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Molecular mechanisms of cardiomyocyte regeneration and therapeutic outlook

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Differently from some lower vertebrates, which can completely regenerate their heart, in higher vertebrates cardiac injury generally leads to progressive failure. Induction of cycle re-entry in terminally differentiated cardiomyocytes and stem-cell transplantation are strategies to increase the regenerative potential of the heart. As experimental and clinical studies progress, demonstrating that adult stem-cell administration has a favorable impact on myocardial function, the identification of cardiac stem cells suggests that some endogenous repair mechanisms actually exist in the mammalian heart. However, a deeper understanding of the mechanism that drives cardiomyocyte proliferation and stem-cell-mediated cardiac repair is required to translate such strategies into effective therapies.

Introduction

Myocardial regeneration is of obvious clinical relevance given the increasing prevalence of acute and chronic heart disease leading to contractile mass loss. However, the knowledge of the molecular mechanisms underpinning cardiomyocyte regeneration is still primordial compared with that of other cell types such as skeletal muscle cells. Among many reasons for this delay, there is the absence of suitable cell lines enabling easy gene expression manipulation and the notion, which has been ignored until a few years ago, that cardiomyocytes can be replaced in the adult mammalian heart.

The aim of this review is to describe recent progress in the field and to highlight the most promising approaches that might lead to effective therapeutic heart regeneration.

Mechanisms regulating spontaneous cardiomyocyte regeneration: from lower vertebrates to mammals

In contrast to mammals, lower vertebrates such as newt and zebrafish are able to regenerate their heart completely, even after a cardiac mass loss as high as 20% [1–3]. The regenerative process depends on the plasticity of cardiomyocytes (see Glossary), which revert to proliferating progenitor cells and later re-differentiate to replace the injured tissue. However, the existence of undifferentiated cardiac progenitor cells in the damaged heart of adult zebrafish has been recently suggested [4].

Glossary

Apoptosis: a tightly regulated form of cell death. At morphological level, it is first characterized by chromatin condensation and cell shrinkage. Then, the nucleus and cytoplasm fragment, forming membrane-bound apoptotic bodies that can be engulfed by phagocytes without triggering inflammation.
Cardiac hypertrophy: an adaptative process of the heart to increased workload caused by mechanical stress, growth factors, cytokines or catecholamines.
Differentiation: process by which cells acquire specific functional and morphological characteristics activating a specific gene expression profile.
Dominant negative mutant: an allele of a gene that, when overexpressed, is both non functional *per se* and capable of inactivating the function of the endogenous wild-type counterpart.
Failing heart: a heart that has lost contractility, functional vasculature and electrical integrity.
Fusion: originally, this term referred to the process through which two or more cells of the same origin join. Fusion normally occurs between skeletal muscle cells. Recently, this term has been used to indicate the merging between stem and differentiated cells. If fusion does not involve nuclei, a binucleated cell is formed with the phenotypic features of the differentiated cell. If fusion is accompanied by nuclear fusion, a mononucleated tetraploid cell is obtained.
Hibernating myocardium: condition of regional ventricular dysfunction that is characterized by the presence of hypocontractile areas of viable myocardium. It reverses gradually after revascularization.
Hoechst 33342: a vital dye that stoichometrically binds to AT-rich regions of DNA minor groove.
Master gene: gene, the expression of which promotes a specific differentiation pathway.
Microarray analyses: technologies to investigate the expression levels of thousands of genes simultaneously.
MicroRNA: small RNA (21 bp) involved in the regulation of gene expression.
Niches: microenvironments that provide supporting cells and signals necessary for self-renewal and differentiation of stem cells.
Oncoprotein: the product of an oncogene. An oncogene is a gene that, when mutated or overexpressed, causes or increases the malignancy of a cancer cell. Most oncogenes either increase the rate of cell proliferation or decrease the rate of apoptosis.
Plasticity: ability of cells to differentiate into a cell type that is different from that of the tissue in which the cell resides or derives during embryonic development.
Proteomic profile: technology to investigate total protein content of cells or tissues.
Scar: fibrous tissue resulting from the biological process of wound repair.
Side population (SP): population of stem cells identified by cytofluorimetric analysis as low fluorescent cells after staining with the Hoechst 3342. The expression, on membrane of pumps, including the ABCG2 and MDR1, enables the efflux of the dye causing the low staining of the cells. The activity of the membrane pumps is blocked by verapamil inducing SP fraction disappearance.

