this "measured" self-renewal is far from being very robust, its physiological significance would be highly doubtful. However, the conclusion of Bergmann et al. (2009) on the rate of turnover depends on the validity of a complex mathematical formula, whose impact on the results dwarfs that of the measured data. Their calculations identify the highest turnover rate during youth and early adulthood followed by a steady decrease with age. The latter conclusion, which is contrary to most or all the turnover values measured for all other human tissues, including the heart (Nadal-Ginard et al. 2003), has passed without a ripple. Similar discrepancy exists with respect to the origin of the new myocytes and their physiological significance. Three main sources of origin of the new myocytes have been claimed: (a) circulating progenitors, which through the bloodstream home to the myocardium and differentiate into myocytes (Quaini et al. 2002); (b) mitotic division of the pre-existing myocytes (Boström et al. 2010; Bersell et al. 2009; Kühn et al. 2007) and (c) a small population of resident myocardial and/or epicardial multipotent stem cells able to differentiate into the main cell types of the heart: myocytes, smooth and endothelial vascular and connective tissue cells (Torella et al. 2007; Rasmussen et al. 2011). It is clear now that the blood borne precursors, although well documented as a biological phenomenon (Eisenberg et al. 2006), might be limited to very special situation (Orlic et al. 2001) and their direct regenerative import is very limited, if any (Loffredo et al. 2011). Myocyte replacement, particularly after injury, was originally attributed to differentiation of a stem-progenitor cell compartment (Beltrami et al. 2003) a source confirmed by genetic cell fate mapping (Hsieh et al. 2007). However, more recently this last investigator group, using the same genetic tools, claims

that myocytes in the border zone of an infarct are actually replaced by the division of pre-existing post-mitotic myocytes (Senyo et al. 2013). Pre-existing cardiomyocyte division has not been convincingly documented and/or remains to be confirmed by different authors. This result, in addition to being contrary to most known biology of terminally differentiated cells, would shift the target of regenerative therapy towards boosting mature cardiomyocyte cell cycle re-entry. However, Senyo et al. (2013) only document that a very small fraction of cardiomyocyte DNA replication occurs in cells that have already activated the αMHC gene, a well-documented part of myocyte development, which falls short of documenting mature myocyte re-entry into the cell cycle. This evidence is indeed equally compatible with new myocyte formation from the pool of multipotent cardiac progenitor cells because it is a well-documented fact that newly born myocytes are not yet terminally differentiated and are capable of a few rounds of mitosis before irreversibly withdrawing from the cell cycle (Nadal-Ginard et al. 2003). Undoubtedly, the best documented source of the regenerating myocardial cells in the adult mammalian heart, including the human, is a small population of cells distributed throughout the atria and ventricles of the young, adult and senescent mammalian myocardium, that have the phenotype, behaviour and regenerative potential of bona fide cardiac stem cells (eCSCs) (Torella et al. 2007; Rasmussen et al. 2011; Srivastava and Ivey 2006). In 2003, we identified the first population of eCSCs in the adult mammalian rat heart (Beltrami et al. 2003). These cells express the stem cell marker c-kit (c-kit^{pos}), are positive for Sca-1 and MDR-1 (ABCG2), yet are negative for markers of the blood cell lineage, CD31, CD34 and CD45 (described as Lin^{neg}). They are self-renewing, clonogenic and multipotent and exhibit significant regenerative potential when injected into the adult rat heart following a myocardial infarction (MI), forming new myocytes and vasculature and restoring cardiac function (Beltrami et al. 2003). c-kit^{pos} eCSCs with similar properties to those originally identified in the rat have been identified and characterized in the mouse (Messina et al. 2004; Fransioli et al. 2008), dog (Linke et al. 2005), pig (Ellison et al. 2011) and human (Messina et al. 2004; Torella et al. 2006a, b; Bearzi et al. 2007; Arsalan et al. 2012). These cells are present at a similar density in all species (~1 eCSC per 1,000 cardiomyocytes or 45,000 human eCSCs per gram of tissue) (Torella et al. 2007). Similar to the rodent heart, the distribution of c-kit^{pos} eCSCs in the pig and human heart varies with cardiac chamber. Although a variety of markers have been proposed to identify eCSCs in different species and throughout development (Messina et al. 2004; Oh et al. 2003; Matsuura et al. 2004; Martin et al. 2004; Laugwitz et al. 2005; Moretti et al. 2006; Kattman et al. 2006; Wu et al. 2006; Smart et al. 2011), it still remains to be determined whether these markers identify different populations of eCSCs or, more likely, different developmental and/or physiological stages of the same cell type (Ellison et al. 2010). Recently, another multipotent cell type, present in the epicardium and derived from the pro-epicardial organ has been described (Ellison et al. 2007a). The role of these cells in normal or pathological myocyte turnover remains to be elucidated.

