Regeneration of the heart

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The death of cardiac myocytes diminishes the heart's pump function and is a major cause of heart failure, one of the dominant causes of death worldwide. Other than transplantation, there are no therapies that directly address the loss of cardiac myocytes, which explains the current excitement in cardiac regeneration. The field is evolving in two important directions. First, although endogenous mammalian cardiac regeneration clearly seems to decline rapidly after birth, it may still persist in adulthood. The careful elucidation of the cellular and molecular mechanisms of endogenous heart regeneration may therefore provide an opportunity for developing therapeutic interventions that amplify this process. Second, recent breakthroughs have enabled reprogramming of cells that were apparently terminally differentiated, either by dedifferentiation into pluripotent stem cells or by transdifferentiation into cardiac myocytes. These achievements challenge our conceptions of what is possible in terms of heart regeneration. In this review, we discuss the current status of research on cardiac regeneration, with a focus on the challenges that hold back therapeutic development.

Introduction

Heart failure is often the consequence of cardiac myocyte loss after insult, such as myocardial infarction. Patients hospitalized with an index episode of heart failure have a prognosis as low as patients with common malignancies (Stewart et al, 2001). With the exception of heart transplantation and implantation of mechanical ventricular assist devices, current therapies do not address the central problem of decreased pumping capacity owing to a depleted pool of cardiac myocytes. Thus, there has been enormous interest in the prospect of cardiac regeneration rather than slowing the inexorable decline of heart function.

Until recently, the prospect of cardiac regeneration was largely a subject of science fiction. The longstanding paradigm held that the mammalian heart is a terminally differentiated organ, incapable of replenishing any myocyte attrition. During the past decade, however, studies revealed not only that mammalian cardiac myocytes retain some capacity for division (Beltrami et al, 2001), but also identified endogenous cardiac progenitor cells in the heart (Beltrami et al, 2003) or bone marrow (Orlic et al, 2001). These cells retain some potential for differentiation into the cellular components of the heart, including endothelial cells, smooth muscle cells and cardiac myocytes.

If progenitor cells residing in the adult are capable of producing new heart cells, the therapeutic delivery of such progenitors might facilitate the generation of *de novo* functional myocardium. In this context, cell-based therapies for the heart have been rapidly translated into the clinic to treat heart disease, but randomized clinical trials with bone marrow progenitors have shown at best modest improvements in ventricular function (Martin-Rendon et al, 2008). In short, the promise of complete cardiac regeneration has not yet been realized.

In light of mixed results from clinical trials, it is worth revisiting both the foundations of cardiac regeneration and highlight recent advances that may portend future directions in the field. We will first define the problem, that is elucidating the scope of endogenous mammalian regeneration and, by extension, the scale of the regenerative deficit. We will then summarize current regenerative approaches, including both cell-based therapies and pharmacoregenerative strategies. In this context, we will summarize the many challenges that stand in the way of cardiac regeneration, both endogenous repair processes and exogenous regenerative therapies.

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Heart regeneration in lower organisms

The regenerative deficit of the mammalian heart is obvious when compared with organisms such as zebrafish and newts, which demonstrate a remarkable survival capacity after removal of up to 20% of the heart by transection of the ventricular apex (Oberpriller & Oberpriller, 1974; Poss et al, 2002). Pre-existing cardiac myocytes adjacent to the site appear to undergo a process of dedifferentiation, characterized by dissolution of sarcomeric structures. This is followed by incorporation of deoxyribonucleic acid (DNA) synthesis markers (e.g. nucleotide analogues) consistent with proliferation (Jopling et al, 2010; Kikuchi et al, 2010; Laube et al, 2006). Ultimately, newly generated cardiac myocytes are functionally integrated with the preexisting myocardium. The heart is left with little residual evidence of the injury, thus providing a natural example of complete myocardial regeneration.

Evidence for heart regeneration in mammals

During embryonic development and the early post-natal period, mice also demonstrate a remarkable regenerative capacity. Embryos heterozygous for a cardiac myocyte-specific null mutation in the x-linked holocytochrome *c* synthase (*Hccs*) gene demonstrate cardiac myocyte replacement during foetal development (Drenckhahn et al, 2008): when one of two X-chromosomes is randomly inactivated in each female somatic cell, approximately 50% of the cardiac myocytes are rendered *Hccs*-null and hence dysfunctional. Proliferative functional *Hccs*-expressing cardiac myocytes compensate for dysfunctional *Hccs*-null myocytes, such that, at birth, 90% of the heart is derived from myocytes containing one functional *Hccs* allele. Similar plasticity in post-natal mice is confined to the first week

after birth; apical excision results in myocyte proliferation and structural restoration similar to regeneration in zebrafish and newts (Porrello et al, 2011). Afterwards, however, injured myocardium is largely replaced by fibrosis and scarring. Distinguishing whether the adult mammalian heart is incapable of cardiac myocyte replacement or whether it retains a low-level capacity for repair is therefore fundamentally important because even low-level myocyte turnover raises the specter of amplifying that response for therapeutic ends. Thus, it is worth reviewing both the evidence supporting the evolving view of a more plastic mammalian heart, while closely considering potential artefacts that may affect the interpretation of these results.

The first arguments against the paradigm of the terminally differentiated quiescent cardiac myocyte date back decades; the increasing force of these arguments reflects the emergence of new data made possible by methodological innovations. Early evidence supporting cardiac myocyte plasticity relied on mathematical modelling of the myocyte population based on cytometric indices. For example, the measured average volume increase of cardiac myocytes was calculated to fall short of the increase predicted by the observed volumetric changes in the whole heart (Astorri et al, 1971). These data and similar analyses based on DNA content asserted that changes in heart volume could not be explained by hypertrophy alone, and that cardiac myocyte hyperplasia (increase in cell number) contributed to changes in heart mass. However, these studies were not widely accepted because their conclusions relied on a number of assumptions about myocyte size and DNA content.

