From fish to amphibians to mammals: in search of novel strategies to optimize cardiac regeneration

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Different vertebrate species have different cardiac regeneration rates: high in teleost fish, moderate in urodele amphibians, and almost negligible in mammals. Regeneration may occur through stem and progenitor cell differentiation or via dedifferentiation with residual cardiomyocytes reentering the cell cycle. In this review, we will examine the ability of zebrafish and newts to respond to cardiac damage with de novo cardiogenesis, whereas rodents and humans respond with a marked fibrogenic response and virtually no cardiomyocyte regeneration. Concerted strategies are needed to overcome this evolutionarily imposed barrier and optimize cardiac regeneration in mammals.

In the mammalian heart, a variety of injuries, ranging from ischemia to inflammatory diseases or maladaptive responses, can lead to massive or sporadic loss of myocardial cells. After an infarction, cardiomyocyte death triggers a series of molecular and cellular events that culminate in an inflammatory response, fibroblast accumulation, the production of extracellular matrix, and scarring. In humans, fibrous scar tissue may cause severe contractile dysfunction, even to the point of heart failure, and conventional pharmacological treatments are frequently inadequate. Regenerating the heart has thus become a major challenge, and its potential impact in experimental medicine justifies the growing number of strategies and approaches currently being investigated to achieve therapeutically significant cardiac regeneration.

It is generally agreed that soon after birth, mammalian cardiomyocytes stop dividing and make a transition from hyperplastic to hypertrophic growth. Cell cycle activity is low in adult cardiomyocytes, and their inability to reactivate mitosis may explain why tumors of the myocardium are very rare, and why immortalized cardiomyocyte lines have never been obtained spontaneously. Although regenerating the heart by stimulating the reactivation of cardiomyocyte proliferation remains an attractive prospect, it is probably not enough to bring about a complete functional recovery. Instead, a wide variety of exogenous stem cells have been considered as possible cardiac regeneration aids, both in experimental models and in human trials. Skeletal myoblasts, bone marrow–derived hematopoietic and mesenchymal stem cells, and circulating endothelial progenitor cells all have been tested, but the results have generally been disappointing (for review see Dimmeler et al., 2008). Transient improvements in heart function have been reported; however, it is now clear that this is caused by paracrine mechanisms acting on host tissue, not by transdifferentiation of exogenous cells into cardiomyocytes (Dimmeler et al., 2008). Support for the ambitious goal of cardiac regeneration has come from the recent finding that the adult mammalian heart contains cardiogenic stem and progenitor cells (Beltrami et al., 2003; Oh et al., 2003; Messina et al., 2004; Martin et al., 2004). Why these cells cannot arrest or reverse progressive myocardial loss during disease remains to be explained.

In this review, we first analyze the ability of some vertebrates, specifically zebrafish and salamanders, to efficiently regenerate damaged cardiac tissue. We then focus on the mammalian heart, which responds to cardiac tissue damage not by regeneration but by scarring. In particular, we reconsider the biology of two key cardiac cells in close structural partnership, the cardiomyocyte and the interstitial fibroblast, with a view to identifying the barriers to efficient cardiac regeneration in mammals. In this context, we also reexamine and critically evaluate the role of mammalian cardiac stem and progenitor cells.

The unquestionable regeneration potential of zebrafish and salamander hearts

Unlike wound healing, which is common to all animals, the capacity for regeneration varies between and within species and phyla. Species with the potential to regenerate the heart essentially use two strategies; progenitor cell proliferation, or dedifferentiation and subsequent division of the cells surrounding the injury (here and elsewhere we use the term "dedifferentiation" to mean the condition whereby a cell regresses from a fully differentiated form into a simpler state, an event coupled with cell cycle reentry). Heart regeneration is remarkably efficient in adult zebrafish, as demonstrated by experiments involving resection of the ventricular apex (Poss et al., 2002). New

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Abbreviation used in this paper: EPDC, epicardium-derived cell.

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cardiomyocytes originate from progenitor cells that express epicardial markers, such as raldh2 and Tbx18 (Poss, 2007). Zebrafish cardiac progenitor cells are successfully driven to regenerate by interaction with the epicardium, the thin epithelial layer enveloping the chambers. The epicardium is not simply a bystander to myocardial regeneration after injury; rather, it exhibits a rapid and robust proliferation. Cells close to the resection site invade the regenerating tissue through a process strongly reminiscent of the epithelial-to-mesenchymal transition occurring in the developing heart, contributing endothelial and smooth muscle cells for the new vessels (Lepilina et al., 2006). The epicardium also regulates the addition of new myocardial and epicardial cells during homeostatic cardiac growth (Wills et al., 2008), but it remains unclear whether newly formed cardiomyocytes originate from this source or from progenitor cells after epicardial-mediated activation.

Epicardial and myocardial cross-talk is mediated by FGF signaling, and inhibiting the FGF receptor blocks cardiac regeneration (Lepilina et al., 2006). The same signaling pathway guides cardiac progenitor cells in regulating heart size, atrial-to-ventricular proportions, and ventricular cardiomyocyte numbers at later stages of development (Marques et al., 2008). Other signals may cooperate with FGF to induce myocardial proliferation, as suggested by the up-regulation of growth factors (PDGF, insulin-like growth factor, and Delta-Notch) during regeneration (Poss, 2007).

In adult salamanders and newts (*Notophthalmus viridescens*), larval axolotl (*Amblystoma mexicanum*), and teleost fish (*Danio rerio*, zebrafish), regeneration after heart apex amputation involves blastema formation; i.e., the accumulation of dedifferentiated cells near the edge of the lesion. These blastema cells originate either from the dedifferentiation of resident cardiomyocytes (salamanders) or from resident progenitor cells (zebrafish). In the newt, dividing cardiomyocytes partially disassemble sarcomeric structures (Tate and Oberpriller, 1989) and revert to cells that can renew and also differentiate into other cell types on appropriate inductive signals (Laube et al., 2006). After injury, 75% of cultured cardiomyocytes synthesize DNA and 60% progress to karyokinesis; about half of the latter divide, whereas the other half generate multinucleated cells (Bettencourt-Dias et al., 2003), which indicates a heterogeneity in relation to the regulation of cell division. Dedifferentiation of preexisting cardiomyocytes has also been postulated for the zebrafish heart, but it has not been supported by experimental evidence (Lepilina et al., 2006).

