

# Cardiac repair and regeneration: the Rubik's cube of cell therapy for heart disease

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Acute ischemic injury and chronic cardiomyopathies damage healthy heart tissue. Dead cells are gradually replaced by a fibrotic scar, which disrupts the normal electromechanical continuum of the ventricular muscle and compromises its pumping capacity. Recent studies in animal models of ischemic cardiomyopathy suggest that transplantation of various stem cell preparations can improve heart recovery after injury. The first clinical trials in patients produced some encouraging results, showing modest benefits. Most of the positive effects are probably because of a favorable paracrine influence of stem cells on the disease microenvironment. Stem cell therapy attenuates inflammation, reduces apoptosis of surrounding cells, induces angiogenesis, and lessens the extent of fibrosis. However, little new heart tissue is formed. The current challenge is to find ways to improve the engraftment, long-term survival and appropriate differentiation of transplanted stem cells within the cardiovascular tissue. Hence, there has been a surge of interest in pluripotent stem cells with robust cardiogenic potential, as well as in the inherent repair and regenerative mechanisms of the heart. Recent discoveries on the biology of adult stem cells could have relevance for cardiac regeneration. Here, we discuss current developments in the field of cardiac repair and regeneration, and present our ideas about the future of stem cell therapy.

## The clinical problem

Ischemic and non-ischemic cardiomyopathies eventually lead to left ventricular dysfunction and heart failure. Heart failure impedes quality of life, decreases life expectancy and increases medical costs dramatically, and thus constitutes a major health problem (McMurray and Pfeffer, 2005). As the population ages, the prevalence of heart failure will increase. Current therapy provides some survival benefit and improvement of symptoms, but cannot reverse the condition of cardiac tissue from a diseased to a healthy state. Recent developments in stem cell biology and regenerative medicine may offer ways to manage heart failure by replacing damaged or lost cardiac muscle with healthy tissue, and thus improve the quality of life and survival in patients with various cardiomyopathies.

## The disease process of ischemic cardiomyopathy

Myocardial infarction usually results from coronary artery occlusion owing to acute atherosclerotic plaque rupture and platelet aggregation, which leads to thrombosis within the vessel (Antman and Braunwald, 2001). Severe ischemia downstream from occluded arteries causes cardiomyocytic apoptosis within minutes. The widespread injury and cell death, often exacerbated by re-oxygenation following reperfusion, floods the region with reactive oxygen species and toxic agents (Yellon and Hausenloy, 2007). In response, cells in and around the affected areas upregulate and

secrete cytokines and chemokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-1 $\beta$ , IL-6 or IL-8, which trigger an immediate and massive infiltration of circulating leukocytes into the ischemic core (Frangogiannis, 2008). Adjacent endothelial cells bolster the recruitment of pro-inflammatory immune cells by upregulating their expression of cell adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The initial wave of infiltrating cells is followed by monocytes that home to the damaged area and mature into macrophages. The immune cells gradually clear out cellular debris and matrix degradation products in the injury site, leaving behind sparse tissue with enlarged capillaries.

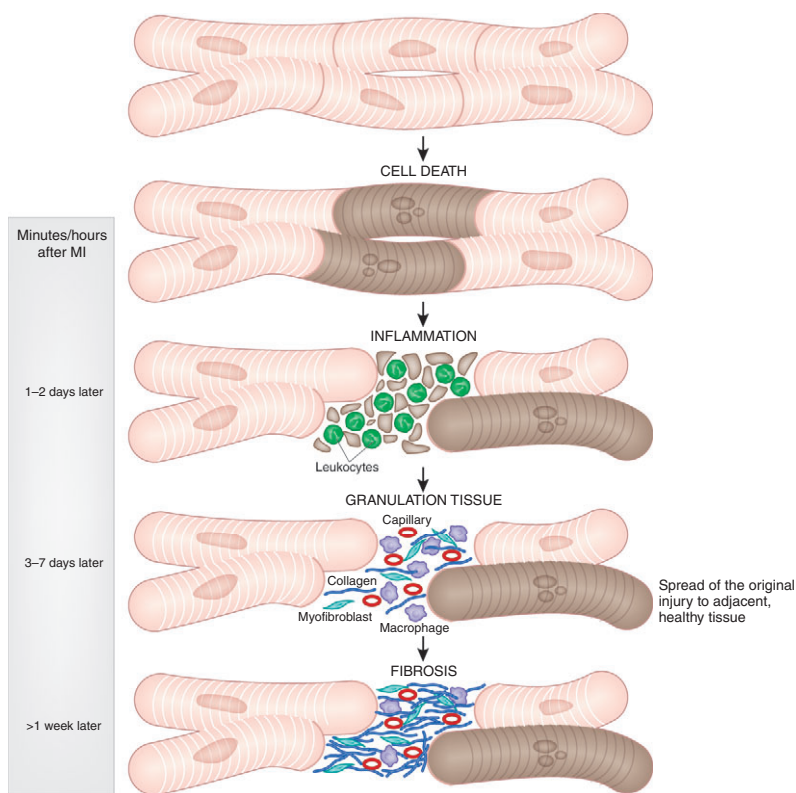
After cellular debris is removed from the injured area, the gap left behind fills with granulation tissue. This process begins with the arrival of myofibroblasts a few days after the initial injury (Frangogiannis, 2008). Granulation tissue is composed mainly of blood vessels, macrophages and myofibroblasts, the latter depositing collagen and other extracellular matrix proteins. A week after the infarction, the granulation tissue starts to develop into a dense scar with collagen deposits intermingled with myofibroblasts (Fig. 1).

The ischemic area is rich in inflammatory cytokines and protease activity, which harms surrounding healthy cells (Vanhouste et al., 2006). The extra mechanical burden on the remaining ventricular cells, further compromises the integrity of the cardiac tissue. Thus, the initial localized injury creates a ripple effect that spreads slowly to larger areas of the heart. The loss of functional tissue and subsequent remodeling eventually causes ventricular dysfunction and electrical instability, leading to heart failure and malignant arrhythmias (Pfeffer and Braunwald, 1990; Sun, 2009).

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**Fig. 1. Schematic representation of cellular events after cardiac ischemic injury.** Severe ischemia downstream from an occluded coronary artery results in cardiomyocytic apoptosis within minutes after the supplying blood vessel is closed. Cell death and the release of toxic products trigger a massive inflammatory response. After the cellular debris is cleared, the injury site fills with granulation tissue that is composed mainly of enlarged capillaries, macrophages and myofibroblasts, which deposit collagen and other extracellular matrix proteins to build a dense scar. The disease milieu affects neighboring cardiomyocytes, leading to a gradual spread of the original injury to relatively healthy tissue.

