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REVIEW

The cardiac stem cell compartment is indispensable for myocardial cell homeostasis, repair and regeneration in the adult

Bernardo Nadal-Ginard[a,c,*], Georgina M. Ellison[a,b,c], Daniele Torella[b,**]

[a] Department of Physiology, School of Biomedical Sciences, King's College, London, UK
[b] Molecular and Cellular Cardiology, Department of Medical and Surgical Sciences, Magna Graecia University, Catanzaro 88100, Italy
[c] Centre for Stem Cells & Regenerative Medicine, King's College, London, UK

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Abstract Resident cardiac stem cells in embryonic, neonatal and adult mammalian heart have been identified by different membrane markers and transcription factors. However, despite a flurry of publications no consensus has been reached on the identity and actual regenerative effects of the adult cardiac stem cells. Intensive research on the adult mammalian heart's capacity for self-renewal of its muscle cell mass has led to a consensus that new cardiomyocytes (CMs) are indeed formed throughout adult mammalian life albeit at a disputed frequency. The physiological significance of this renewal, the origin of the new CMs, and the rate of adult CM turnover are still highly debated. Myocyte replacement, particularly after injury, was originally attributed to differentiation of a stem cell compartment. More recently, it has been reported that CMs are mainly replaced by the division of pre-existing post-mitotic CMs. These latter results, if confirmed, would shift the target of regenerative therapy toward boosting mature CM cell-cycle re-entry. Despite this controversy, it is documented that the adult endogenous c-kit^{pos} cardiac stem cells (c-kit^{pos} eCSCs) participate in adaptations to myocardial stress, and, when transplanted into the myocardium, regenerate most cardiomyocytes and microvasculature lost in an infarct. Nevertheless, the *in situ* myogenic potential of adult c-kit^{pos} cardiac cells has been questioned. To revisit the regenerative potential of c-kit^{pos} eCSCs, we have recently employed experimental protocols of severe diffuse myocardial damage in combination with several genetic murine models and cell transplantation approaches showing that eCSCs are necessary and sufficient for CM regeneration, leading to complete cellular, anatomical, and functional myocardial recovery. Here we will review the available data on adult eCSC biology and their regenerative potential placing it in the context of the different claimed mechanisms of CM replacement. These data are in agreement with and have reinforced our view that most CMs are replaced by *de novo* CM formation through the activation, myogenic commitment and specification of the eCSC cohort.

* Correspondence to: B. Nadal-Ginard, Department of Physiology, School of Biomedical Sciences, King's College London, Shepherd's House, Rm 4.16, Guy's Campus, London SE1 1UL, UK.
** Correspondence to: D. Torella, Molecular and Cellular Cardiology, Dept of Medical and Surgical Sciences, Magna Graecia University, Biosciences Building, Level VIII Rm 09, Campus S. Venuta, Viale Europa, Catanzaro 88100, Italy.
E-mail addresses: bernardo.nadalginard@kcl.ac.uk, bernardo.nadalginard@gmail.com (B. Nadal-Ginard), dtorella@unicz.it (D. Torella).

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Introduction

Despite the remarkable progress made during the past half century in the treatment of cardiovascular disease, which is increasingly effective in dealing with the acute stages of life-threatening pathology, they often extend the life of the patient at the expense of generating a chronic condition. The chronic sequels from an acute myocardial infarction (AMI), such as chronic heart failure (CHF), are frequently without effective treatment or leave organ transplantation as the only alternative to restore function, with all the logistic, economic and biological limitations associated with this intervention (Kahan et al., 2011).

The continuous increase in average human lifespan with progressive aging of the population in all developed countries has generated an increasingly severe epidemic of chronic diseases whose treatment absorbs an ever-larger fraction of human resources and of the healthcare budget. Presently, there are >5 million patients in CHF post-AMI in the USA alone (Roger et al., 2012). More than 550,000 new patients per year are added to this group, which has a similar prevalence in the EU countries. After the first episode, CHF post-AMI has an average annual mortality rate of ~18% and in the USA alone absorbs >$30 billion annually for its care (Roger et al., 2012). The root problem of CHF in general and post-AMI in particular is a deficit of functional myocardial contractile cells (cardiomyocytes) and adequate coronary circulation to nurture them. This combination triggers pathological cardiac remodelling, which, in turn, produces further myocyte death and the late development of cardiac failure in these patients (Jessup et al., 2003). For these reasons, during the past decade a goal of cardiovascular research has been to find methods to replace the cardiomyocytes lost as a consequence of an MI and other insults in order to prevent or reverse the pathological cardiac remodelling. Therefore, stem cell-based therapies have become an attractive experimental treatment for heart disease and failure (Terzic et al., 2010).

Until recently, a paucity of understanding about the cellular homeostasis of most adult solid tissues has been a major factor limiting the expansion to other areas of medicine of the early breakthroughs in adult stem cell research and therapy, such as those applied to the blood and bone marrow diseases. Early in the last decade the prevalent view was that, although tissues like the bone marrow, intestinal epithelium and skin exhibit a robust self-renewal capacity based on the presence of adult (also called "tissue-specific") stem cells (Mercier et al., 2011; Simons et al., 2011; Fuchs, 2009), they were an exception. The established paradigm was that the majority of the remaining solid tissues either renewed very slowly (such as the muscle and the endothelial lining of the vascular system), with renewal being physiologically irrelevant, or not at all. It was firmly believed that starting shortly after birth many tissues did not harbour functional regenerating (stem) cells. A logical consequence of the above paradigm was the belief that for most organs the number and function of their parenchymal cells was in a downward spiral starting in late infancy and continued until death. With the exception of the three main self-renewing tissues mentioned above, it necessarily followed that all therapeutic approaches to disease processes caused by a deficit in the number of functional parenchymal cells could be only directed toward improving and/or preserving the performance of the functional cells remaining in the tissue. Thus, to return the tissue or organ to the *status quo ante* required the transplantation of either identical cells from another individual or transplantation of a cell type capable of differentiating into the cells whose shortage needed to be covered. Because the cells needed for the second option did not exist for the majority of tissues, heterologous/allogeneic organ and cell transplantation became the only possible avenues. In fact, despite the multiple drawbacks of heterologous cell/organ transplantation, its practice has become the cutting edge for several medical specialties (Badylak et al., 2012). However, the extreme shortage of donors, high costs, and the severe side effects of immunosuppression have limited this therapy to a small fraction of candidates in need of treatment. Thus, the positive reception and high expectations that received the successful derivation of multipotent human embryonic stem cells (hESCs) (Evans et al., 1981; Thomson et al., 1998; Murry, 2008) with the capacity to differentiate into most, if not all, known cell types promised an unlimited supply of

donor parts. When the euphoria caused by this development started to dim, because of the ethical and immunological challenges posed by the use of hESCs, came the breakthrough which permitted the conversion of different adult somatic cells, such as fibroblasts, into multipotent cells called induced pluripotent stem (iPS) cells by introduction of a very limited number of genes (now known to be responsible for the multipotent state of stem cells) (Takahashi et al., 2006; Takahashi et al., 2007) or their products. With the development of iPS cells, it became possible to produce different types of parenchymal cells starting with an abundant and easy to obtain cell type from the same patient to be treated. Once converted into the parenchymal type needed, these could be potentially used for autologous cell therapy (Yamanaka, 2007). Although the potential of the iPS cells as therapeutic agents remains high, it is already clear that many hurdles need to be cleared before they can reach clinical application (Robinton et al., 2012). The recent protocols to convert somatic cells directly into some types of parenchymal cells without apparently going through the multipotent stage by introducing tissue-specific transcriptional factors (Qian et al., 2012; Song et al., 2012; Jayawardena et al., 2012) might be too recent to evaluate their clinical potential.

The adult heart is a self-renewing organ

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, even including the brain (Rountree et al., 2012; Reule et al., 2011; Kopp et al., 2011; Kotton, 2012; Buckingham et al., 2008; Suh et al., 2009).

Despite the change in the concept of tissue cell homeostasis, the cardiovascular research community has continued to treat the adult mammalian heart as a post-mitotic organ without intrinsic regenerative capacity. Notwithstanding several reports showing a yet contradictory range from 0.0005% to 3% cardiomyocyte cell cycle activity in normal adult mammalian hearts (Rumyantsev, 1991: 3; Soonpaa et al., 1998; Anversa and Kajstura, 1998), the prevalent view considered the 0.0005% as the most appropriate estimate for cardiomyocyte renewal, establishing in essence its negligent value. The >20-fold increase in cardiac mass from birth to adulthood and in response to different stimuli, was believed to result exclusively from the enlargement of pre-existing myocytes (Hunter et al., 1999; Soonpaa et al., 1998; Laflamme at al., 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 has been based on two generally accepted but erroneous notions: a) all myocytes in the adult heart were formed during foetal life or shortly thereafter, became terminally differentiated and could not be recalled into the cell cycle: therefore, all cardiac myocytes have to be of the same chronological age as the individual (Oh et al., 2001; Chien et al., 2002); b) the heart has no intrinsic parenchymal regenerative capacity because it lacks a stem/progenitor cell population able to generate new myocytes. These concepts were extrapolated into the dogma that from shortly after birth until death no new CM generation was possible and any CMs lost by either wear and tear or injury were not replaced. Thus, from puberty on the myocardium entered an irreversible downslope with a continuously decreasing number of myocytes. This continuous loss was thought to be compensated for some time by hypertrophy of the spared CMs which managed to maintain myocardial mass. In this world there was neither room nor need for endogenous regenerative cardiac biology. Therefore, in the terminally differentiated heart, regeneration could only be accomplished by the replacement of the lost or damaged cardiomyocytes through transplantation of exogenous differentiated CMs or with cells with the potential to differentiate into them.