The questionable regeneration potential of the mammalian heart

At variance with zebrafish and salamanders, the uninjured mammalian myocardium is traditionally considered incapable of self-renewal. Using a sophisticated and very elegant inducible transgenic mouse model, Hsieh et al. (2007) confirmed this hypothesis. They also showed, however, that the adult heart achieves a modest, but nonetheless convincing, self-renewal after injury that was attributed to a pool of resident stem cells. Over the past few years, several reports have described putative cardiac stem and progenitor cells in the adult heart. We give a brief account here of the major contributions in this field, referring the reader to several recent papers for a thorough overview of the topic (Passier et al., 2008; Segers and Lee, 2008; Wu et al., 2008).

Three distinct cardiac stem cell populations have been described in the adult myocardium, based on the expression of the surface markers c-kit (tyrosine kinase receptor-1), Sca-1, and the ATP-binding cassette transporter (also called the "side population" or SP because these cells pump out Hoechst stain; Beltrami et al., 2003; Oh et al., 2003; Messina et al., 2004; Martin et al., 2004). The best characterized of these cells are those expressing c-kit. Beltrami et al. (2003) cloned and expanded these cells from different species, and obtained significant cardiac regeneration (Beltrami et al., 2003), angiogenesis, and arteriogenesis (Tillmanns et al., 2008) after their injection into the infarcted myocardium. Despite these findings, the ability of c-kit$^+$ cells to activate and support spontaneous regeneration in the adult heart remains highly controversial. All studies are based on the transplantation of very large numbers of c-kit$^+$ cells into the injured heart, so a cell lineage approach is required to address this issue conclusively. Two other major concerns about these cells are their precise localization and number. Using genetically engineered mice expressing GFP under the c-kit promoter, Fransioli et al. (2008) demonstrated that c-kit$^+$ cardiac cells decline markedly in the first two postnatal weeks and almost disappear after ten weeks.

Numerous efforts have been put forward in the last few years to investigate the nature of progenitor cells responsible for heart development because it is well established that the multiple programs controlling the onset of cardiogenesis are recapitulated in heart disease. Fate-mapping studies have demonstrated that the developing heart contains Isl1$^+$/Nkx2.5$^+$/Flk-1$^+$ multipotent mesodermal progenitors that can give rise to cardiac muscle, smooth muscle, and endothelial lineages (Fig. 1; Moretti et al., 2006; Kattman et al., 2006; Wu et al., 2006). Isl1-marked cells have been identified in the secondary heart field (Cai et al., 2003; Laugwitz et al., 2005), the embryological anlagen from which the atria, outflow tract, and most of the right ventricle develop (Kelly et al., 2001). However, a recent study suggests that these cells contribute to cardiac, smooth muscle, and endothelial cells in all four cardiac chambers (Ma et al., 2008; for review see Laugwitz et al., 2008). Additionally, a bipotent Nkx2.5$^+$/c-kit$^+$ progenitor isolated from the developing mouse embryo has been shown to generate cardiac and smooth muscle cells in vitro and in vivo (Wu et al., 2006).

A second potential source of stem cells in the developing heart is the epicardium (Fig. 1). Investigations on the fate and differentiation of epicardium-derived cells (EPDCs) indicate that they generate smooth muscle cells and fibroblasts of the coronary vessels, and the interstitial fibrous skeleton of the heart (Winter and Gittenberger-de Groot., 2007). In avians, the origin of endothelial cells in the coronary vessels is still being debated (Pérez-Pomares et al., 2002), as is the myocardial potential of EPDCs (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Gittenberger-de Groot et al., 1998). In the developing mouse heart, recent data have shown that epicardial progenitors expressing Tbx18 (Cai et al., 2008) and Wt1 (Zhou et al., 2008), possibly derived from a common Isl1$^+$/Nkx2.5$^+$ ancestor (Ma et al., 2008), generate new cardiomyocytes.