Non-ischemic cardiomyopathies (caused by genetic mutations, viral infections, drug abuse, chemotherapy, etc.) can also destroy cardiomyocytes leading to inflammation, scar formation, ventricular remodeling and heart failure (Schönberger and Seidman, 2001).

### Stem cells repair damaged cardiac tissue

Until a few years ago, damage to cardiac tissue and the gradual deterioration towards heart failure were regarded as irreparable. A series of studies in animal models, showing that transplantation of various progenitor cell preparations of bone marrow, skeletal, or embryonic origin improve ventricular function after injury, are challenging this view (Segers and Lee, 2008). Some reports document significant rates of stem cell differentiation to cardiovascular tissue, whereas other studies do not. Thus, there is some controversy about the benefits of stem cell transplantation. It is likely that the positive outcome of stem cell application depends on two factors: (1) the ability of transplanted progenitor cells to differentiate, even with low frequency, to cardiac and vascular cells; and (2) the secretion of survival factors by progenitor cells to stimulate tissue recovery after ischemic injury and minimize the infarct size (Kupatt et al., 2005a; Kupatt et al., 2005b; Gnechchi et al., 2005; Uemura et al., 2006; Gnechchi et al., 2008). Recent studies show that products of transplanted progenitor cells such as  $\beta 4$  thymosin, which promotes wound healing, or the Wnt antagonist secreted frizzled-related protein 2 (sFRP2), have favorable effects on cell survival and preservation of cardiac function after myocardial infarction (Mirotsov et al., 2007; Hinkel et al., 2008; Alfaro et al., 2008).

### Cardiac repair using exogenous progenitor cells

As the isolation and characterization of embryonic and adult stem cells increased over the last decade, it became evident that many cell types have cardiogenic potential in vitro, either as part of the natural course of their differentiation program (e.g. embryonic stem cells) (Doetschman et al., 1985) or under specific inductive conditions (e.g. mesenchymal stem cells) (Reinecke et al., 2008). These findings led to numerous experimental studies in animal models of cardiovascular disease to assess the potential of progenitor cell populations to replace lost ventricular tissue and improve cardiac function. The most prominent cell types now considered for cardiac repair are discussed briefly below.

### Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are found in the bone marrow stroma and adipose tissue, as well as in other organs (Uccelli et al., 2008). They can be isolated readily and expanded in culture to obtain large numbers that are adequate for cell therapy. MSCs are multipotent and give rise to adipocytes, chondrocytes, osteoblasts and endothelial cells (Pittenger et al., 1999; Jiang et al., 2002). In culture, they can also differentiate into contracting cardiomyocytes, but only with low efficiency and specialized culture conditions (Makino et al., 1999; Tomita et al., 1999; Shiota et al., 2007). An interesting aspect of MSCs is that they appear to have low immunogenicity, which promotes their use in allogeneic recipients (Aggarwal and Pittenger, 2005; Amado et al., 2005; Dai et al., 2005).

Transplantation of MSCs in animal models of myocardial infarction and dilated cardiomyopathy significantly improves left ventricular cardiac function (Tomita et al., 1999; Toma et al., 2002;

Kudo et al., 2003; Amado et al., 2005; Dai et al., 2005; Silva et al., 2005; Grauss et al., 2008). Besides functional improvement, mortality also decreases in animals after cell transplantation (Miyahara et al., 2006). However, despite enhanced cardiac function, the differentiation of MSCs to cardiomyocytes is limited (Fazel et al., 2005; Silva et al., 2005; Noiseux et al., 2006; Rose et al., 2008). The beneficial effects of MSCs are postulated to come from their influence on neovascularization of the ischemic tissue and their protection of resident cells (Caplan and Dennis, 2006; Gnecci et al., 2008). This 'cell-help-cell' effect is enhanced further when MSCs are engineered to express the pro-survival kinase Akt1 (Mangi et al., 2003; Gnecci et al., 2005; Noiseux et al., 2006). Akt1-expressing MSCs secrete sFRP2 that protects cardiomyocytes from hypoxia-induced apoptosis (Mirotsoy et al., 2007; Zhang, Z. et al., 2009).

The positive effects of transplanted MSCs, and the ability to genetically modify them, promotes their use as a cellular vehicle to deliver angiogenic proteins [e.g. angiopoietin-1 (Ang1)] (Sun, L. et al., 2007), survival factors [insulin-like growth factor-1 (IGF-1)] (Haider et al., 2008), chemokines [stromal cell-derived factor-1 (SDF-1)] (Zhang et al., 2007) or Wnt antagonists (sFRP2) (Alfaro et al., 2008) to further enhance the recovery of injured myocardium. However, it should be noted that MSC transplantation after myocardial infarction produced calcification and ossification islands inside the ventricular tissue in some mice (Breitbach et al., 2007). In one study, this was also observed in approximately 28% of rats analyzed, raising concerns about the direct use of MSCs in cardiovascular cell therapy (Yoon et al., 2004).

### Skeletal myoblasts

Skeletal myoblasts, or satellite cells, are found in the basal membrane of muscle fibers and maintain the homeostasis of skeletal tissue (Shi and Garry, 2006; Buckingham and Montarras, 2008). Myoblasts are easy to isolate from small muscle biopsies as they can proliferate and expand substantially in culture. Obvious similarities between skeletal and cardiac muscle tissue suggest that satellite cells may adopt a cardiomyocytic fate once inside ventricular tissue. Moreover, they are resistant to hypoxia-induced apoptosis, providing another potential advantage for them in repopulating the ischemic myocardium (Menasché, 2007).

Animal studies show that myoblasts that are injected into cardiac tissue after ischemic injury cause global and regional functional improvements (Murry et al., 1996; Taylor et al., 1998; Ghostine et al., 2002). In a chronic heart failure dog model, transplantation of myoblasts also led to a significant recovery in left ventricular hemodynamics (He et al., 2005). In similar fashion, intramyocardial delivery of myoblasts in rabbits with an acute myocardial infarction demonstrated an increase in regional left ventricular wall thickness by magnetic resonance imaging and a decrease in the deleterious effects of post-infarction cardiac remodeling (van den Bos et al., 2005). However, even though myoblasts seem to incorporate into the infarct site and develop contraction-like characteristics, they generally fail to form intercalated disks and appropriate gap junctions with resident cardiomyocytes (Reinecke et al., 2002). The lack of electromechanical coupling with the surrounding host cardiac tissue caused arrhythmias in a number of cases (Leobon et al., 2003).

Although straightforward application of skeletal myoblasts may have limited future use in cardiovascular cell therapy, satellite cells

could offer an ample source of cellular material that is similar to cardiac progenitor cells and that might be reprogrammed with cardiac-specific regulatory factors. Skeletal myoblasts may also be engineered to form connections with resident cardiomyocytes by expressing appropriate gap junction proteins before transplantation (Roell et al., 2007).