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The transcription profile of the regenerating zebrafish heart, examined using microarray analyses, has recognized several genes that are activated during the regenerative process [5]. Among the secreted molecules that seem to have a key role during the remodeling of the damaged heart, vascular endothelial growth factor (VEGF)-C, platelet-derived growth factor (PDGF)-A and PDGF-B, thymosin β4 and metalloproteinases (MMPs) have been identified. Notably, VEGF-C is required for vascular development of zebrafish [6], whereas PDGF-A and PDGF-B have a crucial role in initiating cardiomyocyte proliferation by inducing DNA synthesis [5]. Thymosin β4 might be involved in promoting cardiac cell migration and survival, as described in a mouse model of myocardial infarction [7,8]. Finally, MMPs, which are involved in the degradation of the extracellular matrix, might exert their effects by preventing scar formation. Indeed, in agreement with the hypothesis that scar formation and regeneration in lower vertebrates represent mutually exclusive events, it has been shown that MMP inhibitors enable scarring and abolish limb regeneration in newts [9]. Similarly, in the zebrafish heart, mutation of the Mps1 mitotic checkpoint kinase leads to scar formation instead of regeneration [3].

It is worth noting that heart regeneration in zebrafish results from the activation of a specific program and might not derive from the re-activation of cardiac development program. In fact, members of the Msx transcription factor family and components of the Notch pathway, the activation of which controls the switch between proliferation and differentiation in adult stem cells, are upregulated in regenerating but not in developing zebrafish heart [10].

The mammalian heart has long been considered an organ that is unable to regenerate, in which all cardiomyocytes terminally differentiate and irreversibly exit the cell cycle shortly after birth. The identification of regenerating cardiomyocytes after acute [11,12] and chronic infarction [13] and in failing hearts [14] changed this dogma and supported the view of the heart as a dynamic organ constituted by myocytes that can be replaced. Cycling myocytes are thought to originate from cardiac stem cells (CSCs) that are resident in the heart [11]. CSCs, first isolated by the group of Anversa and characterized by the expression of the stem-cell factor c-KIT [together with stem-cell antigen-1 (SCA-1) and multidrug resistance 1 (MDR1)], are clonogenic, self-renewing and multipotent cells that can give origin to cardiomyocytes, endothelial cells and smooth muscle cells [11]. Many following studies documented the presence of CSCs in the mouse [15–22], dog [12] and human heart [13,19]. In these studies, different methods of isolation and different stem-cell markers have been used to identify CSCs. In mouse hearts, Schneider's group described stem cells that express SCA-1 and are negative for c-KIT [16]. Recently, cardiac progenitors that express the homeobox gene islet 1 (ISL1) have been described in the postnatal rat, mouse and human myocardium [21,23]. ISL1$^+$ cells can be considered cardiomyocyte precursor cells that can give rise to fully differentiated cardiomyocytes; however, it is currently unknown whether they exist in adult hearts. More details about the different CSC populations are reported in Box 1 and Table 1.

Box 1. Cardiac stem cells

Different methods of isolation and characterization documented the presence in the heart of several stem-cell populations that have been identified for the expression of different stem-cell markers. Here, we used CSCs to indicate different populations of heart stem cells, even if this term was originally attributed to cells that had a determined clonogenicity, self-renewal and multipotency (capacity to give origin to cardiomyocytes, smooth muscle cells and endothelial cells) [11]. However, at present these properties have not been investigated to the same extent among the different heart stem cells. Further studies are needed to assess whether they belong to independent pools or represent differentiation steps of the same lineage, and also to determine whether technological differences in the detection of a limited number of markers leads to unnecessary distinctions.

To simplify, we grouped CSC populations based on the expression of three specific antigen markers: c-KIT, SCA-1 and ISL1.

c-KIT$^+$ cell populations. CSCs that express c-KIT have been identified by several groups using different techniques [11,18,19]. The c-KIT$^+$ cells can be divided in subgroups based on the stem-cell antigen markers that are used in association with c-KIT for their characterization. At present, it is unclear whether these cells are different functional populations or the same cell population at different stages of differentiation. Cells that express c-KIT together with SCA-1 differentiate *in vitro* into the main cardiac lineages and regenerate myocardium after infarction.