The progeny of a single eCSC is able to differentiate into cardiac myocytes, smooth muscle and endothelial vascular cells and when transplanted into the

border zone of an infarct regenerates functional contractile muscle and the microvasculature of the tissue (Beltrami et al. 2003). In a normal adult myocardium, at any given time, most of the eCSCs are quiescent and only a small fraction is active to replace the myocytes and vascular cells lost by wear and tear. In response to stress (hypoxia, exercise, work overload, or other damage), however, a proportion of the resident eCSCs are rapidly activated, they multiply and generate new muscle and vascular cells (Ellison et al. 2007a, 2012a), contributing to cardiac remodelling. Recently, two studies have questioned the in situ myogenic potential of c-kit$^{(pos)}$ cardiac cells in adult life as being significantly reduced compared to their neonatal counterparts (Zaruba et al. 2010), and in a model of myocardial cryo-injury (Jesty et al. 2012). Thus, whether the c-kit^{pos} eCSCs are necessary and/or sufficient for the adult cardiac regenerative response to damage/injury remained unproven. However, recently, using mouse and rat experimental protocols of severe diffuse myocardial damage which unlike an experimental infarct spares the eCSCs (Ellison et al. 2007b) combined with several genetic murine models and cell transplantation approaches, we have shown that the eCSCs, in the presence of a patent coronary circulation, fulfil the criteria as the cell type necessary and sufficient for myocyte regeneration, leading to complete cellular, anatomical and functional myocardial recovery (Fig. 2.1) (Ellison et al. 2013). To follow c-kit^{pos}eCSC physiological response to cardiac injury, we induced severe diffuse myocardial damage in adult rats with a single high dose of isoproterenol (ISO) (Ellison et al. 2007b). This treatment—in the presence of a patent coronary circulation—produces a Takotsubo-like cardiomyopathy (Akashi et al. 2008) (a clinical syndrome affecting up to 2 % of patients with symptoms and signs of acute myocardial infarction (AMI), characterized by catecholamine overdrive and reversible cardiomyopathy) killing-10 % of the LV myocytes and resulting in overt acute heart failure (Ellison et al. 2007b). Interestingly, the myocardial damage and heart failure spontaneously reverses anatomically and functionally by 28 days. The anatomical and functional recovery is met through a robust c-kit$^{(pos)}$ eCSC activation and ensuing new myocyte formation, the latter completely balancing the myocyte loss by ISO injury. Using inducible double-transgenic reporter mice to track the fate of adult cardiomyocytes in a "pulse-chase" fashion we compellingly show that new myocytes after diffuse myocardial injury are not generated through the division of pre-existing terminally differentiated myocytes but rather from non-myocyte cells, with the characteristics of a stem-progenitor compartment. Then, to directly identify whether c-kit^{pos} eCSCs replenish cardiomyocytes lost by myocardial damage, we genetically tagged in situ the resident c-kit^{pos}eCSCs and their committed progeny. Through these in vivo genetic cell-fate mapping experiments, we have eventually proved that new myocytes after myocardial injury in the adult mammalian heart originate from resident c-kit^{pos}eCSCs. Furthermore, we have shown in a rat model of severe cardiomyopathy induced by ISO injury and 5-Fluoro Uracil that the ablation of the eCSCs abolishes regeneration and functional recovery. The regenerative process however is completely restored by replacing the ablated eCSCs with the tagged progeny of one eCSC. These eCSCs recovered from the primary host, and re-cloned, retain