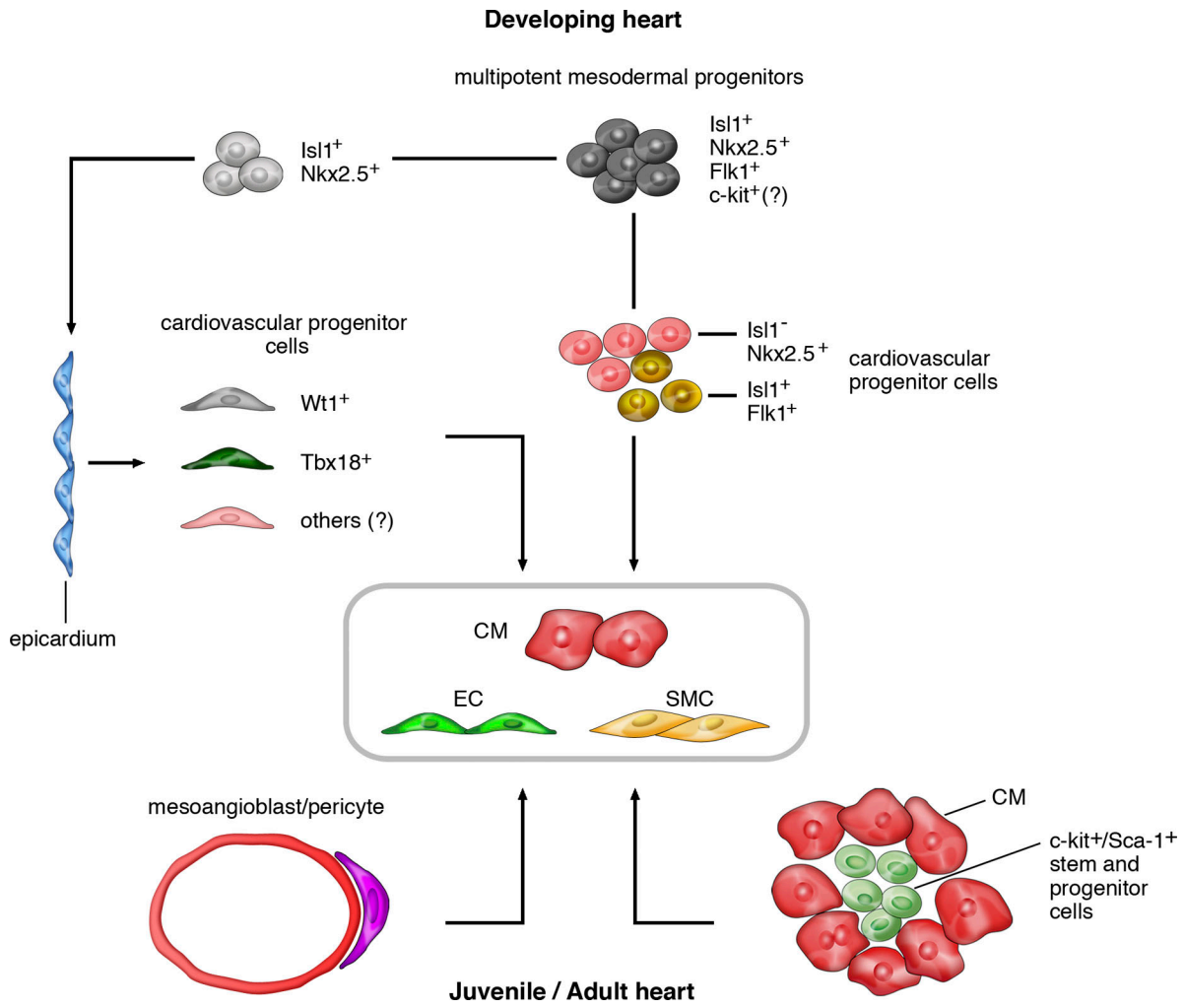

Figure 1. **Schematic representation of stem and progenitor cells in the developing and juvenile/adult rodent heart.** CM, cardiomyocytes; EC, endothelial cells; SMC, smooth muscle cells.

It would be tempting to hypothesize that the essential properties of the epicardium can be recapitulated in the adult heart and used for regeneration. Epicardial c-kit$^+$ multipotent cells expressing Nkx2.5 and GATA4 have been identified in the human and mouse adult heart as a minority of all the epicardial cells. Keeping the pericardial sac intact during infarction by ligating the coronary artery prevents myocardial tissue from deteriorating and results in foci of cardiac regeneration, as demonstrated by the presence of a small population of lentivirus-labeled epicardial cells expressing the cardiac marker α-sarcomeric actin (Limana et al., 2007). Other reports indicate that EPDCs do not acquire a cardiomyocyte phenotype when transplanted into infarcted mouse heart (Winter et al., 2007). To summarize, it seems reasonable to suggest that the mammalian epicardium is composed of heterogeneous populations of cardiogenic progenitors with distinct potentials for differentiation.

As in the zebrafish, the epicardium has a crucial stimulatory role essential for proper development, and possibly also for regeneration. One of the factors that influences this instructive role is thymosine β4 (Tβ4), a G-actin monomer-binding protein implicated in cytoskeleton reorganization. Tβ4 secreted from the developing myocardium stimulates the proliferation, differentiation, and inward migration of epicardial cells (Smart et al., 2007). Similarly, Tβ4 is responsible for enhanced cardiomyocyte survival, and consequently for cardioprotection (Bock-Marquette et al., 2004; Smart et al., 2007), in the event of injury.

A third, and potentially more useful, source of cardiac stem and progenitor cells may be the vasculature. Vessel-associated progenitor cells, called mesoangioblasts, have recently been identified in the juvenile mouse heart (Fig. 1). These cells express endothelial and pericyte markers, and are committed to cardiac differentiation in vitro and in vivo (Galvez et al., 2008). Because they retain a remarkable potential for cardiac differentiation when implanted in the secondary heart field of a chick embryo, it is reasonable to postulate that they are recruited to become cardiomyocytes during postnatal growth. It is worth noting that cardiac progenitor cells, which are identifiable from their ability to grow as cardiospheres, have been obtained using similar technical procedures from both adult mouse heart explants (Messina et al., 2004) and human endomyocardial ventricular biopsies (Smith et al., 2007). In the developing heart, there may be a cell network that, for regeneration purposes, connects the epicardium to the myocardium via the coronary vessels, as shown in Fig. 2. Further

investigations are needed to establish whether such a network persists in postnatal life.

In conclusion, the mammalian heart contains a plethora of potential stem and progenitor cells, but their persistence in adulthood is still unclear. Isl1$^+$ cells progressively disappear in the postnatal heart except where the cardiac autonomic nervous system intersects with the cardiac conduction system (Laugwitz et al., 2008). Vessel-associated progenitor cells, in contrast, persist in the postnatal heart, but their number and potential to regenerate over time has yet to be determined. Cardiac c-kit+ stem cells are found in adult myocardium, but it is unclear whether they are a remnant of development or are recruited postnatally. In addition, it is possible that some of the many progenitor cells found in the developing heart have simply not yet been identified in the postnatal heart.

The fundamental questions in the field are as follows. What are the restrictions to cardiac regeneration and are they species-specific? Can these evolutionarily imposed restrictions be removed? In answer to these questions, two barriers to regeneration in the mammalian heart have been identified: one involving the capacity of cardiomyocytes to proliferate, the other lying in the interactions between cardiomyocytes and interstitial cells.

The intrinsic resistance of mammalian cardiomyocytes to division

Mammalian cardiomyocytes undergo cell cycle withdrawal during late gestation. A final round of incomplete division occurs after birth and results in binucleated or multinucleated cardiomyocytes. Cardiomyocytes can reactivate the cell cycle after various pathological stimuli such as a hypertrophic response (Pasumarthi and Field, 2002; Rubart and Field, 2006; van Amerongen and Engel, 2008). This reactivation is transient and does not always progress to mitosis. Therefore, the histological detection of cell cycle and DNA synthesis markers, such as Ki67, histone H3 phosphorylation, and BrdU incorporation, does not necessarily mean cell division. Only karyokinesis and cytokinesis can be accepted as definitive proof of cardiomyocyte proliferation.

Using a combination of in vitro and in vivo models, it has been demonstrated that the myocardial cell cycle can be reprogrammed, at least to some degree. The majority of proteins that affect the cell cycle activity of cardiomyocytes are involved in transit through the restriction point. CDK4 and CDK6, and their activating partners D-type cyclins (Pasumarthi et al., 2005), the phosphorylation state of the retinoblastoma pocket protein (MacLellan et al., 2005), and the subsequent activation of the transcription factor E2F, have been manipulated to induce cardiac cell division. Striking evidence of the functional impact of reactivating cardiomyocyte proliferation comes from the demonstration that cardiac-specific overexpression of cyclin D2 improves cardiac function after infarction in mice (Hassink et al., 2008).