SCA-1$^+$ cell population. CSCs that express SCA-1 represent ~20% of non-cardiomyocyte heart cells [16]. From this fraction, a subpopulation of cells has been isolated for its capacity to exclude the Hoechst dye 33342 (side population, SP) [16]. Together with SCA-1, these cells are positive for the transport protein ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2) [18]. SCA-1 cells give origin to cardiomyocytes *in vitro* and *in vivo* where they fuse with host cells. Fukuda's group characterized SP cells in the mouse heart that express markers of neural precursor cells and can give origin to cardiomyocytes *in vitro* and *in vivo* [22]. Whether these cells are also positive for SCA-1 and represent a similar population to those indicated as SP is unknown.

ISL1$^+$ cell population. ISL1 is a homeobox gene, the expression of which identifies cardiac precursors cells that contribute to embryonic heart development. Recently, ISL1$^+$ cells have been isolated from rodent and human neonatal hearts and their ability to differentiate into functional cardiomyocytes has been demonstrated in *in vitro* experiments [21].

The number of CSCs that express c-KIT, SCA-1 and MDR1 increases after infarction [13,24] and in aortic stenosis-induced cardiac hypertrophy [14]. By contrast, their number decreases during aging [25,26], diabetes [27] and heart failure [13] because of the imbalance between proliferation and apoptosis. However, mechanisms that regulate CSC proliferation and differentiation are largely unknown. Both physical forces and biochemical signals can regulate their behavior. CSCs reside in niches that are mostly located in the atria and apex, where they are connected via junctional proteins to support cells such as myocytes and fibroblasts [28]. CSCs express receptors for various growth factors, cytokines and chemokines, including the receptors for VEGF, hepatocyte growth factor (HGF), insulin growth factor (IGF), fibroblast growth factor (FGF), serum-derived factor 1 (SDF1) [12] and for the cytokine high mobility group box 1 (HMGB1) [24]. Although the administration of these factors might modulate CSC function (see next section), further studies are required to understand the

Table 1. Characteristics and properties of different CSC population

CSC populations	Markers	Methods of isolations	Species	*In vitro* differentiation	*In vivo* differentiation (in animal models of cardiac tissue damage)	Fusion with cardiomyocytes
c-kit	Sca-1$^+$, MDR-1$^+$	Immunomagnetic sorting FACS	Rat Mouse Dog Human	CM EC SMC	Intramuscular injection after MI and intravascular injection after I/R. Differentiation in CM, EC, SMC	No
	Sca-1$^+$ CD34$^+$ Flk-1$^+$ CD31$^+$	Cardiospheres	Mouse Human	CM EC	Intramuscular injection after MI. Differentiation in CM and EC	ND
Sca-1	CD31$^+$	Immunomagnetic sorting Hoechst 33342	Mouse	CM	CM	Yes, *in vivo*
	CD31$^-$	Hoechst 33342	Mouse	CM	ND	< of 2%
	Abcg2$^+$	Hoechst 33342	Mouse	CM	ND	ND
	Nestin$^+$ Musashi$^+$ MDR-1$^+$	Hoechst 33342	Mouse Rat	CM SMC Neurons Glia	ND	ND
Isl-1	Nkx2.5$^+$ GATA4$^+$	FACS	Mouse Rat Human	CM	ND	ND

CM, cardiomyocyte; EC, endothelial cells; SMC, smooth muscle cells.
MI, myocardial infarction; I/R ischemia riperfusion.

molecular mechanisms that enable the generation of new cardiomyocytes from CSCs.

Mechanisms of induced cardiac regeneration

As mentioned earlier, the postnatal heart displays some regenerative mechanisms, but it is incapable of activating a complete regeneration program in case of extensive injury such as a massive infarction. Consequently, strategies to enhance heart repair are required. Currently, three general approaches are followed: (i) stimulation of endogenous cardiovascular progenitors, (ii) transplantation of regeneration-competent stem cells and (iii) induction of endogenous cardiomyocyte proliferation (Figure 1).