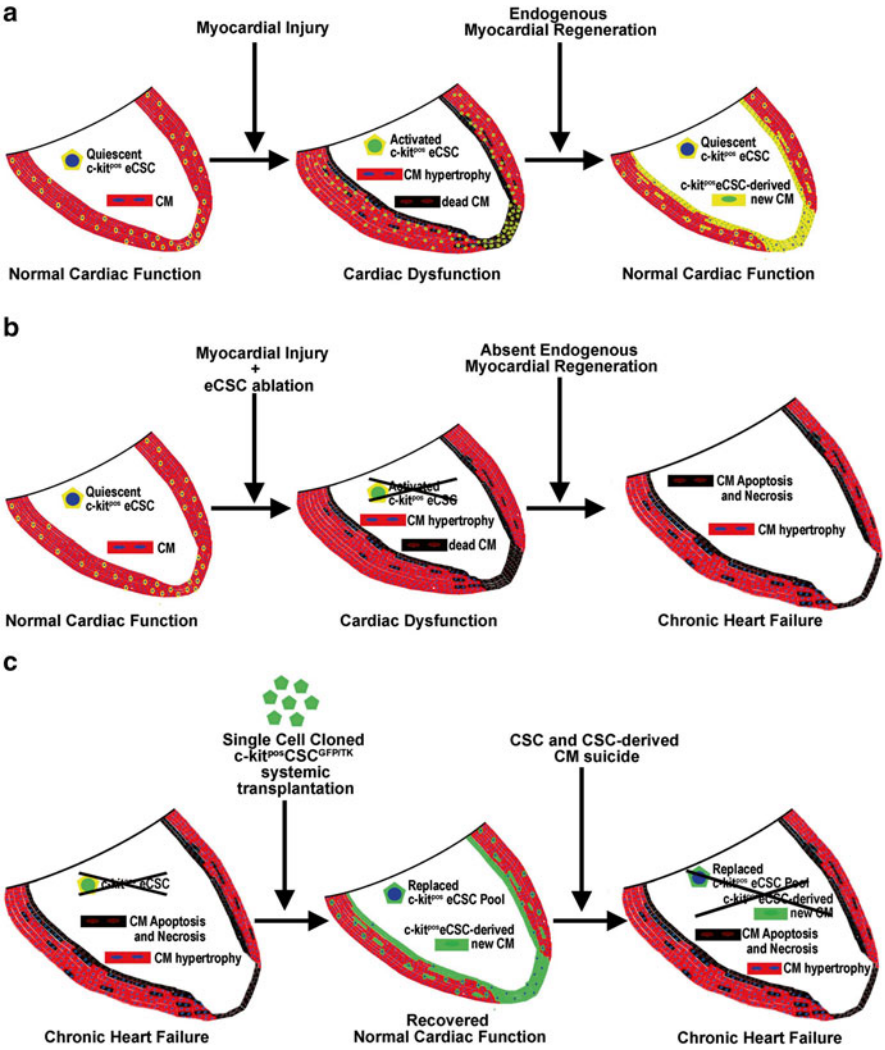

Fig. 2.1 eCSCs are necessary and sufficient for myocardial repair and regeneration. (**a**) Schematic of myocyte damage and cardiac recovery through eCSC activation and new myocyte formation. (**b**) When eCSC are ablated, cardiac regeneration is absent with the development of a severe cardiomyopathy. (**c**) If CSCs are exogenously transplanted, cardiac anatomy and function is restored. However, the selective suicide of the transplanted CSCs and their progeny sets back the animal in heart failure

their regenerative potential in vivo and in vitro. Finally, after regeneration, selective suicide of these exogenous eCSCs and their progeny abolishes the regeneration, severely impairing ventricular performance. Thus, overall these data have provided the ultimate and solid documentation that the resident tissue-specific eCSCs are necessary and sufficient for the regeneration of the adult myocardium and establish these cells as true cardiac regenerative agents.

3 Autologous Cardiac Stem Cell Therapy for Heart Failure

Many questions about eCSC basic biology still remain unanswered, particularly their long-term effectiveness and regenerative potential as well as their origin. It is imperative that such issues be addressed quickly if the full potential of these cells is to be realized, manipulated and applied clinically. In particular, it is imperative to document whether the teratogenic and neoplastic potential of the in vitro expanded eCSCs is low enough to make their use in humans safe. However, clinical trials using autologous cardiac stem/progenitor cells are already underway (Bolli et al. 2011; Makkar et al. 2012). In the SCIPIO (Stem Cell Infusion in Patients with Ischemic Cardiomyopathy; NCT00474461) trial, 16 patients with ischaemic cardiomyopathy with post-infarction LV dysfunction (ejection fraction ≤40 %) who had undergone coronary artery bypass grafting, had 500,000–1 million of autologous c-kit positive, lineage negative, cardiac progenitor cells infused intracoronary, ~4 months after surgery (Bolli et al. 2011). The control group was not given any treatment. LVEF increased by 8 EF points at 4 months after infusion, whereas the LVEF did not change in the control patients, during the corresponding time interval. Moreover, LVEF increased by 12 EF points in eight of the treated patients at 1-year follow-up. cMRI of seven of the treated patients showed that infarct size decreased at 4 and 12 months (Bolli et al. 2011). Furthermore, the interim analysis of myocardial function and viability by magnetic resonance in SCIPIO on a total of 33 patients (20 CSC-treated and 13 control subjects) confirmed the improvement in both global and regional LV function, and a reduction in infarct size at 1 year (Chugh et al. 2012). In the prospective, randomized cardiosphere-derived autologous stem cells to reverse ventricular dysfunction (CADUCEUS) trial, 17 patients (with left ventricular ejection fraction of 25–45 %) were infused into the infarct-related artery with up to 25 million, CD105-positive, autologous cardiosphere-derived cells (CDCs), 1.5–3 months after myocardial infarction (Makkar et al. 2012). Eight patients received standard care and acted as the control group. Compared with controls at 6 months, MRI analysis of patients treated with CDCs showed significant reductions in scar size and mass, increased viable heart mass, regional contractility and systolic wall thickening. However, changes in end-diastolic volume, end-systolic volume, and LVEF did not differ between groups at 6 months (Makkar et al. 2012). Recently, the ALCADIA clinical trial was initiated, which will focus on a hybrid biotherapy approach for treating chronic ischemic cardiomyopathy. This translational study is focusing on the safety and efficacy of autologous clonally amplified CSCs, which have shown to be enriched for embryonic stem cell markers and have mesenchymal cell characteristics (Matsubara and Kyoto Prefectural University School of Medicine 2012). This trial is also investigating cell therapy with the controlled release of basic fibroblast growth factor (bFGF) from a gelatin hydrogel sheet. While this innovative work has proved promising in the respect that transplantation of autologous CSCs has not resulted in any adverse health effects, we now await further studies, which focus on the efficacy of eCSC-based therapies and compare these to results obtained with BMDCs. Indeed, because of the high cost and the long wait for the availability of the cells for autologous cardiac stem/