Despite published evidence that this prevalent view was incorrect (Beltrami et al., 2001; Quaini et al., 2002; Urbanek et al., 2003; Urbanek et al., 2005; Anversa et al., 2002; Nadal-Ginard et al., 2003), it took the publication of Bergmann et al., 2009, to produce a significant switch in the prevalent opinion. This work, based on ^{14}C dating in human hearts concluded that during a lifetime the human heart renews ~50% of its myocytes (Bergmann et al., 2009). However, because this "measured" self-renewal depends on the validity of a complex mathematical formula, whose impact on the results dwarfs that of the measured data which did not appear to be very robust, its physiological significance has remained in doubt. Furthermore, their calculations identify the highest turnover rate during youth and early adulthood followed by a steady decrease with age. This 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. It is a well-documented fact that cell death of all solid tissues increases with chronological age in all mammal, including humans. Without replacement of many of the lost cells, aging would irreversibly lead to the disappearance of the tissue/organ particularly in the most long-lived individual. Yet, the regenerative capacity of most tissues, including the myocardium also decreases with age and, in most cases, results in a cellular deficit because the rate of cell loss is higher than that of regeneration (Nadal-Ginard et al., 2003; Torella et al., 2004). Despite all this confusing and often contradictory evidence, the adult mammalian heart's capacity for self-renewal appears to have finally settled with a consensus that new cardiomyocytes (CMs) are indeed formed throughout adult mammalian life (Hsieh et al., 2007; Bergmann et al., 2009) Nevertheless, the physiological significance of this renewal, the origin of the new CMs, and the rate of adult CM turnover are still highly debated. While Bergmann et al., (2009) calculated a yearly CM turnover of about 1%, others have calculated 4-10% (Senyo et al., 2013; Malliaras et al., 2013) and some as high as 40%/year (Kajstura et al., 2012).

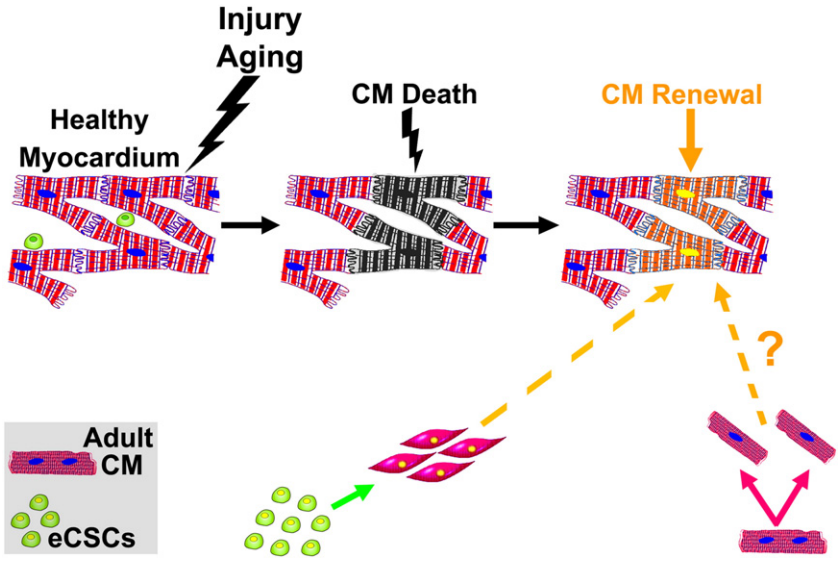

Figure 1 Adult cardiomyogenesis. Adult myocardial tissue during lifespan adds new cardiomyocytes (CMs) in response to CM loss due to different triggers like aging or ischemic/non-ischemic injury. Resident endogenous cardiac stem/progenitor cells (eCSCs) give rise to new functional CMs. However, it has been claimed that adult CMs can actually replicate to form new CMs.

This large spread raises significant scepticism about the validity of the methodologies used and leaves unanswered the physiological significance of CM replacement due to normal wear and tear in adulthood and after injury. Whether the newly formed myocytes originate from precursor cells or from the division of pre-existing myocytes was not addressed (Bergmann et al., 2009) (Fig. 1).

Where does the regenerative potential of the adult mammalian heart reside?

As discussed above, what still remains controversial is not only the amount and physiological significance of cardiomyocyte turnover but also the origin of the newly formed myocytes (Laflamme et al., 2011; Kajstura et al., 2010). Three main sources 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; Senyo et al., 2013) 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 (Beltrami et al., 2003; Torella et al., 2007; Rasmussen et al., 2011; Ellison et al., 2013). It is clear now that the blood borne precursors, although documented as a biological phenomenon (Quaini et al., 2002; Eisenberg et al., 2006) might be limited to very special situations (Orlic et al., 2001) and their direct regenerative import is, if any, very limited (Loffredo et al., 2011).

Is re-entry of pre-existing adult cardiomyocytes into the cell cycle responsible for myocardial cell homeostasis and the regenerative response to increased workload and injury?

The mammalian myocardial response to increased workload and to injury is conditioned by the fact that, shortly after the post-natal period, CMs permanently withdraw from the cell cycle (Nadal-Ginard., 1978). The molecular mechanism of terminal differentiation in CMs has not yet been completely defined. Different experimental models indicate that cardiac muscle lineage determination genes directly interact with the retinoblastoma (Rb) pocket proteins to produce and maintain the terminally differentiated state (Tam et al., 1995; MacLellan et al., 2005). After more than 3 decades of research, there are literally hundreds of papers in reputed journals documenting that mature myocytes in and from the adult myocardium do not re-enter the cell cycle and in the very rare occasions they do, do not undergo mitosis but apoptose, at least in part, due to a deficit in centriole formation (Schneider et al., 1994). This block of cell division (which does not necessarily blocks bi-nucleation and DNA endo-reduplication) has been shown both *in vivo* and *in vitro* in different mammalian species from mouse to human. This behaviour is in contraposition to the replication capacity of foetal and neonatal mammalian myocytes, which do not yet express pRB, and in the mouse can extend up to 7–8 days post-natally (Porrello et al., 2011) as well as those of adult invertebrates, fishes and reptiles (Kikuchi et al., 2012). Furthermore, adult mammalian myocytes can also be co-axed to re-enter the cell cycle at a significant rate through genetic manipulations at the expense of the stability of their differentiated function (MacLellan et al., 2005). Notwithstanding the elegant studies documenting

that adult cardiomyocytes divide in species other than mammals (Zhang et al., 2013), these findings cannot and should not be extrapolated to the adult mammalian heart.

Despite the overwhelming observational and experimental evidence summarized above, a provocative recent publication by Senyo et al., (2013) claimed that adult myocyte renewal is mostly accomplished by division of pre-existing cardiomyocytes, which was estimated to be a more relevant source than resident (or circulating) stem/progenitor cells. Indeed, the authors stated that the birth of cardiomyocytes from pre-existing cardiomyocytes has a projected rate of roughly 0.76% per year in the young adult mouse under normal homeostatic conditions, a rate that increases after myocardial injury in the border region. Also the authors interpreted their findings as a demonstration that cardiac progenitors do not have a significant function in myocardial homeostasis in mammals and thus their role after injury is also limited (Senyo et al., 2013). This publication has been taken by some as a demonstration of the negligible or secondary role of resident CSCs in adult mammalian myocardium homeostasis and regeneration. Interestingly, two previous publications from the same laboratory on the