Novel signaling pathways that control cardiac cell division have recently been revealed with a view to developing new pharmacological strategies to counteract myocardial

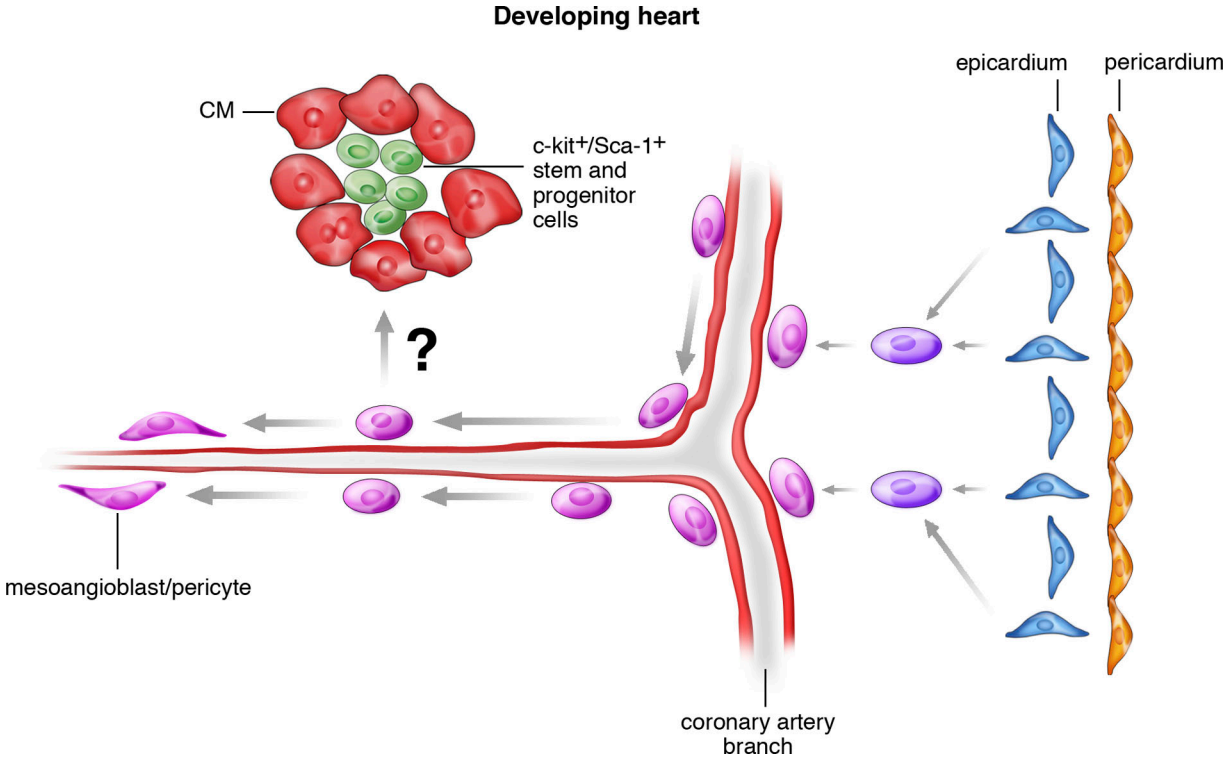

Figure 2. **Schematic representation of the hypothetical link between epicardial progenitor cells and the mesoangioblast/pericyte cell population surrounding the coronary vessels.** A subpopulation of epicardial cells delaminates from the subendocardial space and migrates to the underlying myocardium using the vascular network as a guide. These progenitors may also be able to generate the intramyocardial clusters of c-kit$^+$/Sca-1$^+$ stem and progenitor cells that persist into adulthood, but alternative sources of these cells cannot be excluded. CM, cardiomyocytes.

dysfunction. The extracellular matrix protein periostin reactivates mitosis in mouse cardiomyocytes via β-integrin and phosphoinositide 3-kinase (Kühn et al., 2007), but why high periostin levels in the infarcted myocardium are unable to promote cell cycle activation in the resident cardiomyocytes remains to be explained.

Cardiac cell division is accompanied by structural modifications, resulting mainly in sequential myofibrillar disassembly and reassembly (Ahuja et al., 2004). This complex process might provide a mechanistic explanation as to why postnatal cardiomyocytes stop dividing as the contractile apparatus matures. It may be that dedifferentiation favors cell cycle reentry, as in naturally dividing newt cardiomyocytes (Bettencourt-Dias et al., 2003). Indeed, dedifferentiated cardiomyocytes (characterized by disassembly of the contractile apparatus, the acquisition of fetal markers, and anomalous mitochondria) have been seen in human diseases such as chronic hibernating myocardium or chronic atrial fibrillation, and in the border zone of infarction (Heusch and Schulz, 2000). We have found that adult cardiomyocytes cocultured in the presence of cardiac fibroblasts undergo dedifferentiation and cell cycle reentry (Zaglia et al., 2008). The fine-tuning that enables cell cycle progression and completion after dedifferentiation remains to be elucidated.

Regeneration versus scarring

The differing abilities of zebrafish, salamander, and mammals to generate new cardiomyocytes might also depend on the microenvironment. A comparative discussion on the influence of the microenvironment on cardiac regeneration is complicated because different types of injury have been studied in different species. In mammals, cardiac regeneration has been studied mainly in acute or chronic ischemia, or ischemia/reperfusion induced by coronary artery ligation. In newt and zebrafish, the model used is partial cardiac amputation because zebrafish have only subepicardial vessels that open into lacunes (Hu et al., 2000), and the newt has no coronary vessels at all.

Fibroblast distribution also varies: fibroblasts account for the majority of nonmuscle cells in mammals, but they are rare in zebrafish and newts, and occur randomly interspersed within the subepicardial layer. On the whole, the myocardial histology of these lower vertebrates is quite simple and its 3D structure resembles that of the trabeculated fetal heart of mammals. Tissue organization is more complex in the adult mammalian heart: single cardiomyocytes are in contact with capillaries and interconnected with fibroblasts (Fig. 3). Fibroblasts have an important role in developing the local and global myocardial response to mechanical, electrical, and chemical signals generated by cardiac damage (Baudino et al., 2006).