### Bone marrow-derived progenitor cells

Bone marrow (BM)-derived progenitor cells have been used widely in animal models to enhance the repair of injured myocardium. This area was fueled by results which show that the hearts of female patients, who received male BM, contain Y-chromosome-positive cardiomyocytes, indicating that the new cardiac cells originated from BM (Deb et al., 2003; Bayes-Genis et al., 2007). Transplants of female hearts in male recipients were also found to contain Y-chromosome-positive cardiomyocytes, suggesting an extracardial source of new cells (Laflamme et al., 2002; Bayes-Genis et al., 2002; Quaini et al., 2002; Müller et al., 2002). Moreover, certain subpopulations of BM cells, or of circulating BM-derived peripheral blood progenitor cells, can differentiate into cardiomyocytes, endothelial cells or smooth muscle cells under certain conditions in culture (Badorff et al., 2003; Yeh et al., 2003; Belema Bedada et al., 2005; Koyanagi et al., 2005; Koyanagi et al., 2007; Pallante et al., 2007; Flaherty et al., 2008). Consistent with these *in vitro* results, some transplantation studies suggest extensive differentiation of donor BM-derived cells to both vascular and cardiac cells in the host (Orlic et al., 2001a; Orlic et al., 2001b; Yeh et al., 2003; Kajstura et al., 2005; Rota et al., 2007). By contrast, other studies find that transplanted BM cells only make a significant contribution to vascular structures (Jackson et al., 2001), or show that there is very little, if any, differentiation of BM cells to cardiovascular cell types *in vivo* (Balsam et al., 2004; Murry et al., 2004). Future work may resolve the controversy surrounding the regenerative potential of BM cells for cardiac repair.

Despite the discrepancies regarding the differentiation of BM cells into cardiovascular tissue *in vivo*, most experimental studies demonstrate that transplanted BM cells have beneficial effects, including a robust stimulation of angiogenesis (Zhao et al., 2008). The pioneering work of T. Asahara and the late J. Isner first showed that CD34<sup>+</sup> cells from the vascular circulation could differentiate into endothelial cells in culture and promote neovascularization *in vivo* (Asahara et al., 1997). It is possible that the CD34<sup>+</sup>/CD133<sup>+</sup>/VEGFR2<sup>+</sup> subpopulation of BM cells, also described as endothelial progenitor cells (EPCs), contributes directly or indirectly to neovascularization, thus promoting angiogenesis and reperfusion of ischemic tissue (Kocher et al., 2001; Kawamoto et al., 2003; Young et al., 2007; Lamparter and Hatzopoulos, 2007). The potential of EPCs for cell therapy of vascular disease has been reviewed recently (Gulati and Simari, 2009).

A second possible explanation for the positive action of BM-derived cells on cardiac regeneration is that BM isolates often contain MSCs, which, as discussed already, produce beneficial effects on their own. Also, monocytic cells are part of the BM mononuclear cell population and may promote arteriogenesis and growth of collateral vessels (Kamihata et al., 2001; Kinnaird et al., 2004). Thus, BM mononuclear cells may improve the perfusion index of ischemic areas, which shrinks the infarct territory and

improves cardiac function. Although BM cells might have limited regenerative capacity on their own, they could be useful as an accessory transplant in combination cell therapies, together with stem cells of high cardiomyocytic potential, to enhance angiogenesis and promote stem cell survival in the ischemic environment.

### Embryonic stem cells

Embryonic stem (ES) cells originate from the inner cell mass of pre-implantation blastocysts. The first ES cells were isolated from mice at around day 3.5 of development (Evans and Kaufman, 1981; Martin, 1981). Since then, ES cell lines have been derived from other mammalian species, including humans (Thomson et al., 1998). Although in mice the inner cell mass consists of only about 15–20 cells, these few cells replicate almost indefinitely without differentiating in culture, providing an almost inexhaustible source of stem cells. Injection of culture-expanded mouse ES cells into blastocysts proved that they are pluripotent; that is, they are able to give rise to all embryonic lineages including germ cells.

Mouse and human ES cells can differentiate into a broad variety of organ-specific cell types *in vitro*, offering a rich source of cells for regenerative purposes (Doetschman et al., 1985; Keller, 2005; Murry and Keller, 2008), including cardiovascular cell lineages (Kehat et al., 2001; Xu et al., 2002; Sachinidis et al., 2003; Kattman et al., 2006; Moretti et al., 2006; Wu et al., 2006; Yang, L. et al., 2008).

After transplantation, ES cells and ES cell-derived cardiac cells have been shown to integrate into heart tissue and improve cardiac function in ischemic or cryoinjury models (Klug et al., 1996; Kehat et al., 2001; Kehat et al., 2004; Behfar et al., 2002; Min et al., 2002; Roell et al., 2002; Hodgson et al., 2004; Kofidis et al., 2005; Laflamme et al., 2005; Laflamme et al., 2007; Yan et al., 2009). In myocardial infarction mouse models, ES cells that were injected directly into the infarcted myocardium differentiated into cardiomyocytes, vascular smooth muscle cells and endothelial cells. The ES cell transplant caused a significant increase in left ventricular systolic function and a decrease in cardiac tissue remodeling, suggesting that the size of the injury was reduced (Singla et al., 2006).

ES cell therapy might also be beneficial in non-ischemic heritable cardiomyopathies. In *Kir6.2*-knockout mice, which lack functional  $K_{ATP}$  channels (recapitulating human dilated cardiomyopathy 10), ES cell transplant results in an improvement in systolic dysfunction and electrical synchronization; a decrease in left ventricular remodeling; and an increase in survival (Yamada et al., 2008).

It appears that the differentiation rate of ES cells into new cardiovascular tissues is the most efficient among the stem cell types tested so far. Still, there are several disadvantages that may complicate their use in clinical investigations. First, ES cells have the propensity to develop teratomas, tumors that contain a wide array of different cell types (Nussbaum et al., 2007). Studies show that the number of ES cells used in treatments may need to be titrated carefully to avoid uncontrolled tumor growth. Interestingly, the tumorigenicity is curtailed in mice that overexpress TNF- $\alpha$  in the heart (Behfar et al., 2007). Second, ES cells differentiate into heterogeneous populations. This means that the number of cardiac cells created under normal differentiation conditions is rather low (usually less than, or around, 1% of the total cells in the culture).