Activation of the local regenerative system of the heart by cytokines and growth factors

The existence of growth-factor receptor systems on CSCs indicates that it is possible to stimulate these cells to

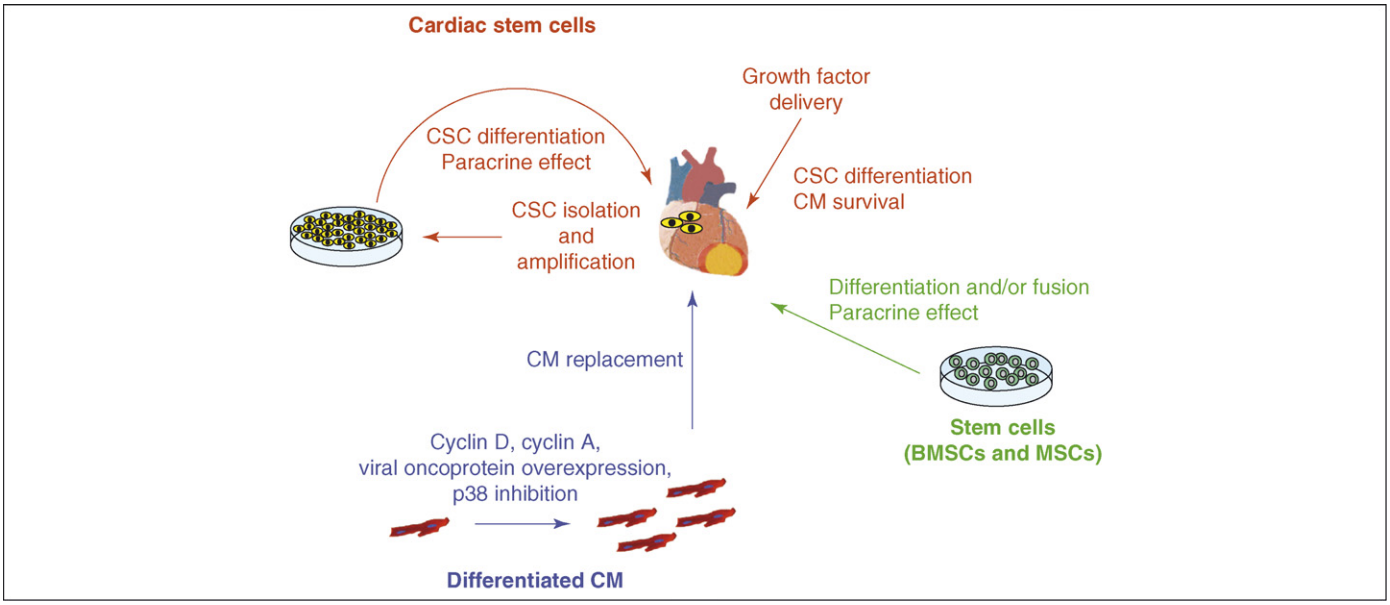

Figure 1. Cellular and molecular approaches for cardiac repair. Three possible strategies are schematically depicted: stimulation of endogenous cardiac stem cells, adult stem-cell transplantation and induction of endogenous cardiomyocyte proliferation. Cardiac stem cells can be locally activated by growth-factor administration. Alternatively, cardiac stem cells and stem cells that are isolated from other tissues might be isolated, *in vitro* amplified and used for autologous transplantation. Transplanted cells might exert their effect by differentiating into cardiovascular lineages (cardiomyocytes, endothelial cells and smooth muscle cells) and/or by secreting paracrine mediators. These factors might improve vessel formation, cardiomyocyte survival and cardiac stem-cell activation. Terminally differentiated cardiomyocytes might be induced to proliferate by direct targeting of cell-cycle modulators. Possible applications of CSCs and other adult stem cells are indicated in red and green, respectively. The strategy to manipulate and induce adult cardiomyocyte proliferation is depicted in blue. Abbreviations: BMSCs bone-marrow stem cells; CM, cardiomyocyte; CSCs, cardiac stem cells; MSCs, mesenchymal stem cells.

promote their activation. Accordingly, IGF-1 and HGF delivery in the mouse [12,17] and dog [12] infarcted heart stimulates CSC migration to the infarcted area and promotes both their proliferation and survival.