The modulation of fibroblast activity can impact the balance of regeneration versus healing after cardiac injury. In zebrafish and newt, regeneration is accompanied not only by progenitor cell activation or cell dedifferentiation, but also by the upregulation of genes encoding matrix metalloproteinases (Vinarsky

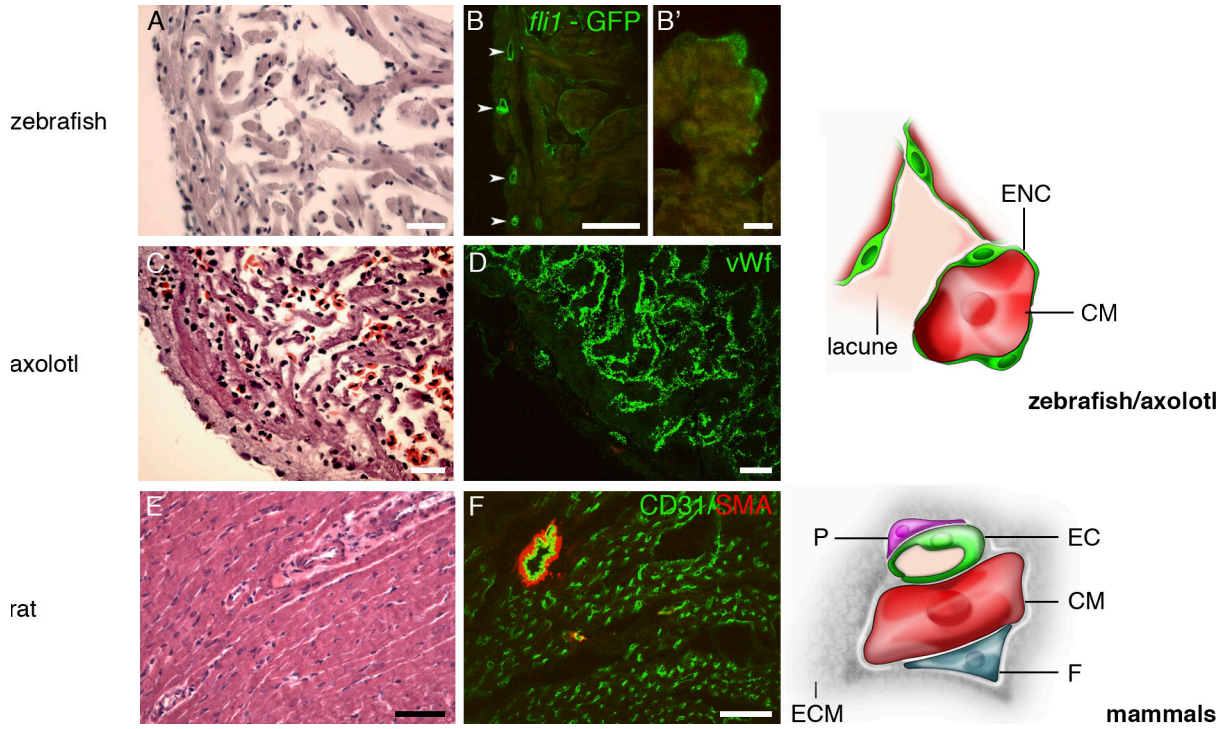

Figure 3. **Myocardial structure in different species.** Hematoxylin and eosin staining shows tissue organization in zebrafish (A), axolotl (C), and rat (E) hearts. Vascular-specific GFP expression in transgenic *fli1* (*friend leukemia integration 1*)-GFP zebrafish (Lawson and Weinstein, 2002) or immunostaining with specific antibodies show the distribution of the vascular network in these species. Zebrafish contain epicardial vessels (B, arrows) and endocardial cells that circumvent the cardiomyocytes (B′). Axolotl have no epicardial vessels, whereas endocardial cells expressing the von Willebrand factor (vWf) surround the cardiomyocytes (D). In the rat, the coronary vessels deepen into the myocardium, as shown by staining with antibodies to CD31 (endothelial marker) and SMA (smooth muscle cell marker). (right) Two schematic representations show the positional relationships between cardiomyocytes (CM) and surrounding endothelial (EC) or endocardial (ENC) cells, fibroblasts (F), and pericytes (P). B, B′, D, and F are confocal images of tissue sections. Bars: (A, C, E, and F) 100 μm; (B and D) 50 μm.

et al., 2005; Lien et al., 2006) and tissue inhibitors of matrix metalloproteinases (Stevenson et al., 2006). In limb reconstruction, these genes are known to control extracellular matrix remodeling and regeneration. After heart injury in zebrafish, fibroblasts and cardiomyocytes are activated in a reciprocal manner. When cardiogenesis is blocked by a dominant-negative FGF receptor or mutation of the *Msp1* mitotic dominant checkpoint kinase, fibrin is retained, and a collagen-rich scar forms (Poss et al., 2002; Poss, 2007). This indicates that regeneration and repair sense each other and that the balance can be tipped toward one or the other, even when evolution has preserved the genetic capacity for regeneration. It also suggests that the extensive, well-organized network of fibroblasts in the mammalian heart is, by itself, an unfavorable condition for regeneration purposes for both cardiac stem cell differentiation and cardiomyocyte division.

A second major player in the damaged myocardium is the immunoinflammatory response. Although this response gives the injured heart the chance to surround the damaged area with granulation tissue and extracellular matrix, thus limiting expansion of the injured area, it can damage the surviving cardiomyocytes and cardiac stem cells. Salamander and zebrafish have innate immunity but incomplete adaptive immunity. It has been postulated from experiments in limb reconstruction that this less sophisticated adaptive immunity may be involved in conferring a marked regenerative potential to these species (Harty et al., 2003; Godwin and Brockes, 2006; Mescher and Neff, 2006). Unfortunately, studies on cardiac regeneration in newt/axolotl and zebrafish have failed to provide a detailed analysis of the inflammatory response. Blocking inflammation would be particularly interesting in zebrafish, for which mutants with an aberrant accumulation or deficiency of some inflammatory cells are available (Mathias et al., 2007).

Finally, the immature immunoinflammatory response and limited number of fibroblasts might also explain the great potential for regeneration of the mammalian heart in its early stages of development (Blewett et al., 1997). If a 14-d-old fetal mouse heart is explanted, surgically damaged, and kept in serum-free medium as an organ culture, its tissue architecture is rapidly reestablished with no inflammatory response or scarring. By 18 or 22 d of gestation, fibroblasts and myofibroblasts have accumulated in the heart, and the trabeculated structure of the ventricle has been replaced by compact myocardium. If the organ is explanted at this point in development, a wound will heal by scarring.

Concluding remarks and future directions

We reasoned that the differing regenerative capabilities of hearts from teleost fish and urodele amphibians compared with mammals stems from the existence of species-specific barriers. In particular, adult mammalian cardiomyocytes have an inherent inability to regenerate, either via stem cell–mediated mechanisms or by reentry of the cell cycle, and, additionally, they show a marked interstitial response. We suggest that the limited regeneration potential of the mammalian heart is caused by an evolutionary prioritization of hemostasis and fibrosis. Bleeding from the heart in a high-pressure circulatory system, which is practically unique to higher vertebrates, can seriously jeopardize survival. Selective pressure probably favored the more rapid fibrous healing response because survival would be compromised in the time it took to seal the defect by regeneration. Small vertebrates have a low-pressure circulatory system and incompletely oxygenated blood. The fetal cardiac microenvironment is characterized by a low oxygen tension in mammals as well, in contrast with the high oxygen-based metabolic demand of the postnatal heart. Because progenitor cell activation requires low oxygen levels in the microenvironment (Simon and Keith, 2008), the mammalian adult heart may be inherently incapable of activating its own resident cardiogenic progenitors after injury.