precursor cell therapy, it will become imperative to compare the beneficial effects of this approach to that obtained with BMDCs because of their easier availability, accessibility and lower cost of the procedure. Furthermore, the widespread use and applicability of autologous cardiac stem cell therapy is highly debatable. Firstly, the procedure for cell acquisition, scale-up and transplantation is complex, time consuming and very expensive. The isolation and expansion of eCSCs to the number needed from catheter and surgical biopsies takes 1–3 months. Therefore, the cells are not available to be administered when they would be most effective, that is when a patient with an AMI in progress arrives at the hospital. Furthermore, the cost of the procedure in human and material resources would make it unavailable to patients beyond those few required to establish proof-of-concept for the therapy and to a small group of individuals with abundant economical resources. Finally, eCSCs undergo senescence with severe pathological consequences (Torella et al. 2004, 2006a; Matsubara and Kyoto Prefectural University School of Medicine 2012; Chimenti et al. 2003). Accordingly, for the cohort of patients (the aged population) most likely candidates for the regenerative therapy, >50 % of their eCSCs can be senescent and unable to participate in the regenerative process (Torella et al. 2004, 2006a; Matsubara and Kyoto Prefectural University School of Medicine 2012; Chimenti et al. 2003). Thus, if eCSC "aging" is an age or cell cycle dependent process, which affects all or most of the eCSC population, most or all regenerative therapies based on eCSC isolation and expansion will likely result in further exhaustion of the self-renewal capability of these cells with an accelerated loss of their regenerative capacity.

4 Stimulation of the Myocardial Endogenous Capacity for Repair and Regeneration

As above mentioned, while autologous eCSCs undoubtedly hold great promise for cardiac repair, their isolation and expansion prior to cell transplantation can be complex, time consuming and costly. This has raised the question of whether it may be advantageous to target the activation and regenerative capacity of the resident eCSCs to reconstitute damaged myocardium in the absence of cell therapy. One potential therapeutic mechanism of action by which the different forms of transplantation cell therapy are now receiving a lot of attention, is the activation, through a paracrine mechanism, of survival pathways in the cohort of cells at risk together with the endogenous regeneration compartment, represented by the eCSCs. A corollary of this hypothesis is that the identification of the molecules secreted by the transplanted cells should make possible the design of therapies, which eliminate the use of the cells and concentrate on the administration of the principal effector molecules these cells had identified. Myocardial regenerative cell-free therapies effective on the in situ activation, multiplication and differentiation of the resident eCSCs should have many advantages over those based on cell transplantation. First, therapeutic components should be available as "off-the-shelf" and ready to use at all

times without the lag time required for the cell therapy approaches; second, they should be affordable, in terms of the production costs of the medicinal product; third, such a therapy should be easy to apply and compatible with current clinical standard of care for AMI, including the widespread use of percutaneous coronary interventions (PCI); and fourth, because of the robustness of the regenerative response produced it should be able to produce and/or recover ~50–60 g of functional myocardial tissue, which is the minimum needed to change the course of the disease in a seriously ill patient. In an attempt to move towards cell-free, protein-based therapies, various growth factors and cytokines have been identified as potential candidates for therapeutic cardiac regeneration and as this list expands so too does our awareness of growth factor-mediated regenerative potential. Vascular endothelial factor (VEGF) is one such factor, which has been identified as central in promoting neo-vascularization post-MI (Crottogini et al. 2003). Initially phase II clinical trials suggested that limited functional benefits were observed upon direct administration of VEGF post-MI (Henry et al. 2003). However, this is now known to be due to the short half-life of VEGF and goes some way to demonstrate how important initial experimental studies are when designing clinical trials. Recent studies have focused on delivering VEGF in combination with various scaffolds and have achieved much greater success in stimulating angiogenesis and restoring cardiac function (Wu et al. 2011; Formiga et al. 2010). Neuregulin 1 (NRG-1) is another key factor implicated in stimulating cardiac repair and regeneration (Wadugu and Kühn 2012; Waring et al. 2012). An Ig-domain containing form of NRG-1β, also known as glial growth factor 2 (GG2) has been shown to improve LVEF and remodelling in pigs post-MI, compared to controls (Kasasbeh et al. 2011). It is thought that NRG-1 imparts functional benefits by activating and increasing c-kit^{pos} eCSC proliferation (Waring et al. 2012), inducing cardiomyocyte replacement (Bersell et al. 2009), protecting cardiomyocytes from apoptosis and improving mitochondrial function (Kasasbeh et al. 2011). Testing regenerative therapies in mouse models of human diseases, although a necessary step in pre-clinical assays, is not an accurate predictor of their human effectiveness. This is so not only because of the potential biological differences between the two species but because of the three order of magnitude difference in mass between the two organisms, which make the challenges not only quantitatively but qualitatively different. Therefore, it is necessary that pre-clinical testing of therapies be carried out in a model, which is more similar in tissue biology, size and physiology to the human than the rodent models commonly used. The pig, because of its size, rapid growth rate, well-known physiology and availability, has proven a very useful and frequently used pre-clinical large animal model for many pathologies, particularly those involving tissue regeneration. Thus, we have recently tested the regenerative effects of intracoronary administration of two growth factors known to be involved in the paracrine effect of the transplanted cells (Ellison et al. 2011). Insulin-like growth factor I (IGF-1) and hepatocyte growth factor (HGF), in doses ranging from 0.5 to 2 μg HGF and 2 to 8 μg IGF-1, were intracoronary administered, just below the site of left anterior descendent occlusion, 30 min after AMI during coronary reperfusion in the pig. This growth factor cocktail triggers a regenerative response