Modern microscopic analyses to detect cell cycle markers such as Ki67 or the incorporation of nucleotide analogues (e.g. iododeoxyuridine or ^{3}H-thymidine) into newly synthesized DNA further support the notion that the mammalian heart may generate new myocytes both during normal homeostasis and after injury. Multiple studies have demonstrated that murine or

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Glossary

Cardiac myocytes
Heart muscle cells.

Fibrosis
Development of excessive fibrous connective tissue in an organ.

Gastrulation
Early phase of embryonic development during which the germ layers form.

Heart failure
Condition in which the heart is not able to pump enough blood into the rest of the body. Also called congestive heart failure.

Ki67
Marker protein for cell proliferation.

Mesodermal
Pertaining to the mesoderm. Mesoderm is one of the three germ layers, which forms skeletal muscle, the skeleton, the dermis of skin, connective tissue, the urogenital system, the heart, blood (lymph cells), the kidney and the spleen.

Nucleotide analogues
Nucleotides, which contain chemical modifications and are incorporated into the DNA during synthesis and cell proliferation. They can thus be easily detected.

Polyploid
Cells or organisms that contain one or more extra sets of chromosomes.

Progenitor—ancestor
A cell that can differentiate into a specific cell type.

Sarcomeric
Pertaining to the sarcomere. Sarcomere is one of the segments into which a striated muscle is divided.

Stromal cells
Cells of the connective tissue of any organ.

Teratomas
Congenital, encapsulated tumours, which contain one or more germ layers.

Transcription factors
Proteins, which are involved in regulation of gene expression after binding to a specific DNA sequence.

Transdifferentiation
The conversion of one differentiated cell to another cell type.

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human cardiac myocytes can reenter the cell cycle, but the described rates of this phenomenon differ by more than one order of magnitude (Beltrami et al, 2001; Kajstura et al, 2010a,b; Soonpaa & Field, 1997).

Recent dating experiments, made possible by nuclear arms testing in the middle of the 20th century, provide the most convincing evidence for post-natal human cardiac myocyte turnover. Since the Partial Test Ban Treaty of 1963, the marked spike in atmospheric ^{14}C levels has dissipated as the biosphere absorbed the carbon. Thus, the period of nuclear testing serves as a historical DNA labelling pulse, and the period after the test ban treaty serves as a chase. The genomic DNA of cells generated during either the pulse or the chase reflect the earth's atmospheric ^{14}C concentration at that point in time, which allows investigators to date the age of cardiac myocytes by measuring the concentration of ^{14}C in their nuclei (Bergmann et al, 2009). It showed that the adult heart contains some cardiac myocytes generated during the human life-span; their model predicted an approximate turnover rate of 1% per year at age 25, declining to a rate of 0.45% by the age of 75.

Confounding factors in the quantitation of cardiac myocyte turnover

The data generated by these diverse methodologies support the notion that the mammalian heart has conserved some capacity for cardiac myocyte turnover though there is lingering controversy about the scope of the mammalian heart's endogenous regenerative capacity. Studies relying on the identification mitotic cardiac myocytes (Kajstura et al, 1998), immuno-detection of cell cycle markers such as Ki67 (Beltrami et al, 2001) or of IdU incorporation into newly synthesized DNA (Kajstura et al, 2010b) suggest that nearly 30% of the heart can be replaced within 1 year during normal homeostasis, a rate that increases 50-fold after injury. These remarkably high rates imply that the entire heart is replaced approximately every 3 years during normal homeostasis, and that all cardiac myocytes lost to an infarction could be replaced within 3 weeks. In contrast, data from nuclear fallout studies (Bergmann et al, 2009) and quantitative studies of DNA synthesis using autoradiographic measurement of ^{3}H-thymidine incorporation (Soonpaa & Field, 1997) indicate turnover rates of 1% or less per year.

The actual scale of the endogenous regenerative response has important implications for rational therapeutic design. If the turnover rate is relatively low, therapeutic interventions should focus on replacing lost or dysfunctional myocytes. If the mammalian heart indeed has a robust intrinsic capacity for myocyte repletion, the therapeutic focus should shift to promoting the assembly of endogenously generated myocytes into functional myocardium. Thus, in the context of discrepant evidence about the true scale of endogenous mammalian regeneration, it is worth reviewing potential sources of bias inherent to all existent methodologies that are used to study cell fate.

The difficulty of measuring cardiac myocyte turnover in the heart is largely a result of the slow rate of myocyte turnover relative to adjacent stromal cells (Bergmann et al, 2009). Within

a timeframe that is practical to directly measure the generation of cardiac myocytes, the number of newborn cells (numerator) is much smaller than the total number of cells (denominator). As a result, small errors may magnify projections of absolute yearly or lifetime myocyte turnover. Another source of error is the occasional mis-identification of cellular components, which is an inevitable consequence of analyzing a three-dimensional organ with two-dimensional tissue sections, particularly when relying on light microscopy which has limited fidelity of resolving cellular borders in complex tissues like the heart (Laflamme & Murry, 2005). The problem is further exacerbated by the autofluorescence of myocardium, which complicates any method that relies on the detection of a fluorescent signal, including the detection of nucleotide analogues, Ki67 or fluorescent labels as used in genetic lineage-mapping experiments.

Such potential confounders could also affect the ^{14}C dating method, because it requires the isolation of cardiac myocyte nuclei by digestion and flow cytometric sorting. Any impurities in the isolated nuclear preparation could introduce bias to the analysis; preferential isolation of senescent myocytes could result in an underestimate of myocyte turnover, whereas an impure nuclear isolate that includes non-myocyte nuclei might result in an overestimate.