We have recently shown that HMGB1 can modulate CSC function [24]. HMGB1 is a nuclear protein secreted by monocytes and/or macrophages in response to pro-inflammatory cytokines and released from necrotic cells. In a mouse model of myocardial infarction, HMGB1 administration enhanced CSC proliferation and differentiation into cardiomyocytes, improving cardiac function.

Other factors might affect CSC function. FGF-2 and its receptor FGF receptor 1 have an important role in cardiogenic differentiation *in vitro* and *in vivo*. Notably, CSCs derived from FGF-2 knock out (*FGF-2*$^{-/-}$) mice exhibit cardiac homing when injected intravenously both in wild-type and in *FGF-2*$^{-/-}$ mice, but differentiate into cardiomyocytes only when the recipient animals can produce FGF-2 [29]. Other members of the FGF family might have similar properties. FGF-5 delivery to a swine model of hibernating myocardium improves blood flow and induces cell-cycle re-entry of small cardiomyocytes; whether these small myocardial cells derive from CSCs remains to be determined [30].

Recent observations demonstrate that VEGF administration in both the dog [31] and sheep [32] infarcted heart restores cardiac function improving neovascularization and myocardial tissue viability. Besides the angiogenic effect, VEGF might enhance CSC recruitment, activating these cells both directly and in a paracrine manner. Grunewald *et al.* [33] reported that VEGF-mediated neovascularization in uninjured target organs, including the heart, involves the release of SDF1 by perivascular fibroblasts. SDF1, in turn, is required for tissue-specific retention of bone-marrow-derived stem cells (BMSCs). Notably, SDF1 delivery in a rat model of ischemic cardiomyopathy enhanced BMSC recruitment and new blood vessel formation into the damaged tissue [34]. Whether SDF1 can induce CSC proliferation and differentiation is still unknown. Nonetheless, the possibility to activate endogenous CSCs by cytokines and growth factors that are delivered into the heart without the need of their *ex vivo* manipulation might represent a promising tool for the treatment of heart-related degenerative diseases.

Cardiac regeneration by stem-cell therapy

Stem-cell transplantation is an established therapeutic approach for cardiac repair. Currently, various stem-cell populations have been analyzed for their ability to induce cardiac regeneration. *In vitro* expanded CSCs from heart biopsies might represent an obviously promising tool for autologous cell transplantation therapy [11,35]. However, little is known about signals and molecular mechanisms regulating their proliferation and differentiation into cardiovascular cells (Box 1 and Table 1). Understanding these events might enable harnessing their clinical potential. In this respect, lessons from embryonic stem cells (ES) might help to deepen the understanding of molecular mechanisms that regulate cardiac and other adult stem-cell differentiation.

Embryonic stem cells

The differentiation of ES into cardiomyocytes and other lineages is a spontaneous event that follows the formation of aggregates called embryoid bodies. Interestingly, ES differentiation depends, at least in part, on regulatory mechanisms directing normal early embryo development. Members of several growth factor families that are known to be involved in cardiac mesoderm induction, such as transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), FGFs and WNTs, are effective in stimulating the cardiomyogenic differentiation of ES lines and have been employed to enrich ES cultures for the cardiogenic lineage [36]. Embryoid bodies that are stimulated with such growth factors show an increased potential for cardiac differentiation that is concomitant with an increase in beating areas. Nonetheless, scarce evidence is available about the exact mechanisms that mediate ES cardiomyocyte differentiation. Experiments conducted in our laboratory show that ES exposure to shear stress causes chromatin remodeling and induces the expression of cardiac and vascular markers [37]. Other recent studies have provided evidence that both BMP inhibition by noggin (NOG) [38] and the absence of NOTCH-1 signaling [39] before embryonic-body formation greatly enhances cardiogenesis and the induction of the mesodermal marker Brachyury. These results suggest that BMP and/or NOTCH-1 signaling are required for mesodermal induction of ES and are therefore essential for their subsequent cardiogenic differentiation. Moreover, because in several systems NOTCH and BMP cooperate to block downstream differentiation events [40], it is possible that these two pathways crosstalk in the same way during ES cardiac induction.