from the c-kit^{pos} eCSCs, which is potent and able to produce anatomically, histologically and physiologically significant regeneration of the damaged myocardium without the need for cell transplantation (Ellison et al. 2011). IGF-1 and HGF induced eCSC migration, proliferation and functional cardiomyogenic and microvasculature differentiation. Furthermore, IGF-1/HGF, in a dose-dependent manner, improved cardiomyocyte survival, and reduced fibrosis and cardiomyocyte reactive hypertrophy. Interestingly, the effects of a single administration of IGF-1/HGF are still measurable 2 months after its application, suggesting the existence of a feedback loop triggered by the external stimuli that activates the production of growth and survival factors by the targeted cells, which explains the persistence and long duration of the regenerative myocardial response. These histological changes were correlated with a reduced infarct size and an improved ventricular segmental contractility and ejection fraction at the end of the follow-up assessed by cMRI (Ellison et al. 2011). Despite their effectiveness, the administration of IGF-1 and HGF has a significant drawback. Although it is very effective in the regeneration of the myocytes and micro-vessels lost, the rate of maturation of the newly formed myocytes is heterogeneous and quite slow. While the newly formed myocytes which are in contact with spared ones mature rapidly and can reach a diameter close to a normal pig cardiomyocyte, there is an inverse correlation between new myocyte size and their distance from the small islands of spared myocardium scattered within the ischemic zone (Ellison et al. 2011). With the exception of those new myocytes in close proximity to spared micro-islands of surviving pre-existing myocytes within the ischemic tissue or those in the border region, at 3 weeks after treatment the length and diameter of the remaining new myocytes (~85 % of those regenerated) is between 1/2 and 1/5, respectively, of an adult myocyte, which means that their volume is significantly less than 1/10th of their mature counterparts (Ellison et al. 2011). Because of this slow maturation process, although the therapy is very effective in restoring the number of myocytes lost by the AMI this is not the case as to the regeneration of the lost ventricular mass which lags behind very significantly. In consequence, the myocardial generation of force capacity, that is the meaningful functional recovery, also lags significantly behind the regeneration of the cell numbers to the pre-AMI state. Despite the beneficial effect of the therapy in reducing the scar area, pathological remodelling and partial recovery of ventricular function, there is little doubt that it would be desirable to obtain a more rapid recovery of the ventricular mass and the capacity to generate force. All the currently proposed autologous cell approaches are very attractive from the theoretical and biological standpoint. For those rare diseases with chronic and long-term evolution affecting hundreds or even thousands of potential patients to be treated, these personalized therapies, despite their high cost in medical and material resources, might even make sense from an economic standpoint. Unfortunately, this is not the case for diseases of high prevalence, such as the consequences of ischaemic heart disease, with millions of patients/candidates for regenerative therapy. Not even the developed world has the resources needed to start a program of personalized regenerative medicine for the patients already in CHF who presently are left with heart transplantation as the only realistic option for recovery. Therefore, although the cell