The heart also presents a unique challenge compared to other organs owing to the propensity of cardiac myocytes to synthesize DNA during S-phase without completing either mitosis and/or cytokinesis (Fig 1). During early post-natal development, for example, the majority of rodent cardiac myocytes (Li et al, 1996) and an estimated 25–57% of human cardiac myocytes (Olivetti et al, 1996; Schmid & Pfitzer, 1985) become binucleated. By adulthood, most cardiac myocyte nuclei have also become polyploid with at least one (4n: tetraploid) or two (8n: octoploid) additional rounds of chromosomal replication (Bergmann et al, 2010). Although some studies demonstrated that the polyploidy rate in the cardiac myocyte pool is not affected by ageing or injury (Olivetti et al, 1996), others suggested that the ploidy state is more dynamic. The ploidy state of cardiac myocytes may increase with myocardial hypertrophy or injury (Adler & Friedburg, 1986), which could be mistaken for myocyte division. Conversely, hearts that have been unloaded by implantation of a ventricular assist device may have a lower percentage of polyploid myocytes, because more 2n cardiac myocytes are being generated (Wohlschlaeger et al, 2010). These aspects of cardiac myocyte biology inevitably represent potential confounders that must be considered in any quantification of cardiac myocyte formation. As with any controversial hypothesis, achieving consensus regarding adult mammalian cardiac myocyte turnover will likely require multiple lines of evidence using multiple different methodologies.

Defining the cellular source of new cardiac myocytes

The majority of reports suggest some endogenous capacity for cardiac myocyte renewal, which has generated a broad focus on finding the cellular source of newly generated cardiac myocytes.
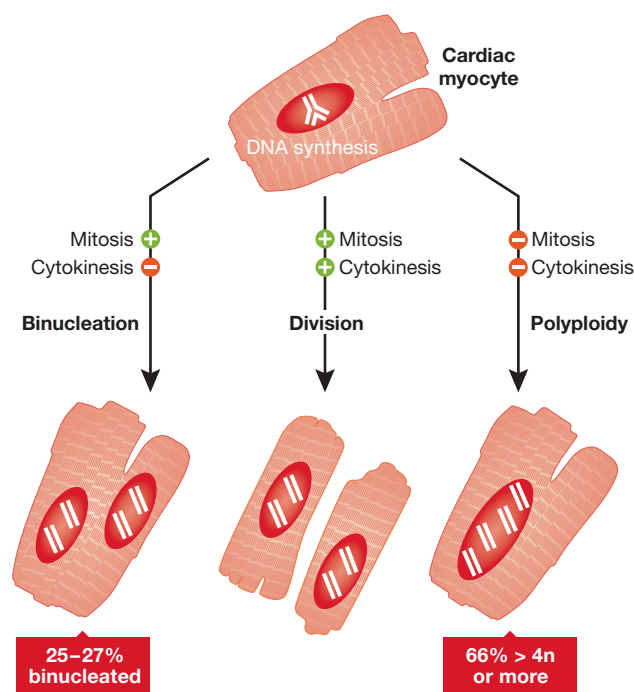
Figure 1. The majority of post-natal human DNA synthesis in the heart does not lead to new myocyte formation. Cardiac myocytes can complete S-phase, followed by mitosis and cytokinesis (centre) resulting in myocyte doubling. Cardiac myocytes can also complete mitosis without cytokinesis (left), resulting in a binucleated cell. Cardiac myocytes can also undergo chromosomal replication without completing either mitosis or cytokinesis (right), resulting in polyploidy nuclei. By the completion of post-natal development, the majority of human myocyte nuclei contain ≥4n chromosomal copies.

Three main pathways for new cardiac myocyte generation have been proposed: division of preexisting mature cardiac myocytes (Kajstura et al, 1998), amplification of dedifferentiated cardiac myocytes (Jopling et al, 2010; Kikuchi et al, 2010) and differentiation of progenitor cells (Hsieh et al, 2007). Evidence exists for all three processes in different species and under different conditions. It may be possible that more than one of these mechanisms can operate within a given species at different developmental or pathological stages.

Although there are many reports of mature cardiac myocyte division in adult mammals, the majority of those reports suggest that it is a rare event. This is not surprising, given the densely packed and highly organized sarcomeric contractile apparatus. In fact, cardiac myocyte replication after injury in the neonatal mouse, newts and zebrafish is preceded by molecular and/or cytoskeletal evidence of dedifferentiation (Jopling et al, 2010; Kikuchi et al, 2010; Oberpriller & Oberpriller, 1974; Porrello et al, 2011). Cardiac myocytes near the site of injury can reexpress gene markers of immaturity, like the zinc-finger transcription factor GATA binding protein-4 (GATA4), together with disassembly of sarcomeric structures that is evident by electron microscopy. This population of dedifferentiated cardiac myocytes expresses markers of DNA synthesis during

a proliferative phase before redifferentiating into mature cardiac myocytes.