Elaborate protocols may be needed to increase the production of cardiac cells so that they can be selected from cells of other lineages. So far, a number of ways have been described to manipulate the differentiation process of ES cells in order to increase cardiac cell output (Yuasa et al., 2005; Qyang et al., 2007; Ueno et al., 2007; Hao et al., 2008; Yan et al., 2009). The next challenge is to direct ES-derived cardiac progenitors to monotypic cultures of specialized cell types, such as ventricular and atrial myocytes, or to pacemaker and conduction system cells (Behfar et al., 2008; Chien et al., 2008). Third, transplantation of allograft ES cells can create an immunologic response and cell rejection (Swijnenburg et al., 2005). These technical difficulties are in addition to the ethical issues voiced by some people, which further preclude the straightforward application of ES cells in clinical studies. New developments regarding the possibility to generate ES-like cells, called inducible pluripotent stem (iPS) cells, by reprogramming adult somatic cells (Takahashi et al., 2007; Wernig et al., 2007; Okita et al., 2007) or by adapting adult spermatogonia (Guan et al., 2006), may bypass some of these technical and ethical issues.

### Inducible pluripotent stem cells

iPS cells are generated by expressing specific pluripotency transcription factors in somatic cells, such as fibroblasts or epithelial cells, using viral vectors (Takahashi et al., 2007; Wernig et al., 2007; Okita et al., 2007). Pluripotency factors include proteins that are crucial for the maintenance of the stem cell phenotype, for example Oct3/4, Sox2, Klf4, Nanog or *c-Myc*. The pluripotency factors gradually suppress the expression of genes that are associated with somatic cell function, shutting down differentiation programs and activating the expression of endogenous stem cell pluripotency factors (Jaenisch and Young, 2008). Within several weeks, the altered gene expression reprograms the somatic cells to acquire similar morphological, molecular and functional characteristics to ES cells. iPS cells can generate mice after blastocyst injection and can also differentiate into various lineages in culture, including cardiovascular cells (Schenke-Layland et al., 2008; Narazaki et al., 2008; Mauritz et al., 2008; Zhang, J. et al., 2009).

The same technical challenges of ES cell therapy (low cardiogenic output, heterogeneous differentiation, limited differentiation to specialized cell types, and tumorigenicity) must be resolved before clinical applications of iPS cells are realized fully. Already, studies show that reprogramming can be achieved with only two pluripotency factors, eliminating the need for oncogenic *c-Myc*, or without viral integration into the genome, which can lead to pathological complications (Huangfu et al., 2008; Stadtfeld et al., 2008).

### Clinical studies

The experimental success of using progenitor cells to improve heart recovery after ischemic damage in animals led clinicians to test the safety and efficacy of cell therapy in patients with myocardial damage following myocardial infarction (Dimmeler and Zeiher, 2008; Segers and Lee, 2008; Burt et al., 2008). Randomized clinical trials with a variety of cell preparations, including BM-derived cells (Wollert et al., 2004; Ruan et al., 2005; Assmus et al., 2006; Lunde et al., 2006; Schächinger et al., 2006a; Janssens et al., 2006; Ge et al., 2006; Hendrikx et al., 2006), circulating progenitor cells (Erbs et al., 2005; Assmus et al., 2006; Kang et al., 2006), skeletal

myoblasts (Menasché et al., 2008) and MSCs (Chen et al., 2004; Chen et al., 2006), with intracoronary or intramuscular delivery, suggest that these approaches are safe and may improve ventricular function (Table 1). Although some trials failed to demonstrate a significant increase in cardiac function after cell transplantation when compared with controls, several other randomized trials showed measurable improvements that were comparable to established therapeutic regimes (Reffelmann et al., 2009). Non-randomized, smaller-scale trials also produced variable results, ranging from no significant changes in left ventricular ejection fraction to a significant improvement, of up to a 14% increase, during the follow-up period (Strauer et al., 2002; Perin et al., 2004; Fernandez-Aviles et al., 2004; Ince et al., 2004; Strauer et al., 2005; Katritsis et al., 2005; Bartunek et al., 2005; Mocini et al., 2006; Gavira et al., 2006; Ahmadi et al., 2007; Choi et al., 2007; Klein et al., 2007; Li et al., 2007; Stamm et al., 2007; Tatsumi et al., 2007).

To date, the largest clinical trial to assess the benefit of skeletal myoblast therapy is the MAGIC (Myoblast Autologous Grafting in Ischemic Cardiomyopathy) trial, which randomized patients to receive either stem cell injection or culture medium. Although early fears of severe arrhythmias did not materialize, the results have been disappointing, showing no significant benefit with skeletal myoblast cell implantation (Menasché et al., 2008). By contrast, a

meta-analysis of 18 randomized and non-randomized trials involving a total of 999 acute myocardial infarction or chronic ischemic cardiomyopathy patients found that transplantation of adult BM-derived stem cells improved left ventricular ejection fraction by 5.40% ( $P<0.001$ ); decreased infarct scar size by 5.49% ( $P=0.003$ ); and lowered left ventricular end-systolic volume by 4.80 mls ( $P=0.006$ ) (Abdel-Latif et al., 2007). It is also encouraging that a multicenter, randomized clinical trial of 204 patients with acute myocardial infarction showed that intracoronary delivery of BM cells (at 3 to 7 days post-reperfusion therapy) decreased the incidence of myocardial infarction and death, or revascularization, at a 12-month follow-up when compared with placebo ( $P=0.009$ ) (Schächinger et al., 2006b).

Cell therapy also shows promise to relieve chronic angina symptoms. In patients with Canadian Cardiovascular Society (CCS) class III or IV angina, intramyocardial transplantation of autologous CD34<sup>+</sup> stem cells decreased the frequency of angina, resulting in an improvement in CCS class, exercise times and nitroglycerin use when compared with patients who did not receive CD34<sup>+</sup> cells (Losordo et al., 2007).

Perhaps the inconsistent results in clinical trials reflect both the variety of stem cell types used and the route of administration, as well as the quantity and quality of the injected cells. For example,