In vivo studies based on heart transplantation of both murine and human ES demonstrate ES ability to contribute to a consistent number of newly formed cardiac myocytes and, therefore, highlight their therapeutic potential [41–43]. However, several issues limit ES use. In addition to obvious ethical constraints, methods to obtain highly pure and terminally differentiated stable cardiac grafts are not yet available. Therefore, problems related to the presence of unwanted non-cardiac cells, tumorigenicity and immunogenicity need to be solved before clinical application of ES can be possible [44].

Adult stem cells

The most extensively used stem cells for cardiac regeneration include BMSCs, mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) (Table 2). Differently from ES cells and with the exception of CSCs, no methods have been established to induce a functional cardiac phenotype in adult stem cells *in vitro* using physiological growth factors or non-toxic chemical compounds. Also, even if master transcriptional regulators (i.e. MyoD) are known for the skeletal muscle lineage, a cardiac master gene, the overexpression of which imposes a switch towards the cardiac lineage in non-cardiac cells, has not been identified. Interestingly, forced expression of the transcriptional activator myocardin can induce the expression of early and late cardiac markers in human MSCs, although such treatment is not sufficient to obtain a functionally competent cardiac phenotype [45].

Table 2. Possible sources of stem cells for cardiac repair

Stem cells	Markers	Direct effect: Differentiation into functional cardiac cells		Indirect effect		Clinical Trials	
		In vitro	*In vivo*	Paracrine action	Fusion	SC transplanted	Safety/Efficacy
ES		EC CM	EC CM	ND	ND	Yes	ND/ND
BMSC	Mononucleated cells	EC CM	EC CM	Yes	Yes	Yes	Yes/Yes
	MSC	EC CM	EC CM	Yes	Yes	Yes	Yes/ND
	c-kit$^+$	CM EC SMC	CM EC SMC	ND	Controversial	ND	ND
EPC	CD34$^+$	CM EC	CM EC	Yes	Yes	Yes, *in vivo*	Yes/Yes
	AC133$^+$	EC	EC	ND	ND	Yes	Yes/ND
Skeletal myoblasts		Skeletal muscle cells	Skeletal muscle cells	ND	ND	Yes	Uncertain/Yes

ES, embryonic stem cells; BMSC, bone marrow stem cells; EPC, endothelial precursor cells; MSC, mesenchymal stem cells, CM, cardiomyocyte; EC, endothelial cells; SMC, smooth muscle cells.
MI, myocardial infarction; I/R, ischemia riperfusion; ND, not determined.

Nonetheless, numerous *in vivo* studies have shown that transplantation of adult stem cells can improve left ventricular function and reduce adverse remodeling after myocardial infarction [46–48]. Differentiation towards the cardiac and vascular lineages by transplanted stem cells has been originally proposed as a mechanism underlying such therapeutic benefit. Anversa and collaborators showed that BMSCs expressing the c-KIT antigen, when transplanted into the infarcted myocardium, differentiate into cardiomyocytes and vascular cells and significantly improve ventricular function [49]. However, other research groups have subsequently failed to detect permanent engraftment and transdifferentiation of transplanted c-KIT$^+$ cells into cardiac myocytes [50,51]. In addition, fusion of bone-marrow (BM)-derived donor cells with recipient cardiomyocytes or cells of other lineages has been documented in several cases [52–55]. These results have questioned the cardiac plasticity of both endogenous and transplanted BMSCs, indicating cell-fusion events with resident cardiomyocytes as mode of action.

MSCs are present in the BM stroma and in other mesenchymal compartments, including the adipose tissue. The ability of MSCs to acquire a cardiac-like phenotype *in vivo* has been documented [56]. However, in these studies the survival rate of transplanted cells was very low and possible fusion events were not taken into account. In a porcine model of myocardial infarction, immunohistochemical analysis demonstrated the engraftment of human MSCs but, despite several muscle-specific proteins were present, cardiac-specific markers and electromechanical coupling with host cells were not observed [57].

EPCs have been identified in the BM and in the peripheral circulation for the expression of the hematopoietic stem-cell markers CD34, CD133 and VEGF receptor 2. EPCs are able to repair the injured heart, and mechanisms such as therapeutic angiogenesis and induction of endogenous myocardial regeneration have been proposed [58–61]. Moreover, purified human circulating CD34$^+$ cells when implanted in the infarcted myocardium differentiate into cardiovascular lineages through a process that involves both autonomous transdifferentiation and fusion [61,62].