Newly generated adult mammalian cardiac myocytes may arise from an endogenous pool of progenitor cells after injury. Our laboratory developed a genetic lineage mapping approach to quantify progenitor-dependent cardiac myocyte turnover (Fig 2) (Hsieh et al, 2007). In the bitransgenic MerCreMer/ZEG inducible cardiac myocyte reporter mouse, mature cardiac myocytes undergo an irreversible genetic switch from constitutive β-galactosidase expression to green fluorescent protein (GFP) expression upon tamoxifen pulse. During a chase period, we evaluated the effect of myocardial injury on the proportion of GFP+ or β-gal+ cardiac myocytes. Pressure overload or myocardial infarction resulted in a significant reduction in the percentage of GFP+ cardiac myocytes and a corresponding increase in the percentage of B-gal+ cardiac myocytes, consistent with repletion of the myocyte pool by B-gal− expressing progenitors. Though this approach theoretically captures the sum progenitor contribution to myocyte turnover, it cannot directly identify the molecular identity or anatomic location of the progenitor pool.
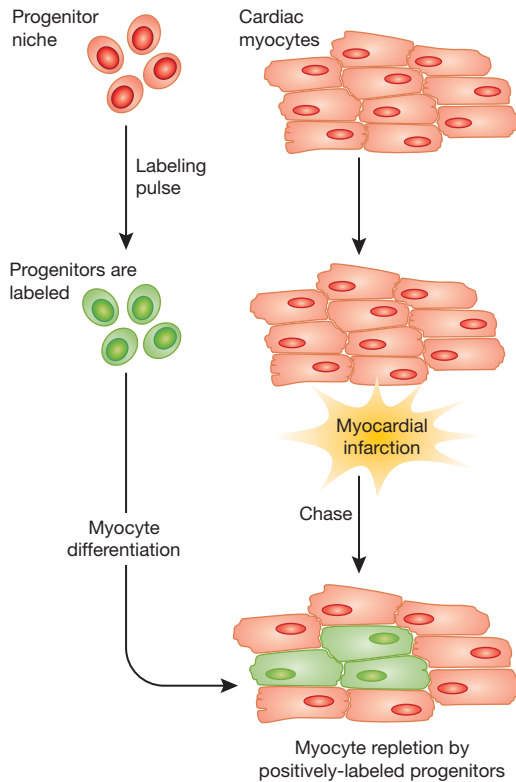
One approach to characterizing the molecular phenotype of cardiac progenitors is to study cardiac embryologic development, guided by the assumption that developmental paradigms are recapitulated during post-natal repair. When examined through a developmental lens, an increasingly detailed picture emerges of the soluble and transcriptional signals that guide the cardiogenic programme from gastrulation (formation of distinct germ layers) through the ultimate maturation of cardiac myocytes (Mercola et al, 2011; Yi et al, 2010). The induction of mesoderm posterior (MESP)-1 expression by brachyury-expressing primitive mesodermal cells is a proximal requirement for the ultimate production of differentiated heart cells (Bondue et al, 2008; David et al, 2008; Lindsley et al, 2008). As the developing embryo grows beyond the germ layer phase, its developing heart receives cells from distinct anatomic progenitor sources: the 1st and 2nd heart fields provide the majority of the myocardium, with some contribution from epicardial progenitors. Certain fields may be preferentially marked by specific transcription factors; for example, the first heart field by T-box transcription factor 5 (Tbx5) (Takeuchi et al, 2003), the second heart field by Lim-homeodomain protein Islet1 (Isl1) (Cai et al, 2003) and epicardial progenitors by Wilms tumour-1 (WT1) or T-box transcription factor 18 (Tbx18) (Cai et al, 2008; Zhou et al, 2008). Other cardiogenic factors identified by embryonic lineage tracing or analysis of gene silencing include homeobox protein nkx2.5 (Wu et al, 2006), the myocyte enhancer factor 2C (Mef2c) (Lin et al, 1997) and GATA4 (Molkentin et al, 1997).

In contrast to the relatively well-defined pre-natal development, there is no consensus yet about the molecular identity of post-natal mammalian cardiac progenitor cells or 'adult cardiac stem cells'. A number of laboratories have identified cell populations within the post-natal mouse, which fulfil some criteria of cardiac progenitors. The common approach of such studies has been to enzymatically digest myocardial tissue and to isolate specific cell populations based on transcriptional
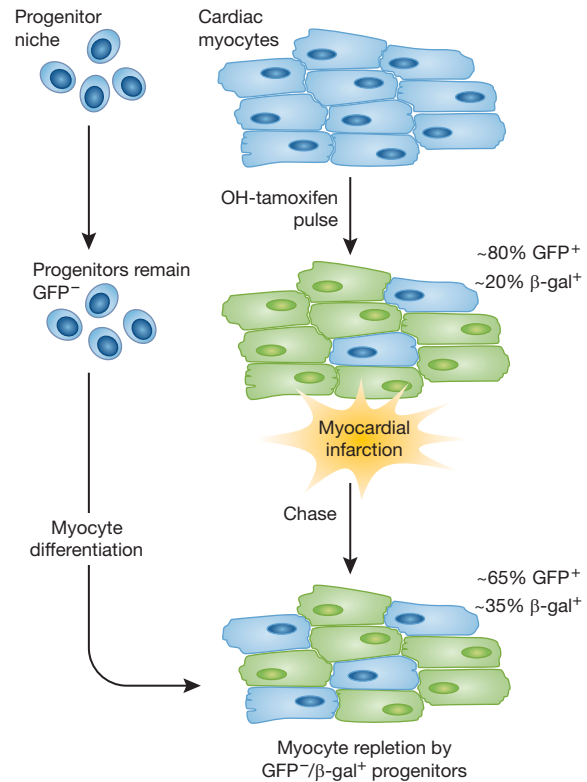
Figure 2. Lineage-mapping in the adult heart. Left: Theoretical progenitor lineage-mapping is depicted. Progenitors would be selectively marked by fluorescent protein expression. After injury, the appearance of fluorescently labelled cardiac myocytes would support the concept that these progenitors were contributing to new myocyte formation. Right: Differentiated cell (cardiac myocyte) lineage-mapping. Upon treatment of the MerCreMer-ZEG mouse with OH-tamoxifen, approximately 80% of the cardiac myocytes undergo a permanent switch from β-galactosidase to GFP expression. The dilution of the GFP$^+$ cardiac myocyte pool after injury is consistent with repletion by β-gal$^+$ progenitors.

expression of a developmentally important gene (isl-1(Laugwitz et al, 2005)), specific cell surface receptor profile (c-kit (Beltrami et al, 2003) or sca-1 (Oh et al, 2003)), the physiologic capacity to actively exclude Hoechst dye (so-called side population cells (Martin et al, 2004)) or based on the outgrowth of typical spherical colonies in tissue culture (Messina et al, 2004; Smith et al, 2007) (Fig 3). In general, the label of 'cardiac stem cell' results from the observation of self-propagation and cardiac myocyte transdifferentiation when exposed to cardiogenic conditions *in vitro* or when delivered *in vivo* after injury.