**Table 1. Stem cell and progenitor cell therapies: randomized clinical trials**

Study	Year	Clinical setting	n	Cell type	Method of cell transplantation	Cell number transplanted	Mean follow-up (months)	% Change in LVEF compared with control
Menasché et al. (MAGIC)	2008	ICMP	97	SMB	Intramyocardial <sup>†</sup>	$4 \times 10^8$ or $8 \times 10^8$	6	-1.0% or +0.8%; NS
Meluzin et al.	2008	AMI	60	BMMNC	Intracoronary	$1 \times 10^8$	12	+7.0%; $P=0.03$
Meyer et al. (BOOST)	2006	AMI	60	BMMNC	Intracoronary	$2.5 \times 10^9$	18	+2.8%; NS
Assmus et al. (TOPCARE-CHD)	2006	ICMP	51	BMMNC	Intracoronary	$2 \times 10^8$	3	+4.1%; $P<0.001$
Schächinger et al. (REPAIR-AMI)	2006	AMI	204	BMMNC	Intracoronary	$2.4 \times 10^8$	12	*
Schächinger et al. (REPAIR-AMI)	2006	AMI	204	BMMNC	Intracoronary	$2.4 \times 10^8$	4	+2.5%; $P=0.01$
Ge et al. (TCT-STAMI)	2006	AMI	20	BMMNC	Intracoronary	$4 \times 10^7$	6	+6.7%; NS
Hendrikx et al.	2006	ICMP	20	BMMNC	Intramyocardial <sup>†</sup>	$6 \times 10^7$	4	+2.5%; NS
Janssens et al.	2006	AMI	67	BMMNC	Intracoronary	$1.7 \times 10^8$	4	+1.2%; NS
Lunde et al. (ASTAMI)	2006	AMI	100	BMMNC	Intracoronary	$8.7 \times 10^7$	6	-3.0%; $P=0.05$
Ruan et al.	2005	AMI	20	BMC	Intracoronary	Not available	6	+9.2%; $P<0.05$
Chen et al.	2006	ICMP	45	MSC	Intracoronary	$5 \times 10^6$	12	-3.0%; NS
Chen et al.	2004	AMI	69	MSC	Intracoronary	$6 \times 10^{10}$	6	+12.0%; $P=0.01$
Assmus et al. (TOPCARE-CHD)	2006	ICMP	47	CPC	Intracoronary	$2.2 \times 10^7$	3	+0.8%; NS
Kang et al.	2006	AMI/ICMP	82	CPC	Intracoronary	$1.4 \times 10^9$	6	-0.2%; NS
Erbs et al.	2005	ICMP	26	CPC	Intracoronary	$7 \times 10^7$	3	+7.2%; NS
Losordo et al.	2007	ICMP	24	CD34 <sup>+</sup>	Intramyocardial <sup>‡</sup>	$5 \times 10^4$ , $1 \times 10^5$ or $5 \times 10^5$	6	**

AMI, acute myocardial infarction; BMC, bone marrow cells; BMMNC, bone marrow mononuclear cells; CPC, circulating progenitor cells; LVEF, left ventricular ejection fraction; ICMP, ischemic cardiomyopathy; MSC, mesenchymal stem cells; NS, non-significant; SMB, skeletal myoblasts. <sup>†</sup>Intramyocardial through surgical transeptal approach. <sup>‡</sup>Intramyocardial through percutaneous transendocardial approach. \*BMMNC therapy resulted in significant decrease in death, myocardial infarction and revascularization at 12 months, whereas LVEF was not included in the pre-specified cumulative endpoint. \*\*In CCS class III or IV angina patients, CD34<sup>+</sup> cell therapy resulted in a trend towards a decrease in the frequency of angina and improvement in CCS class, exercise times and nitroglycerin use.

mononuclear BM cell transplantation improves left ventricular ejection fraction in post-myocardial infarction patients when a higher number of cells are injected (i.e.  $10^8$  cells compared with  $10^7$  cells) (Meluzin et al., 2006). In addition, the ability of BM-derived cells to migrate, or induce neovascularization, might be impaired in heart disease patients, which would affect the efficacy of autologous cell therapy (Heeschen et al., 2004; Walter et al., 2005). The timing of left ventricular assessment after cell transplantation in patients may further explain some of the discrepancies in ventricular function since a number of benefits appear to be transient (Meyer et al., 2006; Meluzin et al., 2008), consistent with findings in animal studies of ES cell transplantation (van Laake et al., 2008). Finally, the portion of stem cells retained at the area of interest may be low, thereby diminishing the beneficial effects, as discussed in the next section.

### Methods of stem cell delivery

In the clinical setting, stem cells have been delivered by intracoronary injection; intravenous infusion; by direct injection into cardiac muscle through the endocardium by a percutaneous approach; or through the epicardium during open-heart surgery.

The infusion of stem cells into the coronary arterial circulation is relatively straightforward. In this situation, the cells are surrounded by nutrients and oxygen, which creates a favorable environment. However, the number of stem cells retained in the ventricular area of interest might be low. For example, in patients who sustained an ST-segment myocardial infarction and received a coronary stent, it was estimated that only 1.2-3.6% of BM cells that were delivered through an intracoronary route remained within the myocardium (Hofmann et al., 2005).

The homing of progenitor cells requires interaction with the vascular wall and transendothelial migration towards injured or ischemic areas, which may involve similar mechanisms to those involved in the recruitment of immune system cells to sites of inflammation (Vajkoczy et al., 2003; Chavakis et al., 2005; Chavakis et al., 2008). Thus, activation of adhesion molecules in the host vasculature and donor progenitor cells before cell delivery may stimulate homing efficiency. For example, adenosine increases the adhesion of EPCs to the vascular wall in experimental models (Ryzhov et al., 2008). Adenosine can be delivered directly into the coronary circulation in humans without significant adverse events and it has a short half-life in the bloodstream; thus, it may increase progenitor cell retention locally in a clinically feasible way (Leesar et al., 1997; Shen and DiCorleto, 2008).

Direct injection of stem cells into injured myocardium ensures that cells are placed in the area of interest. Nevertheless, ischemic conditions along with inflammation and scar tissue make a less than ideal environment for cell survival. Moreover, mechanical loss further diminishes the engraftment of injected cells. Estimates suggest that, during open-heart surgery, the leakage of injected microspheres is 33% and 89% in non-beating and beating porcine hearts, respectively (Teng et al., 2006). Low retention efficiency (approximately 3%) was also recorded for EPCs that were injected directly into the myocardium of rats (Aicher et al., 2003), or for BM cells in infarcted hearts of sheep (Bel et al., 2003); moreover, only a small percentage of myoblasts (approximately 7%) that were injected directly into infarcted mouse hearts survived for longer than 72 hours (Suzuki et al., 2004). Thus, intraventricular delivery

may not improve cell homing compared with intracoronary injection in all cases.

The modest, short-term cardioprotective effects of transplanted cells underscore the need to maximize delivery and differentiation of stem cells in order to produce functional cardiovascular tissue with long-term survival and engraftment within diseased tissue. Current roadblocks to the use of exogenous cells are renewing research interest in the inherent regenerative and reparative mechanisms of cardiac tissue in order to develop novel tools and strategies to enhance the efficacy of current cell therapy protocols.