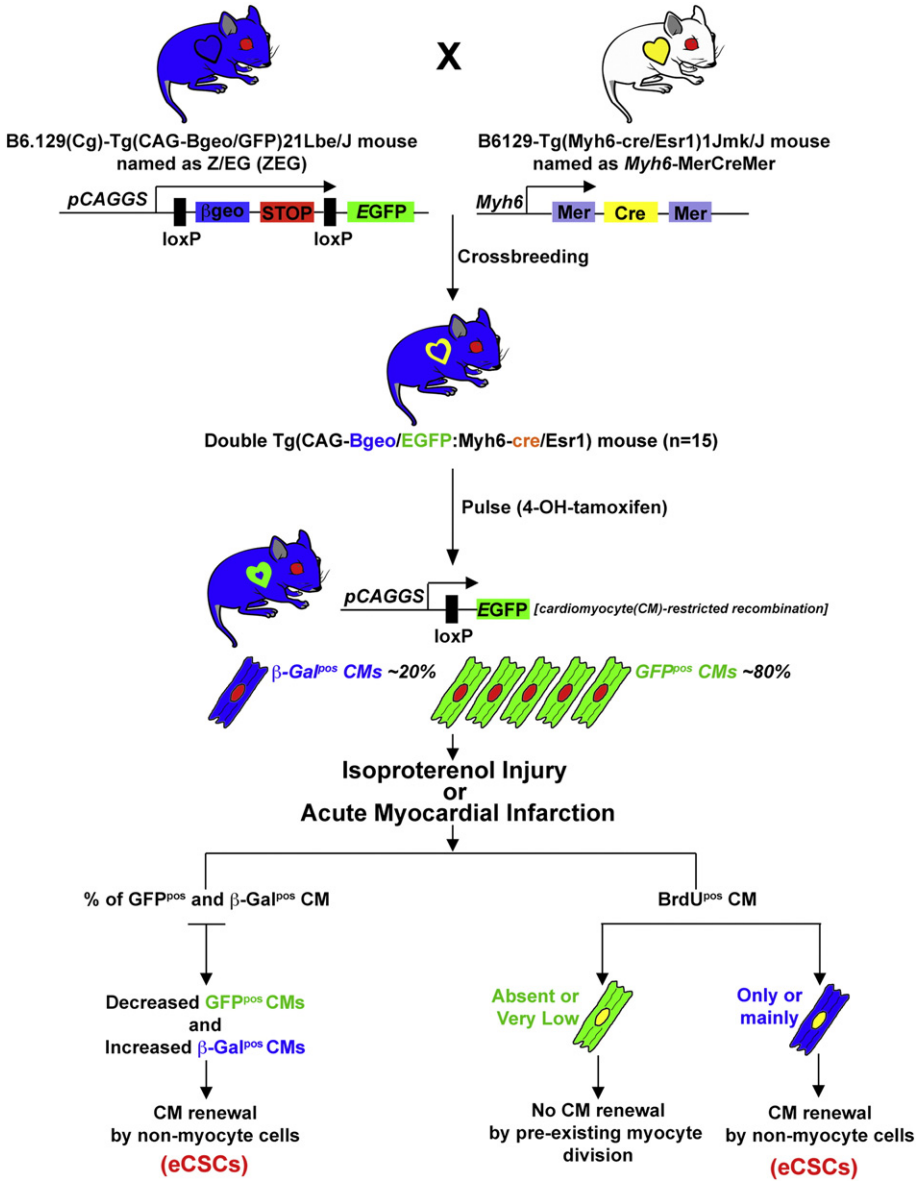

Figure 2 Z/EG×MYH6-MerCreMer mice for adult cardiomyocyte permanent labelling. The cartoon shows the breeding of Z/EG and MYH6-MerCreMEr mice to obtain double transgenic mice with tamoxifen-inducible GFP labelling of adult cardiomyocytes (CMs). Tamoxifen administration, at the most, GFP-labels 80% of the adult CMs leaving 20% of them un-recombined and still expressing β-galactosidase (β-Gal). Either Isoproterenol injury or myocardial infarction in these mice cause GFP^{pos} CM dilution in favour of increased percentage of β-Gal^{pos} myocytes, which indirectly shows new CM formation from non-myocyte cells (that we show to be the eCSCs). BrdU labelling in these mice after the initial injury marks mainly/only β-Gal^{pos} myocytes, which further shows that new myocyte formation in response to myocyte loss is accomplished by non-myocyte cells (*i.e.* eCSCs).
*adapted from Ellison et al. *Cell. 2013; 154:827–42.*

same topic and using the same experimental animal models (Hsieh et al., 2007; Loffredo et al., 2011), reached exactly the opposite conclusions; that is, new CMs originated *not* from pre-existing CMs but from a stem cell population which they did not identify. Additionally, Marban's group using the same genetic cell fate tracking mouse model, concluded that CM renewal after AMI is accomplished by both pre-existing CM division and resident stem cell differentiation, with a higher contribution by the latter which is further amplified by cardiosphere-derived cell therapy (Malliaras et al., 2013). In contrast, we have reported that the resident stem cells are not only necessary but sufficient for full myocardial regeneration after diffuse and segmental damage and we have also ruled out any meaningful contribution of mature CM division (Ellison et al., 2013).

The contradictory results pointed out above are, at least in part, due to a misconception of the strength of the transgenic mouse models used for the test. The so-called Z/EG mouse is a double-reporter mouse line that switches from expressing lacZ to enhanced green fluorescent protein (eGFP) in the targeted cells upon Cre-mediated excision (Hsieh et al., 2007) (Fig. 2). Although powerful and useful, this mouse is not fully effective when applied to the genetic labelling of adult CMs by cross-breeding Z/EG with α-MHC-MerCreMer mice where Cre is expressed under the control of *MYH6* (α-MHC) promoter. Its main drawback is that when the MerCreMer construct is induced by Tamoxifen, only ~80% of the cardiomyocytes recombine the *floxed* transgene and turn on the eGFP marker (Hsieh et al., 2007). Therefore, one of every 5 cells continues to express the constitutive transgene, β-galactosidase, and their origin cannot be ascertained (Hsieh et al., 2007). For this reason, new CM formation has to be quantified indirectly by the "dilution" of the eGFP positive myocytes by the new myocytes, which, because they did not exist at the time of tamoxifen induction, should express the constitutive β-galactosidase reporter. Because all numbers are relative, the formation of new β-galactosidase-expressing myocytes by necessity leads to an increase of the % of β-galactosidase (β-Gal)-expressing cells and the corresponding decrease in the % of the eGFP-expressing cohort. This indirect measurement reduces the sensitivity and accuracy of the test, particularly if the number of newly formed myocytes is small.

On the other hand, a major advantage of this alternative double-reporter genetic model is to provide a clear-cut qualitative answer as to whether new CMs are formed after injury and if so, whether these new CMs originated predominantly from pre-existing CMs or from another cell population. If no new CMs are formed after the tamoxifen induction, the relative ratio eGFP/β-Gal (80/20) should remain constant. However, this eGFP/β-Gal ratio changed from 80/20 to 65/35 after myocardial injury (Hsieh et al., 2007), which indicates a robust formation of new CMs. The genetic labelling and fate tracing by the double transgenic mouse proves that the majority, if not all, new myocytes after injury have been formed by non-pre-existing CMs (or more precisely by cells that were not expressing the α-MHC gene during the Tamoxifen induction of the MerCreMer construct). Thus, these results should have settled the question of whether new CMs are formed after damage from a source other than pre-existing CMs. Yet, it does not exclude that some could have originated from pre-existing CMs and also does not provide information about the "cell cohort" most new CMs originated from.

It follows that, in this transgenic setup identifying a small (negligible?) level of incorporation of a DNA-labelling compound in a few eGFP (in the range of <0.1%) but not in the β-Gal positive cardiomyocytes, as reported by Senyo et al. (2013) it is not sufficient to conclude that adult pre-existing cardiomyocytes can actually divide, because it pushes this DNA-labelling tool beyond its power and sensitivity. In fact, the more sensitive the method of detecting DNA labelling the less reliable becomes its interpretation. The cumulative error of this approach is reported by the authors to be 0.2% (Hsieh et al., 2007) and, therefore, far larger than the measured values (<0.1%) (Senyo et al., 2013). Furthermore, it is assumed that the eCSCs in their quiescent state do not express any detectable level of α-MHC (*Myh6*), so that it could be argued that no CSC-derived myocyte would turn on eGFP expression as result of α-MHC-regulated cre-lox recombination induced by tamoxifen. Unfortunately, the reality is different. We have shown that the eCSCs robustly and rapidly commit to myocyte lineage and induce the expression of myocyte-specific genes after damage (Ellison et al., 2013). This fact suggests that cre induction by administration of Tamoxifen over a two week-long period can induce the β-Gal to eGFP recombination in those eCSCs that have activated expression of the *Myh6* gene after committing to the myocyte lineage (Ellison et al., 2013). In agreement with the above conclusion, it has been reported recently that myocardial injury activates the α-MHC-MerCreMer transgene in c-kit^{pos} cardiac progenitors (Dong et al., 2012).

In the first paper using the above-described genetic model, Lee's group (Hsieh et al., 2007) reported that after myocardial infarction or pressure overload, the percentage of eGFP+ cardiomyocytes decreases from 82.8% in the sham-treated mice to 67.5% in areas bordering a myocardial infarction, indicating that stem cells or precursor cells had replenished the cardiomyocytes. Thus, in the MI border zone 15.3% of the myocytes are new and have been formed after the damage. The authors excluded division of the existent myocytes as main contributors to the myocyte "refreshment". Indeed, most BrdU+ cardiomyocytes were GFP-negative and, therefore, could not be derived from the majority of pre-existing myocytes. Although some BrdU+/Troponin-T+/GFP+ were present in the border zone (none in the remote zone) after myocardial infarction their frequency, 0.07%, is significantly below the reported leakage of the genetic system, *i.e.* 0.2% and cannot be distinguished from background. However, even if 0.07% of the new myocytes had originated from pre-existing myocytes (BrdU+/Troponin-T+/GFP+) it still leaves 15.1%, which have originated from cells, which had not expressed Cre during the tamoxifen induction and, therefore, as the authors conclude, originated from a population of myocardial stem/precursor cells. A following paper from the same laboratory (Loffredo et al., 2011), using the same double transgenic mouse model, concludes that cell therapy with bone marrow-derived c-kit+ cells after myocardial infarction further dilutes the GFP+ pool (56.6% *vs.* 68.8%). A result that is consistent with transplanted c-kit^{pos} cell-mediated augmentation of cardiomyocyte progenitor activity.

The only difference between the study of Senyo et al. (2013) concluding that new myocytes originate from the division of pre-existing ones and the previous two papers from

the same laboratory that concluded that new myocytes after myocardial damage originated not from the pre-existing myocytes but from a pool of stem/precursor cells, is that in the latest publication, instead of using BrdU labelling to identify new myocytes, stable isotope labelling followed by multi-isotope imaging mass spectrometry (MIMS), was used, which appears to be able to localize stable isotope reporters in domains smaller than 1 μm^3. Using this technique, the authors affirm that an important response to myocardial injury is new myocyte production originated from the pre-existing mature myocytes in the infarct/border. This statement is based on finding a total of 6 analogue-labelled nuclei out of 4190 myocytes analysed in the normal adult heart, which are diploid, mononucleated and GFP+. That is, 6/4190 = 0.0014% have originated from pre-existing myocytes in the normal heart. The situation is not more convincing in the myocardial infarction where a total of 11 individual nuclei are identified in the border area, which the authors argue originated from pre-existing myocytes. However, 12% of the putative new myocytes in the same region are not originated from pre-existing myocytes (67% after AMI *vs.* 79% in control mice GFP+ myocytes). So, even taking their data at face value, 11/7063 = 0.0015% new myocytes are coming from pre-existing myocytes, while 12% originated not from adult myocytes.