It seems unlikely that an organ with such limited regenerative capacity would harbour so many biologically distinct progenitors (Laflamme & Murry, 2011; Mercola et al, 2011). Ultimately, the field will benefit from careful *in vivo* lineage tracing studies—without *ex vivo* culture steps—to study if and how a given cell type contributes to cardiac myocyte replenishment during either normal homeostasis or after injury (Fig 2). The lack of such publications to date owes in part to the lack of specificity of many stem cell markers, either because they are

also expressed by non-cardiovascular cells/progenitors or because they may be part of the stress-induced reactivation of 'foetal gene programmes', a phenomenon that is well documented in adult cardiac myocytes (Bisping et al, 2006; Kolodziejczyk et al, 1999; Saadane et al, 1999).

Moving towards a regenerative therapy

The plasticity of the mammalian heart and the identification of cell preparations that demonstrate potency for cardiac differentiation has sparked efforts to develop regenerative therapeutic strategies. The therapeutic challenge is considerable: a typical large myocardial infarction that leads to heart failure will kill around 1 billion cardiac myocytes (Laflamme & Murry, 2005), roughly a quarter of the heart's myocytes (Fig 4). A possible therapeutic approach would coax an endogenous stem cell population or an exogenously delivered cell-based therapy to replace lost cardiac myocytes in a coordinated fashion with
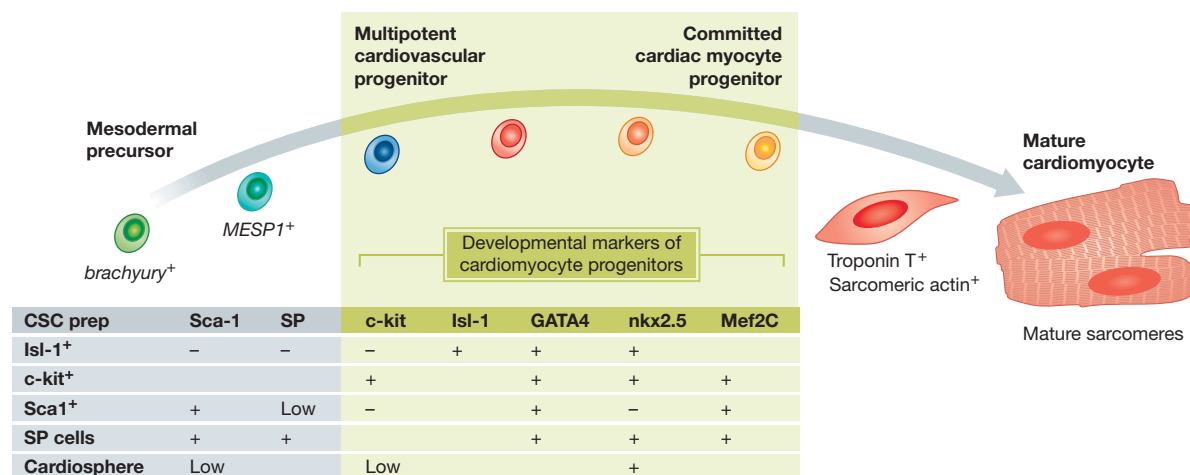
Figure 3. Possible recapitulation of developmental paradigms by endogenous post-natal cardiac stem cells. Between mesodermal development and the emergence of cardiac myocytes, cardiovascular progenitors express a number of markers that have also been detected in the various post-natal cardiac stem cell (CSC) preparations. Expression as measured by messenger RNA (mRNA) or protein expression is denoted with (+). Absent expression is denoted by (−). Blank = untested.

long-term functional integration. Amongst the myriad of potential cell-based therapies, no clear winning strategy has so far emerged (Segers & Lee, 2008).

Bone marrow derived progenitors

A series of nearly simultaneous landmark studies sparked excitement about a possible adult cardiogenic progenitor originating outside of the heart. A post-mortem examination of male heart transplant patients who had received female donor hearts found that approximately 10% of α-sarcomeric actin-positive cardiac myocytes had Y-chromosomes. These cells obviously came from the male patient and from a circulating progenitor outside of the heart graft (Quaini et al, 2002). Two

other contemporaneous studies suggested that the source of circulating progenitors could be the bone marrow. In both cases, a bone marrow cell population with a higher density of the cell surface receptor c-kit, showed repopulation of murine cardiac myocytes after experimental myocardial infarction (Jackson et al, 2001; Orlic et al, 2001). Since these initial reports, a number of studies failed to demonstrate similar rates of chimerism in transplanted hearts (Deb et al, 2003; Hocht-Zeisberg et al, 2004; Laflamme et al, 2002; Muller et al, 2002) or potency of bone marrow stem cells (Balsam et al, 2004; Murry et al, 2004) leading some to postulate that cardiac myocyte transdifferentiation was overestimated owing to an artefact (Laflamme & Murry, 2005).

Nonetheless, some therapeutic effect was observed even in studies with no detectable transdifferentiation (Balsam et al, 2004). Cell-based therapy using autologous bone marrow
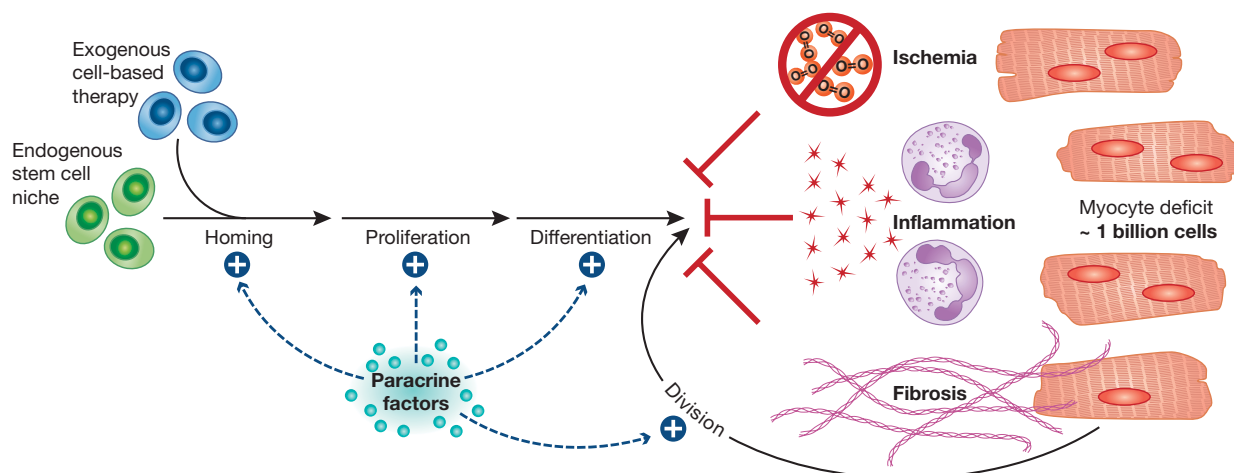