At present, the plasticity towards the cardiac or other lineages of adult stem cells is still a matter of debate. Contrasting results concerning the mechanism through which these cells are supposed to generate new cardiac myocytes continue to appear in the literature.

Recent studies by Anversa's group have confirmed their initial observation: by transplanting green fluorescent protein (GFP)-tagged, c-KIT$^+$ BMSCs from male mice into the infarcted heart of female recipients, they provided evidence for extensive fusion-independent cardiac transdifferentiation of c-KIT$^+$ BMSCs associated to cardiac recovery [63]. In contrast, by BM reconstitution of neonatal mice, it was observed that donor-derived cardiomyocytes were generated exclusively via fusion with host cardiac cells [64]. Finally, several other studies based on direct transplantation in adult recipients suggest that fusion-dependent and cell autonomous events can account for the transdifferentiation towards the cardiac and vascular lineages of adult stem cells both *in vitro* and *in vivo* [61,62,65,66].

Regardless of whether stem cells transdifferentiate via fusion-dependent or -independent mechanisms, it is clear that in many cases the number of newly generated cardiac myocytes is too low to justify functional improvements. It has therefore been proposed that the functional benefits observed after stem-cell transfer in animal models of cardiac injury might in some cases relate to secretion of paracrine factors that can cause the attenuation of pathological ventricular remodeling and the induction of revascularization processes (Figure 1). Accordingly, BMSCs, MSCs and EPCs have been shown to secrete various growth factors with angiogenic and anti-apoptotic properties [67,68]. Using c-KIT mutant mice with an intrinsic defect in BMSC mobilization, Fazel *et al.* [69] observed an accelerated progression to dilated cardiomyopathy following infarction, which is associated to impaired angiogenesis. Notably, both of these phenotypes could be rescued by wild-type BM transplantation. Moreover, by reconstituting

the BM of irradiated mice with GFP-tagged BM cells, these authors provided evidence that c-KIT$^+$ cells of BM origin migrated to the infarcted heart and constituted the majority of heart-resident c-KIT$^+$ cells after infarction [69]. Nevertheless, only few BM-derived cells engraft in the long term and did not give rise to new cardiomyocytes or vessel-associated cells, suggesting that BM-derived c-KIT$^+$ cells might have a positive effect on cardiac repair by promoting neoangiogenesis via paracrine signaling pathways [69].

An interesting study performed with a monolayer of cultured adipose-tissue-derived MSCs shows that transplantation of these cells in rats with myocardial infarction reverses wall thinning in the scar area and improves cardiac function [66]. The engrafted monolayer includes newly formed vessels, undifferentiated cells and few cardiomyocytes, part of which are generated via fusion with resident cells [66]. However, because the presence of donor-derived cardiomyocytes is rare, transdifferentiation is not sufficient to explain the functional improvement. Implanted cells secrete a large amount of angiogenic and anti-apoptotic cytokines, suggesting that transplanted adipose-tissue MSCs, similar to BM MSCs and to a recently described new subpopulation of human BM-derived cells [65], act mostly through paracrine pathways. Interestingly, it has been recently shown that MSCs overexpressing the survival factor AKT (also known as protein kinase B, PKB) exert their effects of cardiac protection and functional improvement through paracrine mechanisms [70]. These conclusions are based not only on the observation that the functional improvement occurs only 72 hours after transplantation, before any possible effect due to cell transdifferentiation can manifest, but also on the fact that most of the benefits can be reproduced by injecting cell-free supernatants from AKT-MSC cultures [70].

In conclusion, regardless of the underpinning mechanisms, there is a general agreement that stem-cell transplantation has beneficial effects on infarcted heart function and vascularization.

Clinical studies

The first clinical studies on cell-based cardiac repair have been performed with skeletal myoblasts [71,72]. These cells have beneficial effects on ventricular contractile function. However, they neither give origin to new cardiomyocytes nor establish electrical coupling with resident cardiac cells [73,74], suggesting paracrine effects and/or scar tissue replacement as potential therapeutic mechanisms.