Cardiomyocyte reentry in the cell cycle. Cardiomyocyte proliferation could provide a mechanism for cardiac regeneration; however, it is unclear whether it is better for cardiomyocytes to revert to proliferating progenitors or to dedifferentiate back to immature cardiomyocytes that should be capable of one or more cell divisions. The former option would enable them to proliferate in abundance and become pluripotent; the latter would have a limited effect, but could prevent tumorigenic outcomes and might also favor cell–cell contact and integration with preexisting tissue.

Evidence from infarcted hearts of MHC-cycD2 transgenic mice demonstrated that cardiomyocyte cell cycle reactivation resulted in newly formed myocardium that participated in a functional syncytium with surviving myocardium from outside of the damaged zone (Hassink et al., 2008).

Cardiac stem cell identification. Cardiac stem cells remain an attractive possibility for regeneration, but the major identifying criteria for these cells in adult heart need to be fixed. First, clonogenic, self-renewal, and differentiation properties should be established in vitro and in vivo. In the latter case, a single cell should be transplanted into a cardiac region depleted of its own putative stem cell reservoir, as this is the gold standard that has been achieved in other systems such as skeletal muscle (Sacco et al., 2008) and bone marrow (Bryder et al., 2006). Second, if cardiac stem cells differentiate according to a hierarchical model, it is necessary to establish the transcriptional signature at different stages of quiescence, amplification, differentiation, and maturation. The combined expression of stem cell markers and cardiogenic transcription factors should be investigated using a cell lineage approach.

Fibrogenic response and fibroblast reprogramming. Slowing the process of fibrosis may tip the balance of cardiac repair from healing to regeneration. Therapeutic options to counteract fibrosis by controlling the inflammatory response have been largely discouraging (for reviews see Hinz et al., 2007; Wynn, 2008); however, new approaches need to be attempted because the mechanisms involved in fibrogenesis are now known to be distinct from those pertaining to inflammation.

Cardiac fibroblasts might also be manipulated genetically to control their capacity for proliferation and transition toward myofibroblasts. Depressing fibroblast proliferation via the p53 and/or p16 retinoblastoma pathways leads to a reduced fibrosis in the liver in mice (Krizhanovsky et al., 2008), which suggests that a similar approach is worth testing in the heart. Other promising research involves reprogramming cardiac fibroblasts to obtain a different phenotype. It has recently been shown that

somatic cells like adult fibroblasts can be reprogrammed to obtain a stable pluripotency using a combination of four transcription factors: c-myc, Oct-4, Sox-2, and Klf4 (Takahashi and Yamanaka, 2006). Cardiac fibroblasts might feasibly be reprogrammed to become contractile cells. We recently found that cardiac fibroblasts differ from those of other organs in that they already express a "repertoire" of cardiac genes, including GATA4 (Zaglia et al., 2008). Whether this particular gene expression program makes them more responsive to cardiac reprogramming remains to be seen. It is worth adding that forcing the expression of myocardin in postinfarcted cardiac fibroblasts induces the expression of cardiomyocyte-specific proteins (van Tuyn et al., 2007).

On the whole, it is probably just as important to block fibroblast proliferation as to provide specific cues to induce myocardial proliferation. We need to reconsider regeneration as a process dependent on a concerted interplay between cardiomyocytes and the surrounding nonmyocardial cells, possibly as a structural and functional unit. It is interesting that in a mouse model of heart failure, specific inhibition of the interstitial fibrogenic response has an impact on cardiac hypertrophy and dysfunction, which suggests that fibroblasts control the cardiomyocyte response to pathological stimuli (Thum et al., 2008). The complexity of this partnership varies considerably from one species to another, ranging from single cardiomyocytes with associated supporting capillaries and fibroblasts, as in mammals, to cardiomyocytes surrounded by endocardium with almost no fibroblasts or capillaries, as in zebrafish and axolotl (Fig. 3).

To summarize, the lesson that we have learned from comparison with regeneration-prone species is that the mammalian heart is inherently resistant to regeneration, possibly as a result of an evolutionary imposition. Therefore we propose that a combined approach encompassing all the three strategies described in this review, namely cardiomyocyte cell cycle reentry, stem cell mediated regeneration, and controlled fibrogenic response, has the greatest chance to succeed in restraining or even reversing the progression of cardiac deterioration in disease.

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References

Ahuja, P., E. Perriard, J.C. Perriard, and E. Ehler. 2004. Sequential myofibrillar breakdown accompanies mitotic division of mammalian cardiomyocytes. *J. Cell Sci.* 117:3295–3306.

Baudino, T.A., W. Carver, W. Giles, and T.K. Borg. 2006. Cardiac fibroblasts: friend or foe? *Am. J. Physiol. Heart Circ. Physiol.* 291:H1015–H1026.

Beltrami, A.P., L. Barlucchi, D. Torella, M. Baker, F. Limana, S. Cimenti, H. Kasahara, M. Rota, E. Musso, K. Urbanek, et al. 2003. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* 114:763–776.

Bettencourt-Dias, M., S. Mittnacht, and J.P. Brockes. 2003. Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes. *J. Cell Sci.* 116:4001–4009.

Blewett, C.J., R.E. Cilley, H.P. Ehrlich, J.H. Blackburn II, P.W. Dillon, and T.M. Krummel. 1997. Regenerative healing of incisional wounds in midgestational murine hearts in organ culture. *J. Thorac. Cardiovasc. Surg.* 113:880–885.

Bock-Marquette, I., A. Saxena, M.D. White, J.M. DiMaio, and D. Srivastava. 2004. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature.* 432:466–472.

Bryder, D., D.J. Rossi, and I.L. Weissmann. 2006. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am. J. Pathol.* 169:338–346.

Cai, C.L., X. Liang, Y. Shi, P.H. Chu, S.L. Pfaff, J. Chen, and S. Evans. 2003. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev. Cell.* 5:877–889.

Cai, C.L., J.C. Martin, Y. Sun, L. Cui, L. Wang, K. Ouyang, L. Yang, L. Bu, X. Liang, X. Zhang, et al. 2008. A myocardial lineage derives from Tbx18 epicardial cells. *Nature.* 454:104–108.