Figure 4. The challenge of regenerating the heart. Both exogenously delivered cell therapies and progenitors in the endogenous niche encounter a similar hostile environment after myocardial injury, often including inadequate blood supply (ischemia), inflammation and fibrosis/scarring. Regenerative pathways may be activated by as yet unknown paracrine pathways, responsible for recruiting progenitors from the niche, stimulating proliferation and coaxing differentiation.

progenitors was rapidly translated into the clinic to treat human ischemic heart disease. A number of randomized trials, using bone marrow mononuclear cells have been performed and most studies demonstrated modest cell therapy-mediated improvements in ventricular function. In the largest study to date, the reinfusion of enriched progenitor cells and infarct remodeling in AMI (REPAIR-AMI) study (Schachinger et al, 2006), 204 randomized patients with acute myocardial infarctions received intracoronary delivery of bone marrow cells or vehicle control. Follow-up after 1 year demonstrated significant improvements in both cardiac function and in a combined clinical endpoint consisting of death, recurrent myocardial infarct or any revascularization procedure. In contrast, analysis of 18-month follow-up data from the similarly conducted bone marrow transfer to enhance ST-elevation infarct regeneration (BOOST) study (Meyer et al, 2006), suggested a waning of early improvements in ventricular function. However, it is worth noting that although improvements in ventricular function have been modest, no pattern of adverse events has emerged from these trials.

Bone marrow-derived mesenchymal stem cells have also been the subject of clinical translation research. These cells are generated from bone marrow samples that are serially passaged and cultured on plastic. Though traditionally defined by their capacity for trilineage differentiation into osteoblasts, chondrocytes and adipocytes, mesenchymal stem cells have also demonstrated evidence for cardiac myocyte differentiation both *in vitro* in some culture conditions and when delivered after myocardial infarction to mice (Toma et al, 2002). Given their reported tendency to evade rejection owing to 'immune privilege', these cells have been administered to humans as an allogeneic treatment after myocardial infarct (Hare et al, 2009). The initial clinical experience is consistent with trials using bone marrow mononuclear cells: patients experience modest improvements in ventricular function with subtle improvements in heart failure symptoms. Overall, the experience with cell-based therapies in humans does not support widespread adoption at this time given their modest benefits, insufficient safety data and the anticipated quality control costs of bringing these therapies to the bedside.

Endogenous cardiac progenitors

Since the initial suggestion that the mammalian heart may contain an endogenous population of progenitor cells (Beltrami et al, 2003), there has been an intense discussion about the therapeutic potential of these cells. There are a number of theoretical advantages to this approach. Autologous transfer may be feasible, even if an *ex vivo* expansion stage would likely be necessary given the small number of stem cells in heart biopsy specimens. Furthermore, other than cells isolated from non-cardiac tissue beds, progenitors derived from the heart itself are theoretically partially pre-programmed to differentiate into heart cells. However, some reports actually question the intrinsic stem-like properties of some of the endogenous adult progenitor preparations, including the 'stemness' of adult cardiac c-kit+ cells (Tallini et al, 2009)

and cardiosphere-derived cells (Andersen et al, 2009), both of which are being used in clinical trials. It is possible that ongoing trials with these putative endogenous cardiac progenitors will demonstrate benefits to cardiac function, but this may not necessarily indicate differentiation into cardiac myocytes.

Pluripotent stem cells

Embryonic stem (ES) cells represent the prototypical stem cells with the hallmarks of clonogenicity, self-renewal and pluripotency. The potency of these cells also represents a real safety concern, given their tendency to form teratomas (Nussbaum et al, 2007), even several years after therapeutic delivery (Amariglio et al, 2009). One approach to overcoming this prohibitive safety problem has been to generate pluripotent-derived progenitors that have already committed to a cardiogenic pathway. As a proof-of-principle example of such a strategy, cells with an expression profile of Oct4, stage-specific embryonic antigen 1 (SSEA-1) and MESP1 demonstrated some regenerative potency when delivered therapeutically in a primate infarct model, without detectable teratoma formation (Blin et al, in press). One could envision a similar strategy using cardiogenic intermediates that express any of the previously mentioned transcription factors associated with cardiac progenitors or cell surface markers such as the receptor for vascular endothelial growth factor (Flk1/KDR) (Yang et al, 2008). Yet, such a strategy should still demonstrate both substantial preclinical efficacy without tumorigenicity before human translation. If such criteria are met, ES-derived therapies have the potential of providing 'off-the-shelf' cardiac myocytes to treat acute myocardial infarctions or chronic heart failure.

A second approach, which may also obviate the risk of teratomas, is to generate a pure population of ES-derived cardiac myocytes for therapeutic delivery either as a cell suspension or after *ex vivo* tissue engineering. There has already been enormous progress during the past decade in defining the factors and transcription signals to differentiate cardiac myocytes from ES-cells. As discussed in greater detail (Noseda et al, 2011), cardiac myocyte development is dictated by the time and dose-dependent exposure to a series of growth factors from the wingless-type MMTV integration site (Wnt), fibroblast growth factor (FGF), bone morphogenetic protein (BMP) and nodal families. Guided by these mechanistic insights, several laboratories have successfully generated ES-derived preparations with more than 50% of functional cardiac myocytes (Laflamme et al, 2007; Yang et al, 2008). Because of the inherent technical challenge of delivering a sufficient number of differentiated cardiac myocytes — not to mention ensuring their survival and electromechanical integration — the most realistic future for such technical advances may be as an unlimited source of cardiac myocytes for engineering myocardial grafts.