The above review summarized the available evidence claiming a major role of pre-existing myocyte replication over eCSC activation followed by myogenic specification as the source of the regenerated myocytes in myocardial cell homeostasis and regeneration after damage. We argue that when analysed closely these reports, taken together and individually, do not support the conclusions of Senyo et al. (2013) but argue against them. In fact, the data do not contradict the main tenets of accepted myocardial cell biology but instead support the main role of eCSC in cardiac

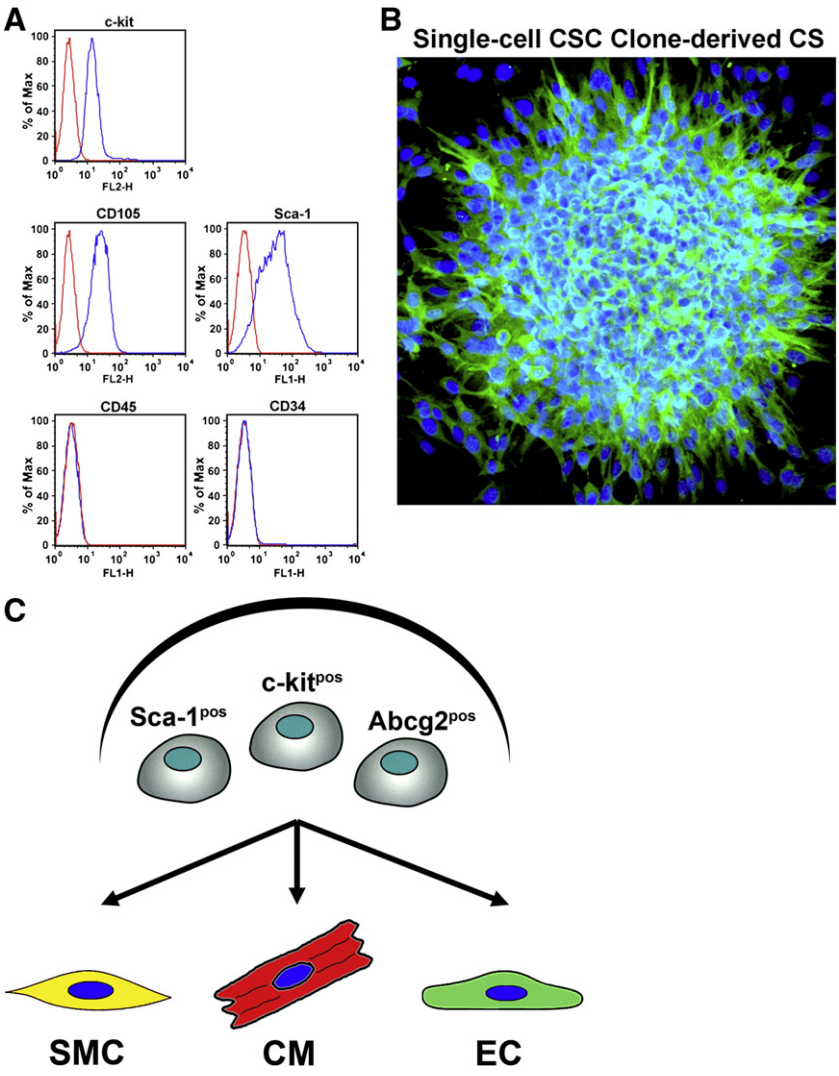

Figure 3 Resident endogenous cardiac stem/progenitor cells (eCSCs). (A) Essential CD membrane-phenotypic profile of c-kit^{pos} CD45^{neg} sorted eCSCs from a wild type adult mouse heart is characterized by FACS analysis. (B) One of the main properties of the eCSCs is the ability to grow in non-adherent culture conditions as pseudo-embryonic bodies, named cardiospheres (CS). The representative confocal image shows a typical cardiosphere derived from a cloned rat eCSC. (C) Cartoon schematics of CSC tri-lineage differentiation potential into cardiomyocyte (CM), smooth muscle cell (SMC) and endothelial cell (EC).
*panels A and B adapted from Ellison et al. *Cell. 2013; 154:827–42.*

cell homeostasis and regeneration in the adult mammalian heart. Furthermore, it is a well-documented fact that newly formed CMs 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).

The eCSCs are necessary and sufficient for adult myocardium cellular homeostasis as well as functional and anatomical repair after major damage

Undoubtedly, the best documented source of 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* endogenous cardiac stem cells (eCSCs) (Fig. 3) (Torella et al., 2007; Rasmussen et al., 2011; Srivastava et al., 2006; Ellison et al., 2013). The first report of these endogenous regenerating myocardial stem cells was in 2003 (Beltrami et al., 2003). Shortly thereafter, several stem/progenitor cell types were identified in the adult mammalian heart by different membrane markers and transcription factors (which often led to claims of a new stem cell type with each new marker used for its identification). Indeed, when referring to eCSCs or cardiac progenitor cells (CPCs), there are at least seven resident heart populations so far identified (mainly according to the single antigen used for their primary isolation) in the adult mammalian heart, including the human (Ellison et al., 2013). These are c-kit^{pos} eCSCs (that have been also defined as CD34^{neg}, CD45^{neg}, Sca-1^{pos}, Abcg2^{pos}, CD105^{pos}, CD166^{pos}, GATA4^{pos}, NKX2-5^{low}, MEF2C^{pos}) (Beltrami et al., 2003; Ellison et al., 2013); Sca1^{pos} eCSCs (CD34^{neg}, CD45^{neg}, FLK1^{neg}, c-kit^{low}, GATA4^{pos}, NKX2-5^{low}, MEF2C^{pos}) (Oh et al., 2003; Matsuura et al., 2004); side population (SP) cells (CD34^{pos}, CD45^{pos}, Abcg2^{pos}, Sca1^{pos}, c-kit^{pos}, NKX2-5^{neg}, GATA4^{neg}) (Martin et al., 2004); Cardiosphere-derived cells (CD105^{pos}, CD34^{pos}, CD45^{pos}, Abcg2^{pos}, Sca1^{pos}, c-kit^{low}) (Messina et al., 2004; Smith et al., 2007; cardiac resident colony-forming unit-fibroblast (cCFU-Fs) (Sca-1^{pos}, PDGFRα^{pos}, CD31^{neg}, c-kit^{low}, CD45^{neg}, FLK1^{neg}, CD44^{pos}, CD90^{pos}, CD29^{pos}, and CD105^{pos}) (Chong et al., 2011); cardiac mesangioblasts (CD31^{pos}, CD34^{pos}, CD44^{pos}, CD45^{neg}, Sca1^{pos}, c-kit^{pos}) (Sampoalesi et al., 2005); and the Isl-1^{pos} cardiac progenitor cells (CPCs) (CD31^{neg}, Sca1^{neg}, ckit^{neg}, GATA4^{pos}, NKX2.5^{pos}) (Laugwitz et al., 2005; Moretti et al., 2006). Another multipotent cell type, present in the epicardium has been described (Smart et al., 2011).

All these claims led to the paradoxical situation whereby the heart, previously described as a non-renewing organ, has turned into the organ with the highest number of distinct types of resident stem/progenitor cells. However, aside from the Isl-1^{pos} cardiac progenitor cells, which are direct progeny of a defined embryonic cardiac progenitor cell population present in only very small numbers in the adult heart (Laugwitz et al., 2005), and the epicardial stem/progenitor cell, which seem originated in the proepicardial organ (Smart et al., 2011), it is evident just from the significant overlap of the main and secondary markers used for their characterization that the different eCSC/CPC populations are closely related. So it is logical to postulate that many, if not all, different putative adult eCSCs reported so far, likely represent different developmental and/or physiological stages of a unique resident stem-progenitor cell type with multipotent regenerative capacity yet to be completely defined (Ellison et al., 2010). Clearly, cell lineage tracking in genetically modified mice, if properly designed, would be an invaluable tool to try to establish the connection among the different eCSC/CPC populations as well as their real contribution to heart development, adult homeostasis and disease.

The progeny of a single eCSC is able to differentiate *in vitro* and *in vivo* into cardiac myocytes, smooth muscle and endothelial vascular cells and when transplanted into the border zone of an infarct or into a myocardium with severe diffuse damage, they regenerate functional contractile muscle, the microvasculature and the connective tissue (Beltrami et al., 2003; Ellison et al., 2013) leaving the myocardium with an anatomical, cellular and molecular phenotype indistinguishable from the spared myocyte cohort (Ellison et al., 2013). In a normal adult myocardium, at any given time, most of the eCSCs are quiescent and only a small fraction is active to replace 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, multiply and generate new muscle and vascular cells (Ellison et al., 2007b; Waring et al., 2012; Ellison et al., 2012) contributing to cardiac remodelling. CSC transplantation can regenerate the contractile cells lost as a consequence of a major AMI affecting up to 25% of the left ventricular mass (Beltrami et al., 2003; Ellison et al., 2013).

The identification of multiple putative cardiac stem cells types raises the question of whether any of them is primarily responsible for the cellular homeostasis of the myocardium throughout life and its repair in response to damage. Koch's postulates,[1] enunciated in 1884 for infectious agents, have been lately used as general criteria to establish that a specific entity is the causative agent of a particular disease or biological phenomenon (Brown et al., 1992; Chien at al., 2000) Despite these postulates being formulated in a scientific environment vastly different from the present, they are still a powerful tool to test a cause-effect relationship between two phenomena (Brown et al., 1992; Chien at al., 2000).

In order to test whether the scepticism towards resident cardiac stem/progenitor cells is justified or whether, on the contrary, adult c-kit^{pos} eCSCs fulfil Koch's postulates as causal agents for cardiac homeostasis and regeneration, we employed experimental protocols of severe diffuse myocardial damage with preserved coronary circulation which, unlike an experimental infarct with permanent coronary ligation, spares the eCSCs (Ellison et al., 2007a). This model was used in several transgenic mice and in combination with

[1] These postulates state that a causal agent must: (a) be present in every case of the disease/phenomenon; (b) be isolated from the disease host and grown in vitro; (c) the disease/phenomenon must be reproduced when the agent is delivered to a susceptible host; and (d) be recovered from the transmitted host.