transplantation approaches outlined are very valuable as proof-of-concept and as research tools with the possibility of greatly improving a narrow subset of patients in need of therapy, we believe that all of the autologous cell strategies taken together, now and in the foreseeable future, are and will continue to be ineffective to favourably impact the societal healthcare problem posed by the consequences of CHF post-AMI (Ellison et al. 2012b). Moreover, as outlined above, a consensus is gaining ground that most of the favourable effects of cell transplantation protocols used until now exert their beneficial effect by a paracrine mechanism of the transplanted cells over the surviving myocardial cells at risk and/or through the activation of the endogenous myocardial regenerative capacity represented by the eCSCs. If this is correct, then there seems to be little advantage in the use of autologous cells because a similar, and perhaps enhanced, effect can be obtained by the administration of the proper cell type isolated from allogeneic sources. These can be produced in large amounts beforehand, kept stored frozen before their use, and remain available at all times, which would allow their use not only for the treatment of the pathological remodelling once it has developed but soon after the acute insult in order to induce early regeneration of the cells lost in order to prevent or diminish the pathological remodelling. Mesenchymal stem cells (MSC) have a broad repertoire of secreted trophic and immunodulatory cytokines, however they also secrete factors that negatively modulate cardiomyocyte apoptosis, inflammation, scar formation and pathological remodelling (Ranganath et al. 2012). Medicetty and colleagues (2012) used a porcine model of AMI and delivered 20–200 million allogeneic, multipotent, adult BMDCs (MultiStem; that are nonimmunogeneic and can suppress activated T-cell proliferation and have anti-inflammatory and angiogenic properties as well), directly to the myocardium via the infarct related vessel using a transarterial microsyringe catheter-based delivery system, 2 days after AMI. Echocardiography showed significant improvements in regional and global LV function and remodelling at 30 and 90 days after myocardial injury (Medicetty et al. 2012). Rapidly following on from this pre-clinical study, Penn et al. (2012) conducted a multi-centre phase I trial of the effects of adventitial delivery of MultiStem in patients 2–5 days after primary PCI. In patients with EF determined to be <45 % before the MultiStem injection, at 4 months after AMI, a 1, 4, 14, and 11 % absolute increase in EF was observed following injection of 20, 50, and 100 million cells, respectively (Penn et al. 2012). Recently, Marban and colleagues (2012) have tested the safety and efficacy of using allogeneic, mismatched cardiosphere-derived Cells (CDCs) in infarcted rats. Rats underwent permanent ligation of the LAD coronary artery and two million CDCs or vehicle were intramyocardially injected at four sites in the peri-infarct zone. Three weeks post-MI, animals that received allogeneic CDCs exhibited smaller scar size, increased infarcted wall thickness and attenuation of LV remodelling. Allogeneic CDC transplantation resulted in a robust improvement of fractional area change (~12 %), ejection fraction (~20 %), and fractional shortening (~10 %), and this was sustained for at least 6 months. Furthermore, allogeneic CDCs stimulated endogenous regenerative mechanisms (cardiomyocyte cycling, recruitment of c-kit^{pos} eCSCs, angiogenesis) and increased myocardial VEGF, IGF-1 and HGF (Malliaras et al. 2012). Unlike other cell types (Janssens 2010;