The generation of induced pluripotent stem (iPS) cells may overcome two important limitations of ES cells: ethical concerns about harvesting ES cells from embryos and graft rejection by the patient's immune system, because iPS cells can be custom-engineered from a patient's stromal cells for autologous transplantation. A recent demonstration of heightened

immunogenecity in syngeneically transplanted iPS cells, however, suggests that the immune system cannot yet be discounted in the development of iPS-based therapies (Zhao et al, 2011).

The initial protocols for iPS cell generation involved retroviral-mediated expression of four stem-cell genes (Takahashi & Yamanaka, 2006; Yu et al, 2007). As virally reprogrammed cells may harbour an associated risk of neoplastic conversion, alternative reprogramming strategies, such as the use of small molecules (Shi et al, 2008) or non-viral gene modifying approaches (Warren et al, 2010) will probably be a necessary component of any future therapeutic strategies. However, the most important lesson from these landmark studies may be the remarkable plasticity of even the most terminally differentiated cells when exposed to the right combination of signals.

Tissue engineering

With stem cells as a potential unlimited source of cardiac myocytes, engineering *ex vivo* myocardial grafts could, in theory, provide another therapeutic approach. The most clinically advanced tissue engineering to date involves thin, simple, relatively avascular tissues, such as tracheas (Macchiarini et al, 2008). Owing to the complexity and physiological requirements of functional myocardial tissue, an abundant source of cardiac myocytes alone is not enough to build a clinically useful graft. Historically, the greatest challenge in tissue engineering has been an adequate supply of oxygen and nutrients for metabolically active tissues such as the heart. One approach has been to engineer thin cardiac sheets, which can then be stacked for *in vivo* delivery (Shimizu et al, 2002). Although these layered sheets demonstrate some degree of electromechanical coordination and neovascularization *in vivo*, it is not clear yet if such an approach can be optimized to yield full-thickness myocardium with an adequate blood supply. The addition of non-myocyte cellular components, such as fibroblasts and endothelial cells, leads to the formation of primitive vascular structures within engineered grafts, but the electromechanical properties are not sufficient for normal functionality (Stevens et al, 2009). Ultimately, success in the engineering of functional, full-thickness myocardium will likely only be possible if the problem of vascularization is solved (Zimmermann et al, 2006).

Circumventing cell-based therapy with pharmacoregeneration?

It is hard to imagine that a single pharmacologic intervention could lead to complete cardiac repair, but a number of recent discoveries lend credence to the underlying concept that paracrine signals may activate repair mechanisms in the heart. In general, the development of small molecule or protein-based therapies raises relatively modest problems with quality control compared to cell-based therapies. Therefore, pharmacoregenerative approaches may be a more practical near-term approach to promote cardiac repair.

Perhaps the most stunning proof-of-principle approach to generating new cardiac myocytes from endogenous cells is the recent transformation of resident cardiac fibroblasts into cardiac myocytes (Ieda et al, 2010). Similar to protocols for generating iPS cells, the forced expression in cardiac fibroblasts of three genes—GATA4, MEF2C and TBX5—resulted in the expression of the cardiac myocyte-specific myosin heavy chain 6 (Myh6) promoter in approximately 20% of the cells and spontaneous beating in approximately 1% of reprogrammed cells. When isolated cardiac fibroblasts were infected with viruses carrying the genes encoding the three reprogramming factors and reintroduced to the heart within 24 h, some transformed directly into cardiac myocytes. Although this approach is still in its infancy, it suggests the exciting possibility that the fibroblast rich post-infarct scar could be transformed into functional myocardium via reprogramming.

A more realistic short-term goal may be to exploit paracrine signalling to amplify the existent endogenous regenerative response. Recent cell transplantation experiments conducted in our laboratory, using an inducible cre-based genetic lineage mapping approach, tested the hypothesis that cell-based therapies might exert proregenerative effects via a paracrine mechanism (Loffredo et al, 2011) (Fig 5). Consistent with some prior studies (Balsam et al, 2004; Murry et al, 2004), we found no evidence for transdifferentiation of exogenously delivered bone marrow cells into cardiac myocytes. However, we did find increased generation of cardiac myocytes from endogenous progenitors in mice, which were administered c-kit+ bone marrow cells but not mesenchymal stem cells. This finding suggests paracrine signalling between exogenously delivered cells and endogenous resident progenitors. It provides a rationale for therapeutic interventions aimed at activating progenitors or recruiting them from their niche to the injury site (Steinhauser & Lee, 2009). One such approach involves the administration of the peptide thymosin β4, which appears to induce WT-1-expressing epicardial cells to differentiate into *de novo* cardiac myocytes (Smart et al, 2011). Interestingly, the WT-1+ cells do not appear to differentiate into cardiac myocytes in the adult mouse without being primed by thymosin β4, which suggests the involvement of a reprogramming mechanism.

An alternative to myocyte generation by pharmacologic means involves stimulating cell-cycle reentry of preexisting cardiac myocytes. Inhibition of p38 MAP kinase increases mitotic activity in cardiac myocytes (Engel et al, 2005), although concomitant growth factor therapy (FGF1) was necessary to achieve a noticeable improvement in cardiac function (Engel et al, 2006). The administration of the growth factor neuregulin-1 stimulates mature cardiac myocyte division *in vitro*, which translated into a modest increase in the division of the mononucleated cardiac myocytes *in vivo* after systemic delivery of neuregulin (Bersell et al, 2009). Similar stimulation of cardiac myocyte mitosis was observed after prolonged local delivery of periostin (Kuhn et al, 2007), an extracellular matrix signalling protein; however, the effect was not replicated in a transgenic model of periostin overexpression (Lorts et al, 2009).