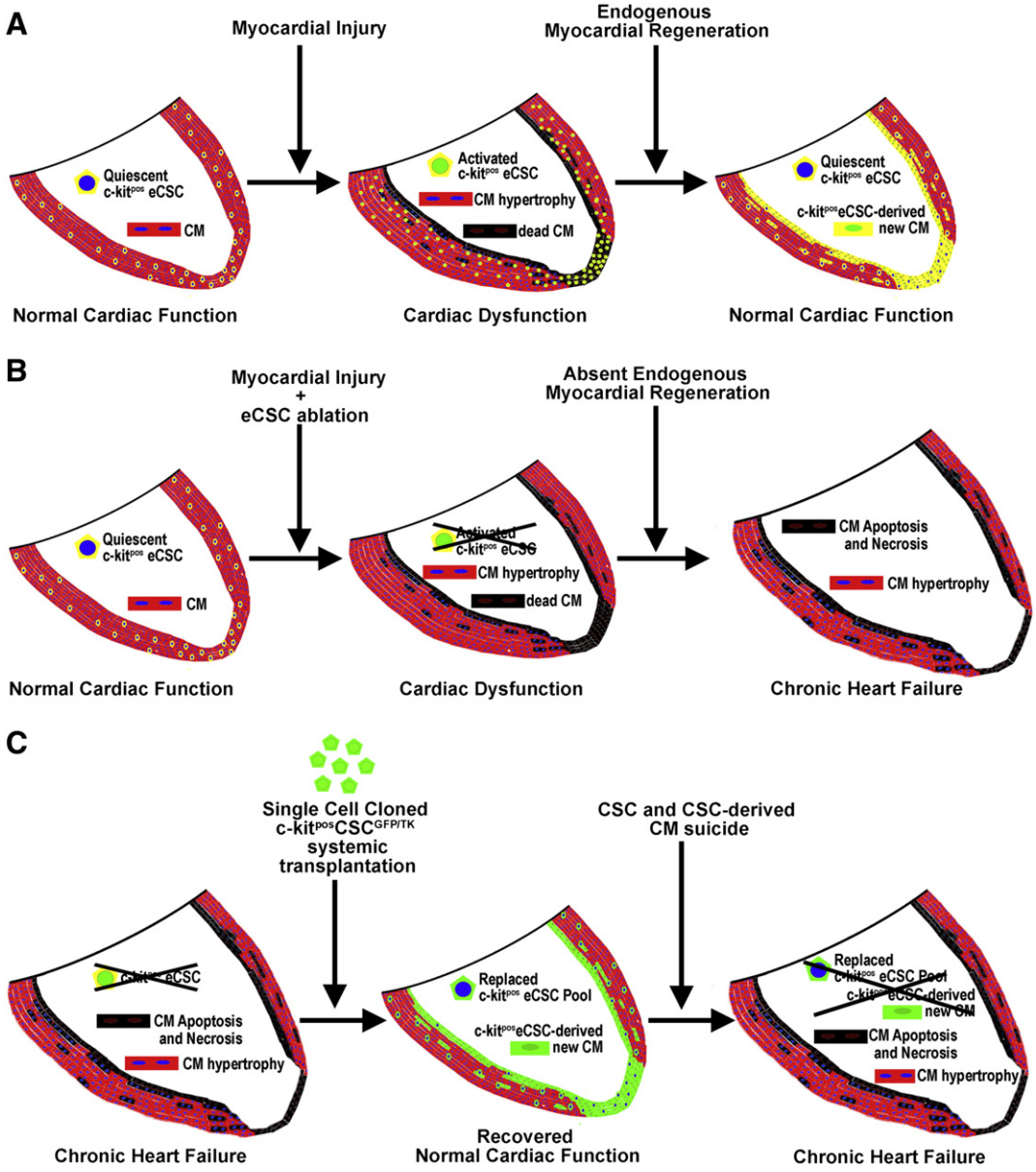

Figure 4 eCSCs are necessary and sufficient for myocardial regeneration. The cartoon depicts cardiac regenerative response following Isoproterenol (ISO) injury. (A)ISO acutely induces cardiomyocyte (CM) death and left ventricular dysfunction, which is followed by CM hypertrophy and endogenous c-kit^{pos} cardiac stem/progenitor cell activation with new CM formation and normalization of cardiac function. (B) When eCSCs are ablated by 5-FU treatment after ISO injury, no new myocytes are generated and the heart goes into chronic heart failure. (C) When rats with failing hearts by ISO+5-FU damage are treated with injection of GFP labeled CSCs carrying the suicide gene TK (CSC$^{GFP/TK}$), myocyte regeneration is restored with recovery of cardiac dysfunction. Finally, if the injected CSCs and their progeny are induced to suicide by Ganciclovir treatment, the heart sets back again to failure.

cell transplantation approaches. These tests have shown that the eCSCs autonomously repair extensive cardiac diffuse damage, leading to complete cellular, anatomical and functional cardiac recovery (Ellison et al., 2013). Furthermore: a) If the eCSCs are ablated, myocardial regeneration is stunted causing heart failure unless they are replaced by exogenous CSCs; b) Selective suicide of these exogenous "replacement" CSCs and their progeny abolishes regeneration and severely impairs ventricular performance; c) The transplanted CSCs can be recovered from the recipient retaining their original characteristics and d) maintain their original regenerative potential when re-transplanted into a new recipient. Therefore, these data confirmed that eCSCs fulfil all Koch's postulates as the cell type necessary and sufficient for myocardial repair and regeneration (Fig. 4).

These conclusions rest mainly on three experimental facts: a model of myocardial injury with patent coronary circulation to test the spontaneous regenerative capacity of eCSCs; *in situ* labelling and genetic tracking the fate of c-kit positive cardiac stem cells and the replacement of the eCSCs by transplantation of genetically tagged CSCs (Ellison et al.,

2013). To that effect, we endeavoured to exclude most of the challenges posed by the potential pitfalls arising from the employed experimental tools. In particular, we demonstrated that newly formed BrdU positive myocytes originate from endogenous and also transplanted CSCs employing multiple and complementary approaches, like genetic tracing, FACS sorting, cell and tissue immunofluorescence, confocal microscopy analysis, and genome pattern of expression. The specificity of the c-kit/cre lentiviral construct to recombine only c-kit expressing cardiac cell lineages in R26/floxed-stop-YFP transgenic mice was demonstrated *in vitro* and *in vivo* by cyto/histo-chemistry, FACS and RT-PCR. The effect of 5-fluoro-uracil (5-FU) and Ganciclovir regime for CSC ablation on neighbouring cells and the essential role of resident CSCs in myocardial regeneration were assessed again by multiple assays. Briefly, the replacement of the endogenous CSCs with cloned genetically labelled CSCs, lead to full anatomical and functional recovery of the transplanted animals while the control cohort (untreated or treated with cardiac fibroblasts) developed lethal heart failure. When the transplanted cells were induced to suicide by Ganciclovir treatment the animals were set back in heart failure. The so-called "bystander effect" elicited by Ganciclovir and cell suicide was ruled out with the best obtainable evidence *in vitro* and *in vivo*. In addition, these transplanted cells were isolated from the transplanted hosts and proven to maintain their CSC properties *in vitro* and in a second round of transplantations.

We documented that 5-FU administration killed most, if not all, the proliferating myocardial cells after ISO injury. It logically follows that the other cardiac progenitor cell populations were most probably also ablated by the ISO+ 5-FU regimen. However, the clone of a single c-kit^{pos} CSC was able to rescue the failing heart phenotype. Myocardial tissue histology and function became dependent on the transplanted cells, which were selectively killed by Glanciclovir. The latter does not exclude a role and participation of the other described stem/progenitor cells in the recovery process. It remains to be proven whether any of the other stem/progenitor cells described so far are equivalent and indistinguishable from c-kit^{pos} CSCs. We would not be surprised if actually the latter hypothesis turn to be at least partially correct as it would reinforce our view that the majority cardiac stem/progenitor cell are different phenotypes of a unique tissue-specific stem/cell population, at least based on the way they have been characterized so far.

Concurrently, a very elegant and well-controlled study by Braun's group, using a triple transgenic mouse approach, has recently lineage traced cardiomyocyte formation in the adult life to be, at least in part, the progeny of eCSCs, expressing Sca-1 (a fraction of which express, as above described, also c-kit) (Uchida et al., 2013). Thus, these data document that the adult heart has a robust autonomous regenerative capacity, which mainly resides in the eCSCs. This capacity, however, spontaneously is not sufficient to significantly repair segmental tissue loses such as in AMIs produced by occlusion of a main coronary artery. This regeneration deficit also affects the segmental loses of any other tissues, no matter the abundance and potency of their tissue stem cells. The challenge is to manipulate the eCSCs and improve their regenerative potential in order to produce an autologous replacement of the cells lost by the insult.

Stimulation of the myocardial endogenous regenerative capacity for damage repair

Most severe cardiac diseases are due to a deficit of contractile cells, the myocytes, in number, function or both. Therefore, most approaches to repair myocardial damage are designed to replace the lost or dysfunctional myocytes. It is surprising that despite the advances of the past decade in understanding myocardial biology in general and cardiomyocyte biogenesis in particular, their impact on the therapeutic attempts to regenerate these losses has been practically nil. Most reported as well as planed clinical trials for cell-based cardiac regeneration remain focused on the formation of new CMs by transplantation of different non-cardiac cell types, mainly bone marrow mononuclear cells and mesenchymal stem cells (Dauwe et al., 2011). This behaviour remains grounded on the concept that the regenerative capacity of the adult heart is negligible. In this intellectual environment it is not surprising that the cardiovascular field has fully embraced the potential of ESCs and iPSs in cardiovascular regenerative medicine (Murry et al., 2008; Yoshida et al., 2010). Unfortunately, the human use of these multipotent cells remains far in the future and faces many hurdles, which they might or might not be able to clear. Curiously, this is happening at the time when the regenerative potential of the endogenous CSCs has been documented at the experimental (Beltrami et al., 2003; Ellison et al., 2013) levels and with very promising but yet preliminary clinical results (Bolli et al., 2011; Makkar et al., 2012).

One potential mechanism of action of transplanting different cell types is a paracrine activation of survival pathways in the cells at risk in the damaged area together with the activation of the endogenous regeneration compartment, the eCSCs. This probable mode of action implies that the sooner after myocardial damage the therapy is applied, the higher the probability to save host cells at risk. Surprisingly, most protocols apply the therapeutic cells 4 to 7 days after damaged when most cells at risk are no longer recoverable (Dauwe et al., 2011). An additional corollary of the paracrine 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 cells and concentrate on the administration of the principal effector molecules produced by them. Interestingly, Smart et al. (2011) found that 'priming' with thymosin β4 (Tβ4) followed by MI in mice resulted in significant activation of Wt1+ epicardial progenitor cells in the infarct and border regions and their subsequent differentiation into cardiomyocytes, albeit at a low level (<1%) relative to the activated progenitor population as a whole (Smart et al., 2011). Therefore, there is urgent need to identify more efficacious factors to induce optimal eCSC activation and drive significant regeneration and maturation of the new myocardium. This point of view is reinforced by the demonstration that tissue stem cells survive long periods of ischemia/hypo-anoxia after all the differentiated cells of the tissue have died (Latil et al., 2012).