### Endogenous stem cells in cardiac regeneration

Classically, the heart is thought of as a terminally differentiated, postmitotic organ without intrinsic mechanisms to replace lost cardiomyocytes. However, a number of recent studies show that the adult heart contains cell populations with stem cell characteristics and that cardiac homeostasis is maintained by replenishing damaged cells (Ellison et al., 2007; Kajstura et al., 2008). Genetic fate-mapping studies show that a considerable number of new cardiomyocytes form in the adult mouse heart after injury (Hsieh et al., 2007), and incorporation of  $^{14}\text{C}$  (which did not exist naturally before nuclear tests) into the DNA of human cardiomyocytes estimates that about 50% of adult cardiomyocytes are exchanged during a normal life span, suggesting a renewal mechanism (Bergmann et al., 2009). The properties of cardiac progenitor cells in the adult heart are discussed briefly below.

### Side population cells

A typical property of some stem cell populations is the exclusion of the vital dyes Hoechst 33342 and rhodamine 123. The cells that do not take up the dyes are called the side population (SP) (Goodell et al., 1996). Dye exclusion in SP cells is the result of high efflux rates mediated by ATP-binding cassette transporters such as ABCG2 and MDR1 (Challen and Little, 2006). SP cells are multipotent and have been identified in various organs including the BM, skeletal muscle and adipose tissue (Challen and Little, 2006).

The dissociation of heart tissue and dye stains show that the adult heart also has a resident pool of SP cells (Hierlihy et al., 2002; Martin et al., 2004). Isolated SP cells from heart tissue can be induced to express cardiac-specific genes *in vitro* when co-cultured with cardiomyocytes or when treated with agents such as oxytocin or the histone deacetylase inhibitor trichostatin A (Pfister et al., 2005; Oyama et al., 2007). The cardiogenic potential is higher in the subgroup of SP cells that express the stem cell antigen-1 (Sca-1<sup>+</sup>), but are negative for the endothelial marker CD31 (Pfister et al., 2005).

The number of SP cells in the mouse heart changes after myocardial infarction through proliferation of resident SP cells, as well as homing of BM-derived SP cells (Mouquet et al., 2005), but this might depend on the injury model used (Oyama et al., 2007). There is limited information about the regenerative potential of SP cells *in vivo*. One study showed homing of SP cells to the injured heart and differentiation of these cells into cardiomyocytes, endothelial cells and smooth muscle cells (Oyama et al., 2007). In another report, isolated SP cells that were allowed to form

cardiospheres in culture displayed features of neural crest (NC) progenitor cells in vitro and in vivo, with the potential to differentiate into glia, neurons, smooth muscle and cardiomyocytes, suggesting that at least a portion of SP cells are of neural crest origin (Tomita et al., 2005).

### c-Kit<sup>+</sup> progenitor cells

A second resident progenitor population is the c-Kit<sup>+</sup> cells, which are located in small clusters within the ventricles and atria of the adult heart (Beltrami et al., 2003). Isolated c-Kit<sup>+</sup> cells do not differentiate fully into cardiovascular cell types in culture, but show impressive regenerative potential after transplantation in the injured rat heart, where they give rise to cardiomyocytes, endothelial cells and smooth muscle cells (Beltrami et al., 2003; Dawn et al., 2005; Rota et al., 2008). Cardiac c-Kit<sup>+</sup> stem cells improve left ventricular ejection fraction after myocardial infarction by 11% compared with control rats that did not receive cell transplantation at a 20-day follow-up (Beltrami et al., 2003). An equivalent c-Kit<sup>+</sup> cell population has been isolated from small samples of human myocardium (Bearzi et al., 2007).

### Sca-1<sup>+</sup> progenitor cells

A third reported stem cell type in the heart expresses Sca-1, but is c-Kit<sup>-</sup> (Oh et al., 2003). Sca-1<sup>+</sup> cells express some of the early regulatory factors of cardiogenesis, such as GATA4, and they can be induced to express Nkx2.5 and sarcomeric proteins after 5-azacytidine (5-Aza-C) treatment. When given intravenously in mice, they home to infarcted myocardium and yield cardiomyocytes around the injury area, although a degree (about 50%) of cell fusion with resident myocytes was noted (Oh et al., 2003). A rare Sca-1<sup>+</sup>/c-Kit<sup>+</sup> cardiac cell type that differentiates into cardiomyocytic cells upon oxytocin treatment was also identified in mice (Matsuura et al., 2004). Finally, a Sca-1<sup>+</sup>/CD31<sup>-</sup> cell population was isolated and induced to differentiate to both cardiomyocytes and endothelial cells using combinations of fibroblast growth factor (FGF) proteins, the Wnt antagonist Dkk-1, and 5-Aza-C treatment (Wang et al., 2006). Transplantation of Sca-1<sup>+</sup>/CD31<sup>-</sup> cells after myocardial infarction in mice improved cardiac function and enhanced neovascularization. However, the benefits seem to be the result of favorable paracrine effects of the transplanted cells on new blood vessel formation and resident cardiomyocytes (Wang et al., 2006).

### Cardiosphere-derived progenitor cells

An elaborate technique for isolation of cardiac progenitors from mouse hearts and human biopsies was described following gentle enzymatic digestion to release round cells that form so-called cardiospheres (CS) in suspension (Messina et al., 2004; Smith et al., 2007). CS-derived cells express endothelial and stem cell markers; show contractile activity in culture; and can differentiate into cardiomyocytes, endothelial cells and smooth muscle cells. Transplantation of these cells improved ventricular function in mice and swine owing to the formation of vascular and cardiac cells, and positive paracrine effects (Smith et al., 2007; Takehara et al., 2008). However, other attempts to obtain cardiac progenitor cells following a similar approach, but without cardiosphere formation, failed to recapitulate the differentiation and regenerative potential of these cells (Shenje et al., 2008).

### Adult cardiac homeostasis and repair

The isolation of various cardiac tissue stem cell types with novel characteristics suggests that they represent either distinct cell populations or different developmental stages of a single cell lineage. Much of the origin and biological properties of cardiac stem cells remain poorly defined. It is unclear whether the cardiogenic stem cells home from the BM, reside in specialized niches, are remnants of embryonic cell populations, or are artifacts of the isolation procedures (Slack, 2008). These possibilities are not mutually exclusive.

### Epithelial- and endothelial-to-mesenchymal transitions generate cardiovascular progenitor cells during embryogenesis

It is reasonable to expect that the mechanisms that give rise to cardiac progenitor cells in the adult heart share similar pathways with the specification and differentiation of embryonic cardioblasts. During development, a key process that generates progenitor cells that differentiate into various tissues is the epithelial-to-mesenchymal transition (EMT). EMT produces mesodermal cells from primitive ectoderm during gastrulation and neural crest stem cells from neuroepithelium (Nakaya and Sheng, 2008; Sauka-Spengler and Bronner-Fraser, 2008). Although cardiac development is a complex morphogenetic operation (van den Berg and Moorman, 2009; Perez-Pomares et al., 2009) involving the well-orchestrated action of scores of genes (Brand, 2003; Olson, 2006), it can also be divided into a series of EMT events, each generating a distinct set of cardiovascular progenitor cells that differentiate into the cellular components of the mature heart.