Dimmeler, S., J. Burchfield, and A.M. Zeiher. 2008. Cell-based therapy of myocardial infarction. *Arterioscler. Thromb. Vasc. Biol.* 28:208–216.

Fransioli, J., B. Bailey, N.A. Gude, C.T. Cottage, J.A. Muraski, G. Emmanuel, W. Wu, R. Alvarez, M. Rubio, S. Ottolenghi, et al. 2008. Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. *Stem Cells.* 26:1315–1324.

Galvez, B.G., M. Sampaolesi, A. Barbuti, A. Crespi, D. Covarello, S. Brunelli, A. Dellavalle, S. Crippa, G. Balconi, I. Cuccovillo, et al. 2008. Cardiac mesoangioblasts are committed, self-renewable progenitors, associated with small vessels of juvenile mouse ventricle. *Cell Death Differ.* 15:1417–1428.

Gittenberger-de Groot, A.C., M.P. Vrancken-Peeters, M.M. Mentik, R.G. Gourdie, and R.E. Poelmann. 1998. Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ. Res.* 82:1043–1052.

Godwin, J.W., and J.P. Brockes. 2006. Regeneration, tissue injury and the immune response. *J. Anat.* 209:423–432.

Harty, M., A.W. Neff, M.W. King, and A.L. Mescher. 2003. Regeneration or scarring: an immunologic perspective. *Dev. Dyn.* 226:268–279.

Hassink, R.J., K.B. Pasumarthi, H. Nakajima, M. Rubart, M.H. Soonpaa, A.B. de la Rivière, P.A. Doevendans, and L.J. Field. 2008. Cardiomyocyte cell cycle activation improves cardiac function after myocardial infarction. *Cardiovasc. Res.* 78:18–25.

Heusch, G., and R. Schulz. 2000. The biology of myocardial hibernation. *Trends Cardiovasc. Med.* 10:108–114.

Hinz, B., T.H. Phan, V.J. Thannickal, A. Galli, M.L. Bochaton-Piallat, and G. Gabbiani. 2007. The myofibroblast: one function, multiple origins. *Am. J. Pathol.* 170:1807–1816.

Hsieh, P.C., V.F. Segers, M.E. Davis, C. MacGillivray, J. Gannon, J.D. Molkentin, J. Robbins, and R.T. Lee. 2007. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat. Med.* 13:970–974.

Hu, N., D. Sedmera, H.J. Yost, and E.B. Clark. 2000. Structure and function of the developing zebrafish heart. *Anat. Rec.* 260:148–157.

Kattman, S.J., T.L. Huber, and G.M. Keller. 2006. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev. Cell.* 11:723–732.

Kelly, R.G., N.A. Brown, and M.E. Buckingham. 2001. The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell.* 1:435–440.

Krizhanovsky, V., M. Yon, R.A. Dickins, S. Hearn, J. Simon, C. Miething, H. Yee, L. Zender, and S.W. Lowe. 2008. Senescence of activated stellate cells limits liver fibrosis. *Cell.* 134:657–667.

Kühn, B., F. del Monte, R.J. Hajjar, Y.S. Chang, D. Lebeche, S. Arab, and M.T. Keating. 2007. Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. *Nat. Med.* 13:962–969.

Laube, F., M. Heister, C. Scholz, T. Borchardt, and T. Braun. 2006. Re-programming of newt cardiomyocytes is induced by tissue regeneration. *J. Cell Sci.* 119:4719–4729.

Laugwitz, K.L., A. Moretti, J. Lam, P. Gruber, Y. Chen, S. Woodard, L.Z. Lin, C.L. Cai, M.M. Lu, M. Reth, et al. 2005. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature.* 433:647–653.

Laugwitz, K.L., A. Moretti, L. Caron, A. Nakano, and K.R. Chien. 2008. Islet1 cardiovascular progenitors: a single source for heart lineages? *Development.* 135:193–205.

Lawson, N.D., and B.M. Weinstein. 2002. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* 248:307–318.

Lepilina, A., A.N. Coon, K. Kikuchi, J.E. Holdway, R.W. Roberts, C.G. Burns, and K.D. Poss. 2006. A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell.* 127:607–619.

Lien, C.-L., M. Schebesta, S. Makino, G.J. Weber, and M.T. Keating. 2006. Gene expression analysis of zebrafish heart regeneration. *PLoS Biol.* 4:e260.

Limana, F., A. Zacheo, D. Mocini, A. Mangoni, G. Borsellino, A. Diamantini, R. De Mori, L. Battistini, E. Vigna, M. Santini, et al. 2007. Identification of myocardial and vascular precursor cells in human and mouse epicardium. *Circ. Res.* 101:1255–1265.

Ma, Q., B. Zhou, and W.T. Pu. 2008. Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity. *Dev. Biol.* 323:98–104.

MacLellan, W.R., A. Garcia, H. Oh, P. Frenkel, M.C. Jordan, K.P. Roos, and M.D. Schneider. 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.

Marques, S.R., Y. Lee, K.D. Poss, and D. Yelon. 2008. Reiterative roles for FGF signalling in the establishment of size and proportion of the zebrafish. *Dev. Biol.* 321:397–406.

Martin, C.M., A.P. Meeson, S.M. Robertson, T.J. Hawke, J.A. Richardson, S. Bates, S.C. Goetsch, T.D. Gallardo, and D.J. Garry. 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.

Mathias, J.R., M.E. Dodd, K.B. Walters, J. Rhodes, J.P. Kanki, A.T. Look, and A. Huttenlocher. 2007. Live imaging of chronic inflammation caused by mutation of zebrafish Hai1. *J. Cell Sci.* 120:3372–3383.

Mescher, A.L., and A.W. Neff. 2006. Limb regeneration in amphibians: immunological considerations. *ScientificWorldJournal.* 6:1–11.

Messina, E., L. De Angelis, G. Frati, S. Morrone, S. Cimenti, F. Fiordaliso, M. Salio, M. Battaglia, M.V. Latronico, M. Coletta, et al. 2004. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ. Res.* 95:911–921.

Mikawa, T., and D.A. Fischman. 1992. Retroviral analysis of cardiac morphogenesis: discontinous formation of coronary vessels. *Proc. Natl. Acad. Sci. USA.* 89:9504–9508.

Mikawa, T., and R.G. Gourdie. 1996. Pericardial mesoderm generates a population of cornary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. *Dev. Biol.* 174:221–232.