Myocardial regenerative cell-free therapies effective on the *in situ* activation, multiplication and differentiation of the resident eCSCs would have many advantages over those

based on cell transplantation. First, therapeutic components should be available 'off-the-shelf' and ready to use at all times without the lag time of many cell therapy approaches; second, they would be affordable, in terms of production costs of the medicinal product; third, such therapy would be easier to apply and compatible with current clinical standard of care for AMI, including the widespread use of percutaneous coronary interventions (PCI); and fourth, if the regenerative response produced were robust enough, it should be able to save and/or regenerate ~50–60 g of functional myocardial tissue, which is the minimum needed to change the course of the disease in a seriously ill patient. Meeting these conditions should make possible the production of a myocardial regenerative therapy which is: a) available at all times and to all candidate patients; b) safe and of easy application in most medical centres; c) affordable to the national health care systems; and d) effective in reducing pathological cardiac remodelling and its late consequence, chronic heart failure.

Myocardial regeneration without cell transplantation: using growth factors to stimulate the growth and differentiation of the eCSCs

Testing regenerative therapies in mouse models of human diseases, although a necessary step in pre-clinical assays is not an accurate predictor of human effectiveness. This is so not only because of 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. Sparing or regenerating a few mgs of myocardium in the mouse has a significant impact in cardiac function and survival. As mentioned above, to obtain similar effect in a human with a moderate AMI would require the sparing and/or regeneration of 50–60 g of tissue. 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 different pathologies, particularly those involving tissue regeneration. We have tested the regenerative effects of intracoronary administration of two growth factors known to be involved in the paracrine effect of transplanted cells (Ellison et al., 2011). Insulin-like growth factor I (IGF-1) and hepatocyte growth factor (HGF), in a single dose ranging from 0.5 to 2 μg HGF and 2 to 8 μg IGF-1, 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, self-sustained and able to produce anatomical, histological and physiological significant regeneration of the damaged

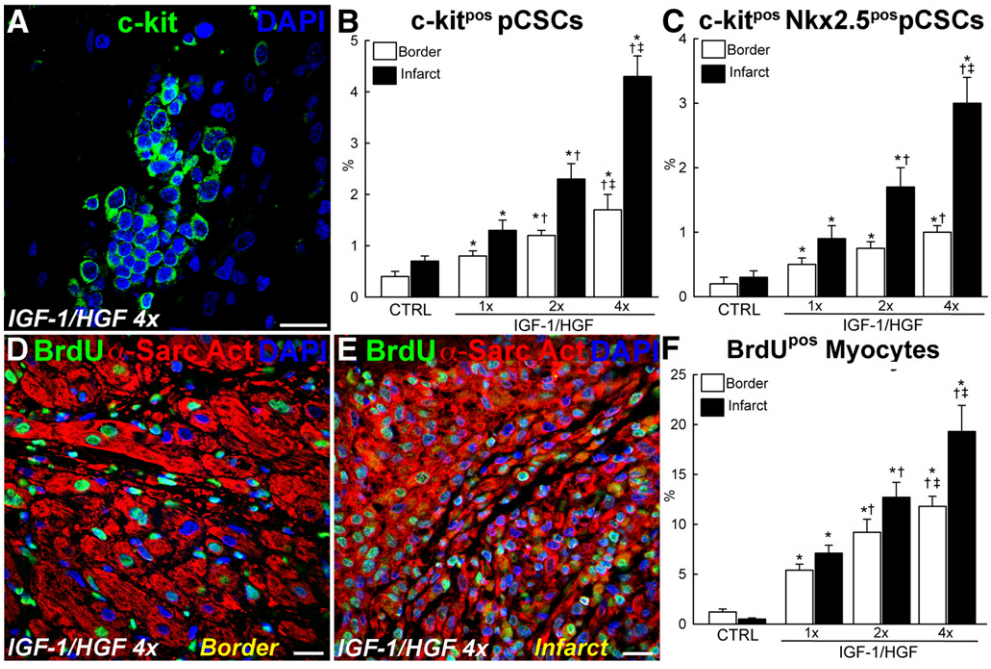

Figure 5 IGF-1/HGF intracoronary injection after AMI in pigs activates endogenous c-kit^{pos} CSCs, giving rise to new cardiomyocytes. (A) A cluster of endogenous c-kit^{pos} CSCs in the infarct zone of an IGF-1/HGF-treated pig heart. (B–C) Number of c-kit^{pos} endogenous CSCs (B) and c-kit^{pos}Nkx2.5^{pos} myocyte progenitor cells (C) in the border (white bars) and infarcted (black bars) regions of IGF-1/HGF-treated and control (CTRL) pigs. (D–E) Newly formed small BrdU^{pos} myocytes (red, α-sarcomeric actin) in the border (D) and infarct (E) zone after IGF-1/HGF administration. (F) The number of newly formed BrdU^{pos} cardiomyocytes significantly increased following IGF-1/HGF administration. All data are mean ± SD; n = 5, 4, 5, and 4 for CTRL, IGF-1/HGF 1×, 2×, and 4×, respectively. *$p < 0.05$ *vs.* CTRL; †$p < 0.05$ *vs.* IGF-1/HGF 1×; ‡$p < 0.05$ *vs.* IGF-1/HGF 2x.
*adapted from Ellison et al. *JACC 2011; 58:977–986.*

myocardium without the need for cell transplantation (Fig. 5) (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, reduced fibrosis and cardiomyocyte reactive hypertrophy. Interestingly, the effect of a single administration of IGF-1/HGF is still measurable 2 months after its application, because a feedback loop triggered by the external stimuli activates the production of growth and survival factors by the targeted cells. The 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 as assessed by cMRI (Ellison et al., 2011). Similar positive effects were obtained when the HGF-IGF1 combination was administered trans-endocardially in pigs with a chronic MI using the NOGA system (Koudstaal et al., 2014).

Despite its effectiveness, the administration of IGF-1/HGF has a significant drawback. Although very effective in regenerating the CMs and micro-vessels lost, the rate of maturation of the newly formed CMs is heterogeneous and slow. Thus, although the CMs and microvasculature lost in the AMI are recovered in the first three weeks, the myocardial mass remains significantly deficient. While the newly formed CMs in contact with spared ones mature rapidly and can reach a diameter close to a normal pig CM, there is an inverse correlation between new myocytes size and their distance from the small islands of spared myocardium scattered within the ischemic zone (Ellison et al., 2011). With the exception of the new CMs in close proximity to spared micro-islands surviving within the ischemic area or those in the border region, at three weeks after treatment the length and diameter of the remaining new CMs (~85% of those regenerated) is between 1/2 and 1/5, respectively, of an adult CM, 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 the myocardial generation force capacity, which is the meaningful functional recovery, also lags behind the regeneration of the cell numbers to the pre-AMI state. A minimum of three months are required for the regenerated myocytes to reach maturity and size comparable to the spared ones (Ellison et al., 2013). 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.

Myocardial regeneration after allogeneic stem cell therapy

All the currently proposed and tested autologous cell therapies are very attractive as proof-of-concept and from a biological standpoint. For rare diseases with chronic and long term evolution affecting hundreds or even thousands of patients to be treated, these personalized therapies, despite their high cost in medical and material resources, might make sense from an economic and health care standpoint. Unfortunately, this is not the case for diseases of high prevalence, such as the sequels of ischaemic heart disease, with millions of patient candidates for regenerative therapy. Not even the richest societies have the resources needed to support a programme 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 autologous cell transplantation therapies have produced some encouraging preliminary results in the clinic with the potential of improving a narrow subset of patients, we believe that all of the autologous cell strategies taken together, now and in the foreseeable future, are and will continue to be incapable to favourably impact the societal health care problem posed by the consequences of CHF post-AMI.

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 (Gnecchi et al., 2008; Hatzistergos et al., 2010). 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 growth factors, the appropriate cell type isolated from allogeneic sources or a combination of both. Allogeneic cells can be produced in large quantities beforehand, stored frozen before their use and made available at all times. That would allow their use not only for the treatment of the pathological remodelling once it has developed but soon after the acute insult to prevent or diminish the pathological remodelling.

Unresolved clinical questions related to the use of allogeneic stem cells in the treatment of patients with AMI remain the identification of the optimal cell population and also the method(s) and time of administration. As previously stated, to be widely available and compatible with current clinical standard of care for AMI, an intracoronary method for delivery at the time of the primary revascularization is the most feasible. Also, direct myocardial injection during revascularization surgery is highly realistic. Mesenchymal stem cells (MSC) have a broad repertoire of secreted trophic and immunodulatory cytokines, however they also secrete factors that negatively modulate CM apoptosis, inflammation, scar formation and pathological remodelling (Ranganath et al., 2012). Moreover, it is questionable whether they are the optimal cell to use in terms of survival and homing to and engraftment in the myocardium since only ~3–4% of the cells administered intracoronary are retained in the myocardium (Dauwe et al., 2011). Furthermore, MSCs can be large and become entrapped in the microvasculature and impede their entry into the myocardium.

Recently, Marban et al. (Malliaras et al., 2012) have tested the safety and efficacy of using allogeneic, mismatched Cardiosphere-Derived Cells (CDCs) in infarcted rats. Allogeneic CDC transplantation resulted in a robust improvement of fractional area change (~12%), ejection fraction (~20%), and fractional shortening (~10%), which was sustained for at least 6 months. Furthermore, allogeneic CDCs stimulated endogenous regenerative mechanisms (recruitment of c-kit^{pos} eCSCs, angiogenesis) and increased myocardial VEGF, IGF-1 and HGF.