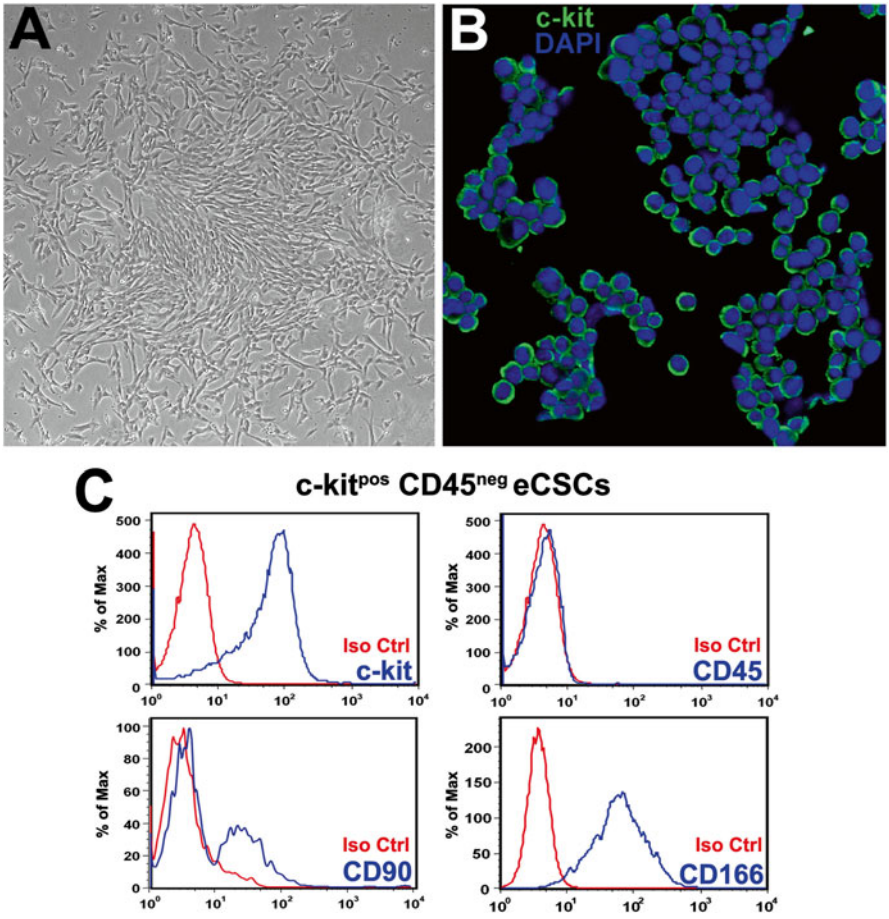

Fig. 2.2 Endogenous cardiac stem cell essential phenotype. (**a**) Light microscopy representative image of long-term cultured pig eCSCs. (**b**) Cytospin preparation and c-kit immunofluorescence of cloned eCSCs. (**c**) Essential CD phenotype of a typical CSC preparation (**b**, **c**) are adapted from Ellison et al. (45)

Hofmann et al. 2005), eCSCs have a very high tropism for the myocardium (Ellison et al. 2013). Under proper culture conditions it is possible to clone and expand a single rodent, porcine or human eCSC to up to 1×10^{10} cells without detectable alteration of karyotype, loss of differentiating properties or the phenotype of the differentiated progeny (Fig. 2.2) (Ellison et al. 2011). These cloned cells produce a repertoire of pro-survival and cardiovascular regenerative growth factors [Our unpublished data]. For this reason, we decided to test whether these in vitro expanded cells, when administered into allogeneic animals, would be the source of a more complex and physiologic mixture of growth and differentiating factors which, through a paracrine effect would produce a robust activation of the eCSCs

with more rapid maturation of their progeny. It was expected that once their short-term effect had been produced and the auto/paracrine feedback loop of growth factor production has been activated in the eCSCs, the allogeneic cells would be eliminated (presumably by apoptosis) and that the regeneration triggered by activated eCSCs would be completely autologous. c-kit^{pos} eCSCs do not express either MHC-I locus or co-activator molecules and have strong immunomodulatory properties in vitro when tested in the mixed lymphocyte reaction [Our unpublished Data]. We therefore expected the expanded cells to survive long enough in the allogeneic host to produce their paracrine effect before being eliminated by the host immune system. Allogeneic, non-matched, cloned male EGFP-transduced porcine eCSCs, were administered intracoronary in white Yorkshire female pigs, 30 min after MI and coronary reperfusion (Ellison et al. 2009). Pig serum was injected to control pigs after MI (CTRL). The cells or sera were injected through a percutaneous catheter into the anterior descending coronary artery just below the site of balloon occlusion used to produce the AMI. We found a high degree of EGFP^{pos}/c-kit^{pos} heterologous HLA non-matched allogeneic porcine CSCs nesting in the damaged pig myocardium at 30 min through to 1 day after MI. At 3 weeks post-AMI, all the injected allogeneic cells had disappeared from the myocardium and peripheral tissues (i.e. spleen). There was significant activation of the endogenous GFP^{neg} c-kit^{pos} CSCs (eCSCs) following allogeneic CSC treatment (Fig. 2.3), so that by 3 weeks after MI, there was increased new cardiomyocyte and capillary formation, which was not evident in the control hearts (Fig. 2.3). Moreover, through paracrine mechanisms, c-kit^{pos} heterologous HLA non-matched allogeneic CSC treatment preserved myocardial wall structure and attenuated remodelling by reducing myocyte hypertrophy, apoptosis and scar formation (fibrosis) (Ellison et al. 2009). In summary, intracoronary injection of allogeneic CSCs after MI in pigs, which is a clinically relevant MI model, activates the eCSCs through a paracrine mechanism resulting in improved myocardial cell survival, function, remodelling and regeneration. A possible risk of using large numbers of in vitro expanded CSCs is the appearance of transformed cells with the potential to form abnormal growths. This risk is completely eliminated by the use of allogeneic cells, with a different HLA allele from the recipient, because they all get eliminated by the immune system without immunosuppression. Claims that some of the transplanted allogeneic cells have a long-term survival in the host, have not been reproduced or thoroughly documented (Malliaras et al. 2012; Quevedo et al. 2009; Huang et al. 2010). If their survival proves to be correct, many of the immunology concepts, which have ruled transplant biology until now, will need to be revised. Furthermore, despite thorough pathological examination and contrary to many iPS- and ECS-derived cell lines, the adult tissue-specific eCSCs have a very low or non-existent capacity to form tumours and/or teratomas in syngeneic or immunodeficient animals [our unpublished data and (Chong et al. 2011)]. Allogeneic CSC therapy is conceptually and practically different from any presently in clinical use. The proposed cell therapy is only a different form of growth factor therapy able to deliver a more complex mixture of growth factors than our present knowledge permits us to prepare. The factors