Moretti, A., L. Caron, A. Nakano, J.T. Lam, A. Bernshausen, Y. Chen, Y. Qyang, L. Bu, M. Sasaki, S. Martin-Puig, et al. 2006. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell.* 127:1151–1165.

Oh, H., S.B. Bradfute, T.D. Gallardo, T. Nakamura, V. Gaussin, Y. Mishina, J. Pocius, L.H. Michael, R.R. Behringer, R.J. Schwartz, et al. 2003. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc. Natl. Acad. Sci. USA.* 100:12313–12318.

Passier, R., L.W. van Laake, and C. Mummery. 2008. Stem-cell-based therapy and lessons from the heart. *Nature.* 453:322–329.

Pasumarthi, K.B., and L.J. Field. 2002. Cardiomyocyte cell cycle regulation. *Circ. Res.* 90:1044–1054.

Pasumarthi, K.B., H. Nakajima, H.O. Nakajima, M.H. Soonpaa, and L.J. Field. 2005. Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. *Circ. Res.* 96:110–118.

Pérez-Pomares, J.M., R. Carmona, M. González-Iriarte, G. Atencia, A. Wessels, and R. Muñoz-Chápuli. 2002. Origin of coronary endothelial cells from epicardial mesothelium in avian embryos. *Int. J. Dev. Biol.* 46:1005–1013.

Poss, K.D. 2007. Getting to the heart of regeneration in zebrafish. *Semin. Cell Dev. Biol.* 18:36–45.

Poss, K.D., L.G. Wilson, and M.T. Keating. 2002. Heart regeneration in zebrafish. *Science.* 298:2188–2190.

Rubart, M., and L.J. Field. 2006. Cardiac regeneration: repopulating the heart. *Annu. Rev. Physiol.* 68:29–49.

Sacco, A., R. Doyonnas, P. Kraft, S. Vitorovic, and H.M. Blau. 2008. Self-renewal and expansion of single transplanted muscle stem cells. *Nature.* 456:502–506.

Segers, V.F., and R.T. Lee. 2008. Stem-cell therapy for cardiac disease. *Nature.* 451:937–942.

Simon, M.C., and B. Keith. 2008. The role of oxygen availability in embryonic development and stem cell function. *Nat. Rev. Mol. Cell Biol.* 9:285–296.

Smart, N., C.A. Risebro, A.A. Melville, K. Moses, R.J. Schwartz, K.R. Chien, and P.R. Riley. 2007. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature.* 445:177–182.

Smith, R.R., L. Barile, H.C. Cho, M.K. Leppo, J.M. Hare, E. Messina, A. Giacomello, M.R. Abraham, and E. Marbán. 2007. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation.* 115:896–908.

Stevenson, T.J., V. Vinarsky, D.L. Atkinson, M.T. Keating, and S.J. Odelberg. 2006. Tissue inhibitor of metalloproteinase 1 regulates matrix metalloproteinase activity during newt limb regeneration. *Dev. Dyn.* 235:606–616.

Takahashi, K., and S. Yamanaka. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 126:663–676.

Tate, J.M., and J.O. Oberpriller. 1989. Primary cell culture and morphological characterization of ventricular myocytes from the adult newt, *Notophthalmus viridescens*. *Anat. Rec.* 224:29–42.

Thum, T., C. Gross, J. Fiedler, T. Fischer, S. Kissler, M. Bussen, P. Galuppo, S. Just, W. Rottbauer, S. Frantz, et al. 2008. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature.* 456:980–984.

Tillmanns, J., M. Rota, T. Hosoda, Y. Misao, G. Esposito, A. Gonzalez, S. Vitale, C. Parolin, S. Yasuzawa-Amano, J. Muraski, et al. 2008. Formation of large coronary arteries by cardiac progenitor cells. *Proc. Natl. Acad. Sci. USA.* 105:1668–1673.

van Amerongen, M.J., and F.B. Engel. 2008. Features of cardiomyocyte proliferation and its potential for cardiac regeneration. *J. Cell. Mol. Med.* 12:2233–2244.

van Tuyn, J., D.A. Pijnappels, A.A. de Vries, I. de Vries, I. van der Velde-van Dijke, S. Knaän-Shanzer, A. van der Laarse, M.J. Schalij, and D.E. Atsma. 2007. Fibroblasts from human postmyocardial infarction scars acquire properties of cardiomyocytes after transduction with a recombinant myocardin gene. *FASEB J.* 21:3369–3379.

Vinarsky, V., D.L. Atkinson, T.J. Stevenson, M.T. Keating, and S.J. Odelberg. 2005. Normal newt regeneration requires matrix metalloproteinase function. *Dev. Biol.* 279:86–98.

Wills, A.A., J.E. Holdway, R.J. Major, and K.D. Poss. 2008. Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. *Development.* 135:183–192.

Winter, E.M., and A. Gittenberger-de Groot. 2007. Towards biomedical applicability: epicardium-derived cells in cardiogenesis and cardiac regeneration. *Cell. Mol. Life Sci.* 64:692–703.

Winter, E.M., R.W. Grauss, B. Hogers, J. van Tuyn, R. van der Geest, H. Lie-Venema, R.V. Steijn, S. Maas, M.C. DeRuiter, A.A. deVries, et al. 2007. Preservation of left ventricular function and attenuation of remodeling after transplantation of human epicardium-derived cells into the infarcted mouse heart. *Circulation.* 116:917–927.

Wynn, T.A. 2008. Cellular and molecular mechanisms of fibrosis. *J. Pathol.* 214:199–210.

Wu, S.M., Y. Fujiwara, S.M. Cibulsky, D.E. Clapham, C.L. Lien, T.M. Schultheiss, and S.H. Orkin. 2006. Developmental origin of a bipotential myocardial and smooth muscle cell precursors in the mammalian heart. *Cell.* 127:1137–1150.

Wu, S.M., K.R. Chien, and C. Mummery. 2008. Origins and fates of cardiovascular progenitor cells. *Cell.* 132:537–543.

Zaglia, T., A. Dedja, C. Candiotto, E. Cozzi, S. Schiaffino, and A. Ausoni. 2008. Cardiac interstitial cells express GATA4 and control dedifferentiation and cell cycle re-entry of adult cardiomyocytes. *J. Mol. Cell. Cardiol.* doi:10.1016/j.yjmcc.2008.12.010.

Zhou, B., Q. Ma, S. Rajagopal, S.M. Wu, I. Domian, J. Rivera-Feliciano, D. Jiang, A. von Gise, S. Ikeda, K.R. Chien, and W.T. Pu. 2008. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature.* 454:109–113.