We have previously shown that eCSCs that express high levels of the transcription factor GATA-4 exert a paracrine survival effect on CMs through increased IGF-1 secretion and induction of the IGF-1R signalling pathway (Kawaguchi et al., 2010). Furthermore, unlike other cell types (Abdel-Latif et al., 2007; Hofmann et al., 2005), CSCs have a very high tropism for the myocardium. When administered through the systemic circulation the majority of CSCs home and nest into the damaged 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 $1x10^{11}$ cells without detectable alteration of karyotype, loss of differentiating properties or the phenotype of the differentiated progeny (Ellison et al., 2011). These cloned cells produce a repertoire of pro-survival, anti-inflammatory and cardiovascular regenerative growth factors such as: IGF-1, HGF, TGF-β1 superfamily, including activins and BMPs, neuregulin-1, periostin, and BMP-10 among others (Waring et al., 2012). For this reason, we decided to test whether these cloned and *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-II locus or the co-activator molecules CD40, CD80, CD86, ICOS-l, and express low levels of MHC-I antigens. They also have strong immunomodulatory properties *in vitro* when tested in the mixed lymphocyte reaction with high expression of PD-L1/programmed cell death-1 [*unpublished*]. We therefore expected the cloned *in vitro* 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. 6), so that by 3 weeks after MI, there was increased autologous new CM and capillary formation, which was not evident in the control hearts (Fig. 6). Moreover, the c-kit^{pos} heterologous HLA non-matched allogeneic CSCs 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

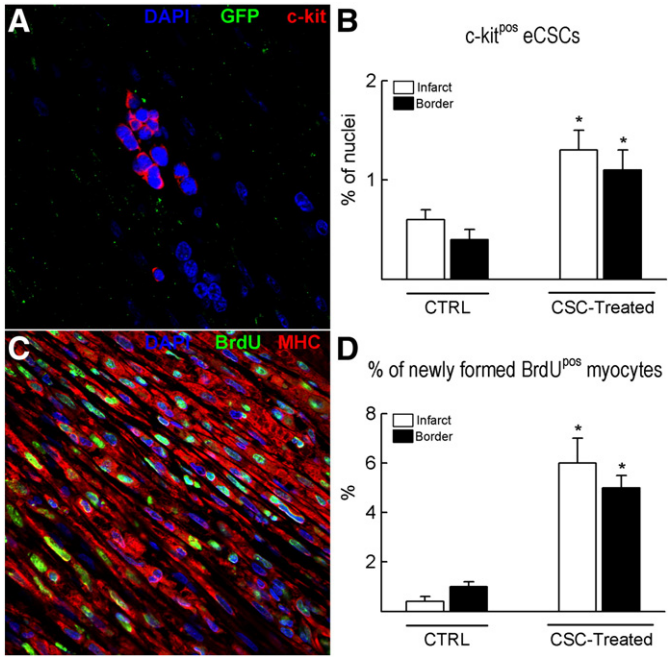

Figure 6 Activation of endogenous CSCs following intracoronary injection of c-kit^{pos} HLA non-matched allogeneic porcine CSCs, after MI in pigs. (A) GFP^{neg}, c-kit^{pos} (red) endogenous CSCs in the 3 week-old infarcted region of the allogeneic treated porcine myocardium. Nuclei are stained by DAPI in blue. (B) The number of c-kit^{pos} endogenous CSCs significantly increased following heterologous HLA non-matched allogeneic CSC treatment. *P < 0.05 *vs.* CTRL. (C) Regenerating band of newly formed BrdU^{pos} (green) cardiomyocytes (red, MHC) in the infarct region, 3 weeks following heterologous HLA non-matched allogeneic CSC treatment. Nuclei are stained by DAPI in blue. (D) New BrdU^{pos} myocyte formation significantly increased following heterologous HLA non-matched allogeneic CSC treatment. *P < 0.05 *vs.* CTRL.
*Adapted from "Ellison et al. *J Cardiovasc Transl Res. 2012;5:667–77*".

CSCs in a clinically relevant MI model activates the eCSCs resulting in improved myocardial cell survival, function, remodelling and regeneration.

A possible risk of using large numbers of *in vitro* expanded autologous CSCs is the appearance of transformed cells with the potential to form abnormal growths. This risk is practically eliminated by the use of allogeneic cells, with a different HLA allele from the recipient, because without immunosuppression all the allogeneic cells get eliminated by the immune system. 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 such survival would prove to be correct, many of the immunology concepts, which have been the basis of transplant biology until now, will need to be revised. In this regard, it is worth noting that in mammals, neoplasias as not transplantable to individuals with a different HLA or MHC haplotype (Welsh, 2011). The only exceptions to this general rule, as far as we are aware, are the case of the oral tumours in the Tasmanian devil, where the reason for the transplantability is under investigation (Siddle et al., 2013). The only other demonstrated cases of long term persistence of cells of a different HLA type in the human are those cases of mothers with microchimerism of cells from her male progeny in different tissues established during pregnancy and persistent thereafter (Bianchi et al., 1996). Whether this microchimerism is causing some of the autoimmune diseases in these women is a matter of dispute. Furthermore, despite thorough pathological examination and contrary to many iPS- and ECS-derived cell lines, the adult tissue-specific eCSCs have demonstrated a non-existent capacity to form tumours and/or teratomas in syngeneic and immunodefficient 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 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. Thus, although the therapeutic cells are allogeneic, the regenerative response is completely autologous because it is carried out by the host CSCs. However, it is clear that despite the clear potential value of such therapy it remains to be yet properly demonstrated endogenous CSC activation is the main mechanism underlying the effect of allogeneic CSC transplantation. To this aim further experiments and a better understanding of the regulatory circuits governing activation, proliferation and differentiation of eCSCs are indeed mandatory.

It is worth noting that it has been demonstrated that the loss of regenerative potential of chronic decompensated human heart is related to aging of CSC (Urbanek et al., 2005). We have found that *in vivo* CSCs are very susceptible to this aging process (Torella et al. 2004). Indeed, in the cohort of patients most likely candidates for autologous hCSC regenerative therapy, >=50% of their CSCs can be senescent and unable to participate in the regenerative process (Urbanek et al., 2005). For that reason, it is also imperative to determine whether the regenerative potential of a particular CSC population in the different pathologies leading to a decompensated heart is determined exclusively by the number of non-senescent CSCs or whether the quality of those that remain functional is also suboptimal because it has deteriorated in response to the stress. In this setting it is important to dissect the factors responsible for CSC aging and to determine whether the aged cells can be "rejuvenated" and co-axed to re-enter the cell cycle and returned to the functional pool. Obtaining specific answers to these un-addressed issues will clearly have an impact on the eventual clinical success of the above-described cell-free regenerative approaches.

Summary and conclusions

The adult heart harbours a regenerative multipotent cell population composed by endogenous cardiac stem progenitor cells (eCSCs) and mammalian, including human, cardiomyocytes are replaced throughout adulthood. That these CSCs are necessary and sufficient for myocardial cell homeostasis throughout live and to regenerate diffuse and segmental myocardial damage represents a paradigm shift in cardiovascular biology. The presence of this regenerative agent within the adult heart supports the view 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, the paracrine effect of allogeneic CSC transplantation or a combination of both approaches.

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References

Abdel-Latif, A., et al., 2007. Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch. Intern. Med. 167, 989–997.

Anversa, P., Kajstura, J., 1998. Ventricular myocytes are not terminally differentiated in the adult mammalian heart. Circ. Res. 83, 1–14.

Anversa, P., et al., 2002. Myocyte renewal and ventricular remodelling. Nature 415, 240–243.

Badylak, S.F., et al., 2012. Engineered whole organs and complex tissues. Lancet 379, 943–952.

Beltrami, A.P., et al., 2001. Evidence that human cardiac myocytes divide after myocardial infarction. N. Engl. J. Med. 344, 1750–1757.

Beltrami, A.P., et al., 2003. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114, 763–776.

Bergmann, O., et al., 2009. Evidence for cardiomyocyte renewal in humans. Science 324, 98–102.