Although the regenerative cardiology field has primarily focused on progenitor cells, these data suggest that controlling
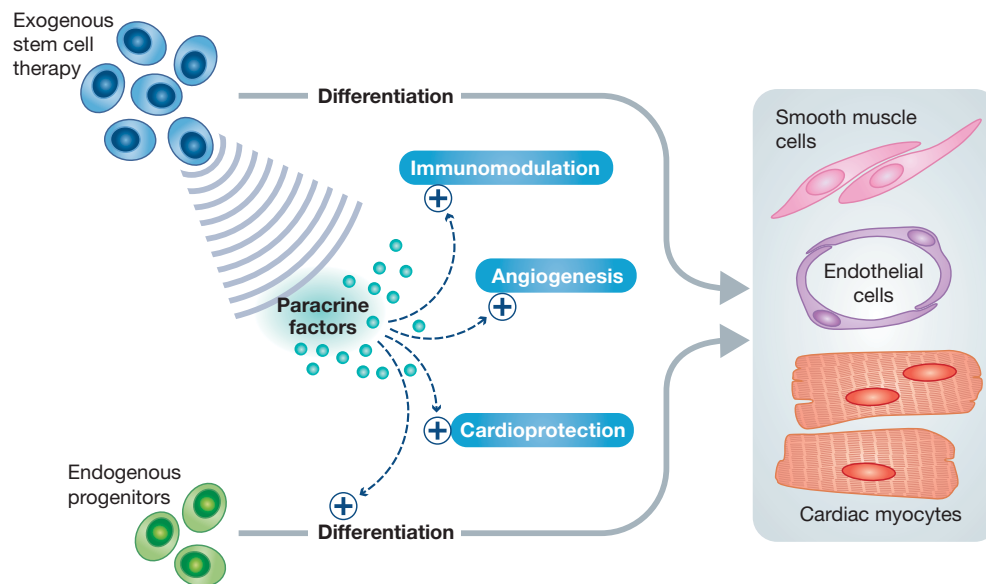
Figure 5. Proposed mechanisms of action for cell-based therapies. In theory, exogenously delivered cells may directly differentiate into endothelial cells, smooth muscle cells and cardiac myocytes. They may also release paracrine factors which may result in non-regenerative effects, such as immunomodulation, angiogenesis or cardioprotection. Recent work from our laboratory suggests that a dominant mechanism achieved with bone marrow progenitor therapy may be via the activation of endogenous progenitor recruitment (Loffredo et al, 2011).

the mitotic activity of mononucleated cardiac myocytes may provide an alternative approach to replenishing cardiac myocytes. A major concern with systemic growth factor therapy, however, is the potential for mitogenic effects that may impact other organs. Thus, the future of pharmacologic regeneration may lie in the local delivery of engineered proteins and small molecules that target specific survival, growth and differentiation pathways.

Pending issues

Dissect the mechanistic differences between adult mammals with limited regenerative capacity and organisms, such as neonatal mice, zebrafish and newts, that demonstrate unambiguous cardiac myocyte regeneration. Understanding these differences may reveal new pathways that can be therapeutically targeted to achieve more robust regeneration.

Complete molecular and functional characterization of endogenous cardiac myocyte progenitors. Multiple laboratories have isolated progenitors from the heart with different molecular characteristics. What are the *in vivo* functional roles of these progenitors? Do the observed molecular differences between these isolated cells represent functionally distinct cell types?

Identify paracrine signalling pathways responsible for activation and recruitment of endogenous cardiac myocyte progenitors. This may facilitate a pharmacoregenerative therapy, in which treatment with a protein or small molecule would hold the promise of amplifying endogenous regeneration.

Refine reprogramming strategies to more efficiently produce mature cardiac myocytes, both *in vitro* and *in vivo*. The ultimate bioengineering goal is to produce a pure population of mature, fully functional cardiac myocytes for *ex vivo* tissue engineering (or) to control the proliferation and differentiation of endogenous cell populations or exogenously delivered cell therapies such that scar tissue is replaced by myocardium. These different strategies are unified by an underlying requirement to understand the fundamental pathways involved in cardiac myocyte differentiation and maturation.

Future directions

In this review, we have described the current status of research on cardiac regeneration, highlighting important recent discoveries and ongoing controversies. The initial hope that a cell progenitor would emerge with the capacity to regenerate the injured mammalian heart in the same manner that bone marrow may be reconstituted has not been realized in animal studies or randomized clinical studies. Even if some studies have questioned the regenerative potency of various endogenous progenitor populations, it is clearly feasible to reprogramme many cell types to differentiate into cardiomyocytes. Interestingly, evolving notions of cellular plasticity may ultimately help to explain the frustration that many in the field have experienced when attempting to replicate the findings of landmark studies or when trying to demonstrate progenitor activity *in vivo*. It may well turn out that *ex vivo* progenitor cultures undergo epigenetic changes that affect cell fate, and that such modifications could account for some of the stem-like properties attributed to endogenous progenitors.

There is reason for optimism for a regenerative medicine approach to heart failure, given the intense research efforts and the capacity of higher organisms, including the neonatal mouse, to regenerate myocardium. Perhaps the most important issue in this field is identifying which findings are consistently supported by multiple experimental approaches. Ultimately, the findings that are easily reproduced by most or all laboratories will most likely benefit patients.

The authors declare that they have no conflict of interest.

For more information

ISSCR Patient Handbook on Stem Cell Therapies
http://www.isscr.org/clinical_trans/pdfs/ISSCRPatientHandbook.pdf

Understanding Stem Cells: Overview from National Academy of Science
http://www.dels.nas.edu/bls/stemcells/

National Institutes of Health Stem Cell Information
http://stemcells.nih.gov/

Stem Cell Research and Applications
http://www.aaas.org/spp/sfrl/projects/stem/main.htm

Stem Cell Information
http://www.tellmeaboutstemcells.org/

Stem Cell Resources
http://www.stemcellresources.org/

Stem Cell Therapies
http://www.closerlookatstemcells.org/

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