The first EMT that gives rise to cardiovascular progenitor cells takes place during gastrulation, when epithelial cells of the epiblast begin to delaminate, adopt mesenchymal characteristics, and migrate to form the mesoderm layer that occupies the space between the two outer epithelia of endoderm and ectoderm (Nakaya and Sheng, 2008). The timing and location of epithelial cell delamination within the primitive streak determines the destination of the migrating mesodermal/mesenchymal cells in the developing embryo and this specifies their subsequent fate (Abu-Issa and Kirby, 2007). Mesodermal cells in the lateral plate adjacent to the foregut differentiate into progenitor cells that express early cardiac regulatory genes, such as *Nkx2-5*, *Mef2c* and *Gata4* (Brand, 2003; Moorman et al., 2007). Early cardioblasts are divided into two types representing the primary and secondary cardiac fields (Buckingham et al., 2005).

The cardiac stem cells of the primary field form the original heart tube that surrounds the endocardial vascular layer, contributing mainly to the future left ventricle; the secondary field cells then migrate and wrap around the primitive heart and contribute to the formation of the atria, the right ventricle, and part, or most, of the left ventricle (Cai et al., 2003; Yang et al., 2006; Sun, Y. et al., 2007; Prall et al., 2007; Ma et al., 2008). The role of the primary heart field progenitors may be limited to the formation of the original cardiac tube during embryogenesis, whereas the secondary field cells, marked by *Isl1*<sup>+</sup> expression, are maintained throughout development, contribute to the neonatal heart growth, and remain present, albeit in small numbers, in the adult heart (Laugwitz et al., 2008). Isolated *Isl1*<sup>+</sup> cells can give rise to cardiomyocytes, smooth muscle and endothelial cells (Laugwitz et al., 2005; Moretti et al., 2006).

The second mesenchymal transformation that shapes the heart takes place when a subpopulation of endocardial cells (the endothelial cells of the original inner cardiac tube) in the atrioventricular canal area undergo endothelial-to-mesenchymal transition (EndMT), migrate into the adjacent cardiac jelly, and build the endocardial cushions that develop into the heart valves (Person et al., 2005; Norris et al., 2008). There is evidence that EndMT continues in the adult valves, supplying cells to maintain and repair the valvular leaflets (Yang, J. et al., 2008).

The third EMT occurs in the epithelium formed by the epicardial cells at the outer surface of the heart. Pre-epicardial tissue, which appears as a cauliflower structure from splachnopleuric mesoderm during development, attaches to the exterior surface of the heart and spreads out over the entire organ in a single epithelial cell layer called the epicardium (Winter and Gittenberger-de Groot, 2007). Shortly thereafter, the epicardial epithelium undergoes EMT, generating a mesenchymal stem cell population named the epicardial-derived progenitor cells or EPDCs. EPDCs invade the cardiac tissue and differentiate into interstitial fibroblasts, perivascular fibroblasts and smooth muscle cells of the developing coronary blood vessels (Reese et al., 2002; Wessels and Pérez-Pomares, 2004). Cell-lineage-tracing experiments using Cre-recombinase technology show a considerable contribution of epicardium to ventricular myocytes, suggesting a more substantial role of EPDCs in heart tissue formation than was thought previously (Cai et al., 2008; Zhou et al., 2008). Transplantation of embryonic EPDCs improved cardiac function after myocardial infarction in mice, but the cells did not differentiate into cardiovascular cells (Winter et al., 2007).

The fourth EMT takes place in the neural tube and generates the cardiac NC progenitor cells, between the cranial and trunk neural crest, that migrate to the heart and take part in the remodeling of the aortic arch arteries and the septation of the common outflow tract into the aortic and pulmonary arteries (Hutson and Kirby, 2007; Snider et al., 2007). Lineage-tracing experiments, using transgenic mice with gene markers that are under the control of NC-specific promoter elements or Cre recombinase technology, suggest that NC cells also contribute to the conduction system and epicardium, but this remains controversial (Stottmann et al., 2004; Stoller and Epstein, 2005; Poelmann et al., 2004; Nakamura et al., 2006). Recent reports show that nestin<sup>+</sup> stem cells of neural crest origin reside in the adult heart and take part in de novo blood vessel formation and reparative fibrosis after ischemic injury (El-Helou et al., 2008).

### EMT and EndMT contribute to cardiac repair and regeneration after injury

Although the importance of EMT in tissue development during embryogenesis is well documented, the contribution of EMT in regenerative processes in the adult organism is not. EMT has been implicated in the emergence of tumor cells that are highly proliferative (or carcinogenic) and motile (or metastatic), suggesting that it plays a key role in the generation and spreading of tumor stem cells (Hollier et al., 2009). A recent study indicates that EMT also yields adult cells with stem cell characteristics (Mani et al., 2008). Therefore, one could predict that, in line with their role during heart development, EMT and EndMT contribute to the pool of cardiovascular progenitor cells to maintain cardiac homeostasis.

Besides the connection of mesenchymal transition to the emergence of stem cells, both EMT and EndMT are recognized as important mechanisms in the generation of the myofibroblasts that take part in fibrosis (Kalluri and Neilson, 2003; Wynn, 2008). Therefore, it is possible that EMT and EndMT contribute to both cardiac regeneration and fibrosis after injury. In the last few years a number of studies have provided supportive evidence for this notion.

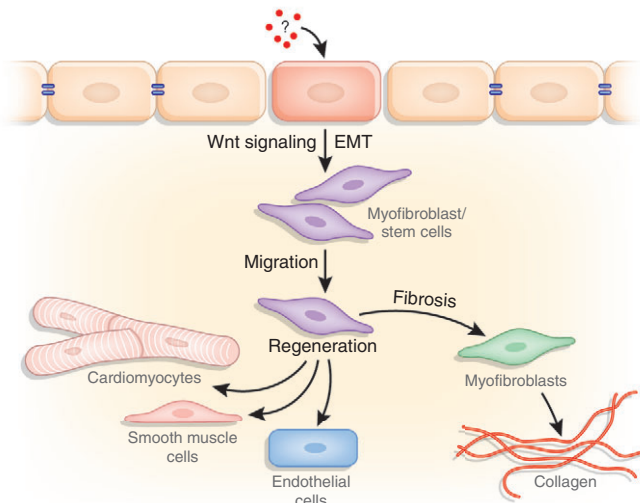
For example, EndMT in the adult mouse heart gives rise to myofibroblasts that migrate and produce scar tissue in mouse models of pressure overload and chronic allograft rejection, recapitulating pathways that take place during formation of the atrioventricular cushions in the embryonic heart (Zeisberg et al., 2007). Moreover, isolated adult epicardial cells can undergo EMT in culture, differentiating to smooth muscle and endothelial cells (van Tuyn et al., 2007; Smart et al., 2007). Epicardial cells that express stem cell markers are induced after injury, migrate to the infarct, and contribute to both cardiac and vascular cell types (Limana et al., 2007). These experiments suggest a function of epicardial cells that echoes their role in the regeneration of the adult zebrafish heart after partial resection (Lepilina et al., 2006). Therefore, both endothelial and epicardial cells become activated after injury and give rise to cardiac, vascular and myofibroblast/smooth muscle cells; this seems similar to their capacity during development.