Bersell, K., et al., 2009. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 138, 257–270.
Bianchi, D.W., et al., 1996. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc. Natl. Acad. Sci. U. S. A. 93, 705–708.
Bolli, R., et al., 2011. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet 378, 1847–1857.
Boström, P., et al., 2010. C/EBPβ controls exercise-induced cardiac growth and protects against pathological cardiac remodeling. Cell 143, 1072–1083.
Brown, M.S., et al., 1992. Koch's postulates for cholesterol. Cell 71, 187–188.
Buckingham, M., et al., 2008. Skeletal muscle stem cells. Curr. Opin. Genet. Dev. 18, 330–336.
Chien, K.R., 2000. Meeting Koch's postulates for calcium signaling in cardiac hypertrophy. J. Clin. Invest. 105, 1339–1342.
Chien, K.R., et al., 2002. Converging pathways and principles in heart development and disease: CV@CSH. Cell 110, 153–162.
Chong, J.J., et al., 2011. Adult cardiac-resident MSC-like stem cells with a proepicardial origin. Cell Stem Cell 9, 527–540.
Dauwe, D.F., et al., 2011. Stem cell therapy for the treatment of myocardial infarction. Curr. Pharm. Des. 17, 3328–3340.
Dong, F., et al., 2012. Myocardial CXCR4 expression is required for mesenchymal stem cell mediated repair following acute myocardial infarction. Circulation 126, 314–324.
Eisenberg, C.A., et al., 2006. Bone marrow cells transdifferentiate to cardiomyocytes when introduced into the embryonic heart. Stem Cells 24, 1236–1245.
Ellison, G.M., et al., 2007a. Acute beta-adrenergic overload produces myocyte damage through calcium leakage from the ryanodine receptor 2 but spares cardiac stem cells. J. Biol. Chem. 282, 11397–11409.
Ellison, G.M., et al., 2007b. Myocyte death and renewal: modern concepts of cardiac cellular homeostasis. Nat. Clin. Pract. Cardiovasc. Med. 4 (Suppl. 1), S52–S59.
Ellison, G.M., et al., 2009. Use of heterologous non-matched Cardiac Stem Cells (CSCs) without immunosuppression as an effective regenerating agent in a porcine model of acute myocardial infarction. Eur. Heart J. 30, 495 (Abstract Supplement).
Ellison, G.M., et al., 2010. Cardiac stem and progenitor cell identification: different markers for the same cell? Front. Biosci. 2, 641–652.
Ellison, G.M., et al., 2011. Endogenous cardiac stem cell activation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and regeneration of the infarcted pig heart. J. Am. Coll. Cardiol. 58, 977–986.
Ellison, G.M., et al., 2012. Physiological cardiac remodelling in response to endurance exercise training: cellular and molecular mechanisms. Heart 98, 5–10.
Ellison, G.M., et al., 2013. Adult c-kit^{pos} cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. Cell 154, 827–842.
Evans, M.J., et al., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156.
Fuchs, E., 2009. The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 137, 811–819.
Gnecchi, M., et al., 2008. Paracrine mechanisms in adult stem cell signaling and therapy. Circ. Res. 103, 1204–1219.
Hatzistergos, K.E., et al., 2010. Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ. Res. 107, 913–922.
Hofmann, M., et al., 2005. Monitoring of bone marrow cell homing into the infarcted human myocardium. Circulation 111, 2198–2202.
Hsieh, P.C., et al., 2007. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat. Med. 13, 970–974.
Huang, X.P., et al., 2010. Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. Circulation 122, 2419–2429.
Hunter, J.J., et al., 1999. Signaling pathways for cardiac hypertrophy and failure. N. Engl. J. Med. 341, 1276–1283.
Jayawardena, T.M., et al., 2012. MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. Circ. Res. 110, 1465–1473.
Jessup, M., Brozena, S., 2003. Heart failure. N. Engl. J. Med. 348, 2007–2018.
Kahan, B.D., 2011. Fifty years in the vineyard of transplantation: looking back. Transplant. Proc. 43, 2853–2859.
Kajstura, J., et al., 2010. Myocyte turnover in the aging human heart. Circ. Res. 107, 1374–1386.
Kajstura, J., et al., 2012. Cardiomyogenesis in the aging and failing human heart. Circulation 126, 1869–1881.
Kawaguchi, N., et al., 2010. c-kitpos GATA-4 high rat cardiac stem cells foster adult cardiomyocyte survival through IGF-1 paracrine signalling. PLoS One 5, e14297.
Kikuchi, K., et al., 2012. Cardiac regenerative capacity and mechanisms. Annu. Rev. Cell Dev. Biol. 28, 719–741.
Kopp, J.L., et al., 2011. Progenitor cell domains in the developing and adult pancreas. Cell Cycle 10, 1921–1927.
Kotton, D.N., 2012. Next generation regeneration: the hope and hype of lung stem cell research. Am. J. Respir. Crit. Care Med. (Apr 19).
Koudstaal, S., et al., 2014. Sustained delivery of insulin-like growth factor-1/hepatocyte growth factor stimulates endogenous cardiac repair in the chronic infarcted pig heart. J. Cardiovasc. Transl. Res. 7, 232–241.
Kühn, B., et al., 2007. Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nat. Med. 13, 962–969.
Laflamme, M.A., et al., 2011. Heart regeneration. Nature 473, 326–335.
Latil, M., et al., 2012. Skeletal muscle stem cells adopt a dormant cell state post mortem and retain regenerative capacity. Nat. Commun. 3, 903.
Laugwitz, K.L., et al., 2005. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433, 647–653.
Loffredo, F.S., et al., 2011. Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell 8, 389–398.
MacLellan, W.R., et al., 2005. Overlapping roles of pocket proteins in the myocardium are unmasked by germ line deletion of p130 plus heart-specific deletion of Rb. Mol. Cell. Biol. 25, 2486–2497.
Makkar, R.R., et al., 2012. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 379, 895–904.
Malliaras, K., et al., 2012. Safety and efficacy of allogeneic cell therapy in infarcted rats transplanted with mismatched cardiosphere-derived cells. Circulation 125, 100–112.
Malliaras, K., et al., 2013. Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart. EMBO Mol. Med. 5, 191–209.
Martin, C.M., et al., 2004. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev. Biol. 265, 262–275.
Matsuura, K., et al., 2004. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J. Biol. Chem. 279, 11384–11391.
Mercier, F.E., et al., 2011. The bone marrow at the crossroads of blood and immunity. Nat. Rev. Immunol. 12, 49–60.

Messina, E., et al., 2004. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ. Res. 95, 911–921.
Moretti, A., et al., 2006. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127, 1151–1165.
Murry, C.E., et al., 2008. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132, 661–680.
Nadal-Ginard, B., 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 15, 855–864.
Nadal-Ginard, B., et al., 2003. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circ. Res. 92, 139–150.
Oh, H., et al., 2001. Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. Proc. Natl. Acad. Sci. U. S. A. 98, 10308–10313.
Oh, H., et al., 2003. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc. Natl. Acad. Sci. U. S. A. 100, 12313–12318.
Orlic, D., et al., 2001. Bone marrow cells regenerate infarcted myocardium. Nature 410, 701–705.
Porrello, E.R., et al., 2011. Transient regenerative potential of the neonatal mouse heart. Science 331, 1078–1080.
Qian, L., et al., 2012. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. Nature 485, 593–598.
Quaini, F., et al., 2002. Chimerism of the transplanted heart. N. Engl. J. Med. 346, 5–15.
Quevedo, H.C., et al., 2009. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc. Natl. Acad. Sci. U. S. A. 106, 14022–14027.
Ranganath, S.H., et al., 2012. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. Cell Stem Cell 10 (3), 244–258.
Rasmussen, T.L., et al., 2011. Getting to the heart of myocardial stem cells and cell therapy. Circulation 123, 1771–1779.
Reule, S., et al., 2011. Kidney regeneration and resident stem cells. Organogenesis 7, 135–139.
Robinton, D.A., et al., 2012. The promise of induced pluripotent stem cells in research and therapy. Nature 481, 295–305.
Roger, V.L., et al., 2012. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics–2012 update: a report from the American Heart Association. Circulation 125, e2–e220.
Rountree, C.B., et al., 2012. Stem cells in liver diseases and cancer: recent advances on the path to new therapies. Hepatology 55, 298–306.
Rumyantsev, P.P., 1991. In: Carlson, B.M. (Ed.), Reproduction of growth and hyperplasia of cardiac muscle cells., 3. Harwood Academic Publishers, Chur, Switzerland, pp. 1–371.
Sampaolesi, M., et al., 2005. Cell therapy of primary myopathies. Arch. Ital. Biol. 143, 235–242.
Schneider, J.W., et al., 1994. Reversal of terminal differentiation mediated by p107 in Rb−/− muscle cells. Science 264, 1467–1471.
Senyo, S.E., et al., 2013. Mammalian heart renewal by pre-existing cardiomyocytes. Nature 493, 433–436.
Siddle, H.V., et al., 2013. Reversible epigenetic down-regulation of MHC molecules by devil facial tumour disease illustrates immune escape by a contagious cancer. PNAS 110, 5103–5108.
Simons, B.D., et al., 2011. Stem cell self-renewal in intestinal crypt. Exp. Cell Res. 317, 2719–2724.
Smart, N., et al., 2011. De novo cardiomyocytes from within the activated adult heart after injury. Nature 474, 640–644.
Smith, R.R., et al., 2007. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 115, 896–908.
Song, K., et al., 2012. Heart repair by reprogramming non-myocytes with cardiac transcription factors. Nature 485, 599–604.
Soonpaa, M.H., et al., 1998. Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ. Res. 83, 15–26.
Srivastava, D., et al., 2006. Potential of stem-cell-based therapies for heart disease. Nature 441, 1097–1099.
Suh, H., et al., 2009. Signaling in adult neurogenesis. Annu. Rev. Cell Dev. Biol. 25, 253–275.
Takahashi, K., et al., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.
Takahashi, K., et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.
Tam, S.K., et al., 1995. Cardiac myocyte terminal differentiation. Potential for cardiac regeneration. Ann. N. Y. Acad. Sci. 752, 72–79.
Terzic, A., et al., 2010. Regenerative medicine advancing health care 2020. J. Am. Coll. Cardiol. 55, 2254–2257.
Thomson, J.A., et al., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.
Torella, D., Rota, M., Nurzynska, D., Musso, E., Monsen, A., Shiraishi, I., Zias, E., Walsh, K., Rosenzweig, A., Sussman, M.A., Urbanek, K., Nadal-Ginard, B., Kajstura, J., Anversa, P., Leri, A., 2004. Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res. 94, 514–524.
Torella, D., et al., 2007. Resident cardiac stem cells. Cell. Mol. Life Sci. 64, 661–673.
Uchida, S., et al., 2013. Sca1-derived cells are a source of myocardial renewal in the murine adult heart. Stem Cell Rep. 1, 397–410.
Urbanek, K., et al., 2003. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc. Natl. Acad. Sci. U. S. A. 100, 10440–10445.
Urbanek, K., et al., 2005. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc. Natl. Acad. Sci. U. S. A. 102, 8692–8697.
Waring, C.D., Vicinanza, C., Papalamprou, A., Smith, A.J., Purushothaman, S., Goldspink, D.F., Nadal-Ginard, B., Torella, D., Ellison, G.M., 2012. The adult heart responds to increased workload with physiologic hypertrophy, cardiac stem cell activation, and new myocyte formation. Eur Heart J. (in press) [Epub ahead of print] PMID: 23100284.
Welsh, J.S., 2011. Contagious cancer. Oncologist 16, 1–4.
Yamanaka, S., 2007. Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1, 39–49.
Yoshida, Y., et al., 2010. Recent stem cell advances: induced pluripotent stem cells for disease modeling and stem cell-based regeneration. Circulation 122, 80–87.
Zhang, R., et al., 2013. In vivo cardiac reprogramming contributes to zebrafish heart regeneration. Nature 498, 497–501.