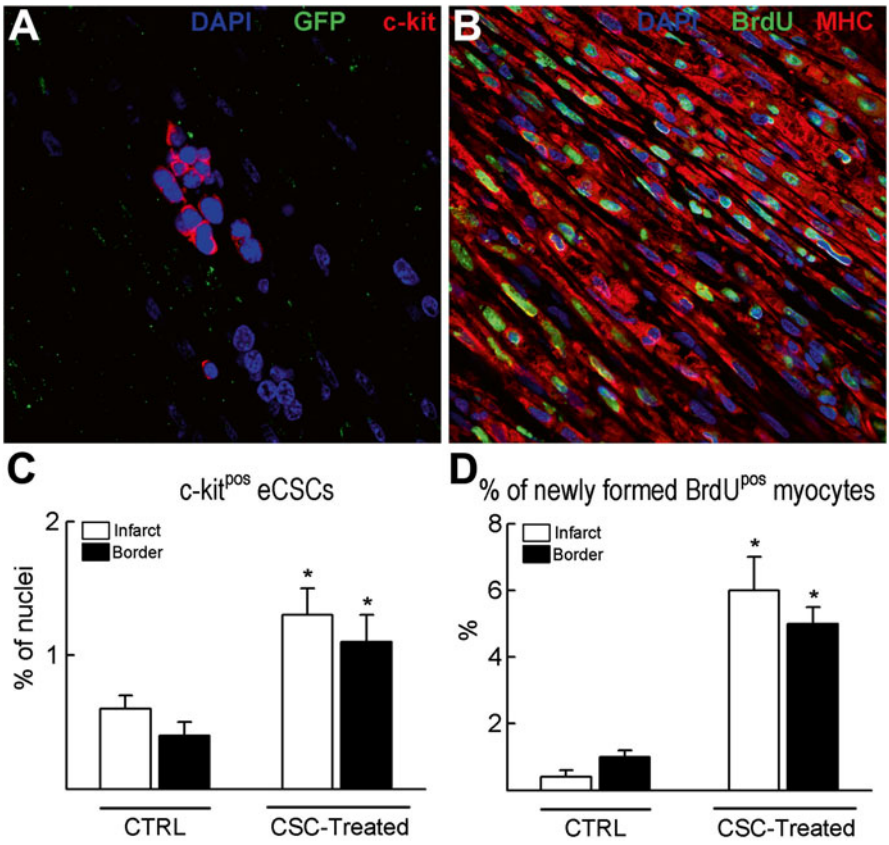

Fig. 2.3 Activation of tissue-specific endogenous resident c-kit^{pos} CSCs following intracoronary injection of c-kit^{pos} allogeneic porcine CSCs after acute myocardial infarction in pigs. (**a**) A cluster of activated GFP^{neg}, c-kit^{pos} (*red*) endogenous CSCs in the 3-week-old infarcted region of the allogeneic CSC-treated porcine myocardium. Nuclei are stained by DAPI in *blue*. (**b**) Regenerating band of newly formed BrdU^{pos} (*green*) cardiomyocytes (*red*, MHC) in the infarct region, 3 weeks following allogeneic CSC treatment. Nuclei are stained by DAPI in *blue*. (**c**) The number of c-kit^{pos} endogenous CSCs significantly increased following intracoronary allogeneic CSC treatment. $*P<0.05$ vs. CTRL. (**d**) New BrdU^{pos} myocyte formation significantly increased following allogeneic CSC therapy. $*P<0.05$ vs. CTRL. Adapted from Ellison et al. J Cardiovasc Transl Res. 2012;5:667–77

produced by the allogeneic cells are designed to stimulate the endogenous stem cells of the target tissue but the transplanted cells themselves survive only transiently and do not directly participate in the production of progeny that contributes to the regenerated tissue. Once more information is available, the allogeneic cells could be used either alone or in combination with the available factor therapy to improve the activation of the eCSCs and the maturation of their progeny.

5 Summary and Conclusions

The findings that the adult heart harbours a regenerative multipotent cell population composed by eCSCs and that mammalian, including human, cardiomyocytes are replaced throughout adulthood represents a paradigm shift in cardiovascular biology. The presence of this regenerative agent within the adult heart supports the view that the heart has the potential to repair itself if the eCSCs can be properly stimulated. Indeed, it is predicted that in the near future it should be possible to replace cell transplantation-based myocardial regeneration protocols with an "off-the-shelf", readily available, unlimited and effective regenerative/reparative therapy based on specific growth factor administration or on the paracrine secretion by allogeneic CSC transplantation able to produce the activation in situ of the resident eCSCs. However, before reaching this optimistic clinical scenario, it is mandatory to obtain a better understanding of eCSC biology in order to fully exploit their regeneration potential. The latter will ultimately lead to developing realistic and clinically applicable myocardial regeneration strategies. Cardiac regenerative medicine is set to revolutionize the treatment of cardiac diseases and such research will have significant and long-term impact on socio-economics and patient well-being. Indeed, therapies which are based on findings from high quality research will undoubtedly cut deaths from cardiovascular disease, reduce recovery times, increase life expectancy and quality of care and save money.

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