### Wnt signaling, stem cells and fibrosis

The experimental evidence outlined above links EMT to the production of stem cells and fibrosis. Although these two processes are controlled by complex regulatory networks (Neilson, 2006; Lowry and Richter, 2007), a common molecular thread that has been studied extensively is the canonical Wnt signaling pathway (Thiery and Sleeman, 2006; Nusse, 2008). The canonical Wnt pathway has been implicated in the generation, maintenance and growth of all stem cell types that have been investigated so far, including neuronal stem cells, hematopoietic stem cells, gut epithelium stem cells, muscle satellite cells, hepatic stem cells, lung stem cells and others (Kalani et al., 2008; Luis et al., 2009; Haegerbarth and Clevers, 2009; Otto et al., 2008; Yang, W. et al., 2008; Zhang et al., 2008). The canonical Wnt pathway is also crucial for the formation of embryonic cardiac stem cells in vitro and in vivo (Nakamura et al., 2003; Kwon et al., 2007; Lin et al., 2007; Qyang et al., 2007; Cohen et al., 2008; Laugwitz et al., 2008). Conversely, blockade of canonical, and activation of non-canonical, Wnt signaling is crucial for the differentiation of embryonic and adult progenitor cells into cardiomyocytes (Koyanagi et al., 2005; Flaherty et al., 2008; Cohen et al., 2008; David et al., 2008).

The canonical Wnt pathway inactivates glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) leading to dephosphorylation and stabilization of cytoplasmic  $\beta$ -catenin, which then translocates to the nucleus where it activates a host of target gene loci. Among them are the transcriptional repressors Slug and Snail that shut down the expression of the cell adhesion protein E-cadherin to loosen the junctions between epithelial cells (Thiery and Sleeman, 2006; Nusse, 2008). Liberated epithelial cells quickly rearrange their cytoskeletal structure and adopt a highly proliferative phenotype with a mesenchymal morphology; this is regulated by  $\beta$ -catenin, which induces genes such as those encoding cyclin D and c-Myc (Clevers, 2006). Thus, Wnt signaling is a central regulatory knot





**Fig. 2. A model of cardiac homeostasis and repair.** Normal wear and tear, as well as acute ischemic injury, induces unknown factors (?) that stimulate Wnt signaling-mediated epithelial-to-mesenchymal transition (EMT), generating mesenchymal cells with dual stem cell and myofibroblast characteristics. Favorable conditions allow stem cells to differentiate to various cardiovascular cell types, whereas the disease environment favors a switch to a pro-fibrotic phenotype leading to scar formation.

that controls two of the landmark events in the biology of stem cells, EMT and cell cycle regulation. Interestingly, Wnt signaling also regulates fibrosis, controlling the generation of mesenchymal cells by EMT as well as their proliferation and motility (Cheon et al., 2002; Bowley et al., 2007; Alfaro et al., 2008). Collectively, these data suggest that proper modulation of Wnt signaling is key in balancing cardiac fibrosis and regeneration after injury.

### Reparative and regenerative cells: two sides of the same coin?

The parallels between the cellular and molecular biology of reparative (myofibroblasts) and regenerative (stem) cells raise the possibility that the two cell phenotypes are connected intrinsically. Moreover, similar to stem cells, myofibroblasts have been shown to derive from multiple sources, such as the BM and resident MSCs, as well as by EMT and EndMT (Hinz et al., 2007). It is possible that mesenchymal cells with stem cell properties follow a default pathway after injury that steers them towards producing scar tissue in order to seal off the necrotic areas and save the surrounding healthy penumbra. These same cells might differentiate to tissue-specific cell types when the conditions favor replacement of malfunctioning or dying cells during organ homeostasis (Fig. 2). This model is consistent with recent findings in skeletal muscle where stem cells switch from a regenerative to a pro-fibrotic phenotype with aging, owing to increased Wnt signaling (Brack et al., 2007).

### Too many stem cells, too little regeneration?

Our current knowledge suggests that a multicellular army of progenitor cells, of BM, endothelial, epicardial and neural crest origin, respond to cardiac tissue injury and take part in the repair process. Why is there a need for so many different cell types? Does a particularly crucial situation, resulting from a catastrophic event,

mobilize all of the available forces at the disposal of the organ? Or, do the different cell types perform distinct functions, such as replenishing specific cell types, including cardiomyocytes, endothelial cells, smooth muscle cells, conduction system cells and neurons, that have been destroyed during ischemic injury?

If progenitor cells are mobilized after injury, why does the function of myofibroblasts and fibrosis predominate? This is particularly disappointing, since the various stem cells that have been analyzed are able to produce cardiomyocytes, smooth muscle cells and endothelial cells in vitro. It is plausible that stem cells perform their respective roles under normal conditions to maintain cardiac homeostasis, but assume a reparative or pro-fibrotic phenotype when confronted with inflammatory proteins, toxic products of apoptotic cells, ischemia and a disintegrating extracellular matrix. Consistent with this, MSCs adopt different fates depending on extracellular matrix tension and other environmental factors (Engler et al., 2006). It is also conceivable that only myofibroblasts survive in the hostile disease environment.

Over the last few years, we have learned a great deal about the individual pieces in the reparative and regenerative puzzle of the heart, but it is unclear how the different components will fit together. Although the findings point to a complex healing process that relies on many different types of repair/stem cells, they reflect the natural program of cardiopoiesis, where a variety of progenitor cells contribute to new cardiac tissue at distinct stages of embryonic development. Our current challenge is to isolate and study the different stem cell-like populations in the heart and identify their individual roles during cardiac repair and regeneration. It is also important to understand how the mission of progenitor cells is adjusted during a severe injury such as a myocardial infarction. If progenitor cells in the heart are confronted with a choice between tissue regeneration and scar formation, it is crucial to recognize the intrinsic molecular mechanisms and environmental factors that determine the outcome of this decision. This information will help devise novel strategies to fortify the endogenous regenerative capacities of the adult heart and to optimize the cardiovascular differentiation of transplanted stem cells.

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The authors declare no competing financial interests.

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