Several trials investigating safety and feasibility of unfractionated BMSCs, MSCs and EPCs have been performed (Table 2) and recently reviewed [46,75]. In spite of controversial results about the long-term efficacy of stem-cell transplantation [76–78], most studies suggest an improvement of cardiac function in the short term, even if it remains unclear whether cardiac muscle regeneration occurs.

Cell-cycle regulation of mammalian cardiomyocytes

Adult mammalian cardiomyocytes are terminally differentiated and the molecular events underpinning their permanent withdrawal from the cell cycle have been extensively studied. Even after major cardiac injury, cardiomyocytes are an abundant cell population, and strategies aimed at forcing their cell-cycle block are of obvious therapeutic interest [79]. Transition through the mammalian cell cycle is a tightly regulated event, involving the sequential and coordinated activation of a complex system of proteins that control and assure the biochemical activities required for cell division [80]. These proteins include members of the retinoblastoma (RB) family, their kinase complexes [e.g. cyclin–cyclin dependent kinases (CDKs)] and inhibitors of these kinase complexes, such as p21^{WAF1} and p27^{KIP1}. Not surprisingly, cyclin–CDK complexes, which exert a positive role in cell-cycle regulation, are expressed at high levels in proliferating cells and are downregulated in the postnatal heart, whereas levels of negative cell-cycle regulators such as CDK inhibitors are increased in the adult heart. Therefore, interventions have been directed either to inhibit negative regulators or to overexpress positive regulators of the cell cycle.

Several reports demonstrated the feasibility of this approach, using viral oncoproteins that target crucial cell-cycle machinery components. For example, targeted expression of the adenoviral protein E1A, which binds to and inactivates RB family members, promoted cardiomyocyte cell-cycle progression [81]. This event was followed by increased cardiomyocyte apoptosis, probably due to the activation of the tumor suppressor p53, as described in other cell systems [82]. Interestingly, cardiomyocyte death could be prevented by disabling p53 and p193 pro-apoptotic proteins using either SV40 large T antigen or dominant negative mutants, yielding G1 arrest override in the absence of apoptosis [83,84].

Direct manipulation of the RB pathway led to equally promising results. Mice that have a simultaneous deletion of RB and p130^{RB2} displayed a striking threefold increase in the heart-to-body weight ratio and persistent adult cardiomyocyte cycling [85].

A complementary approach is represented by the deregulation of cyclin–CDK complexes. D-type cyclins are required for the initiation of DNA synthesis, regulating the progression through the restriction point of the cell cycle [80]. Cardiac-specific overexpression of D1, D2 and D3 cyclins is sufficient to induce DNA synthesis in adult cardiomyocytes [83,86], but only cyclin D2 maintains nuclear localization and cell-cycle promotion activity after myocardial infarction, resulting in infarct regression. By contrast, cyclin D1 and D3 translocate from the nucleus to the cytoplasm following myocardial infarction and lose their ability to stimulate DNA synthesis [83]. In agreement with these results, cardiomyocyte hyperplasia has also been detected with targeted expression of cyclin A2 in the heart [87] and in p27-null mice [88].

Modulation of more-upstream signaling elements also promotes cardiomyocyte proliferation in the adult. Overexpression of nuclear AKT in the heart does not modify cardiac shape and size but results in an increased number of cardiomyocytes and the extension of postnatal proliferation up to three weeks after birth [89,90].

Another important molecule involved in the regulation of cardiomyocyte proliferation *in vivo* and *in vitro* is p38 mitogen-activated protein (MAP) kinase [91]. p38 is linked

to the RB pathway, because it regulates the expression of genes that are required for mitosis in cardiomyocytes, including cyclin A and cyclin B. It seems to be also connected to the phosphoinositide-3 kinase (PI3K)–AKT pathway, because it modulates the expression of relevant upstream modulators. p38 activity inversely correlates with cardiomyocyte proliferation, whereas cardiac-specific 38-α knockout mice show a sharp increase in neonatal cardiomyocyte mitosis. Furthermore, inhibition of p38 in adult cardiomyocytes promotes cytokinesis that is associated with a transient de-differentiation of the contractile apparatus.

Finally, unexpected results were obtained by overexpressing B-cell CLL/lymphoma 2 (*BCL2*) anti-apoptotic gene under a cardiac-specific promoter. A significant increase in the fraction of cycling myocytes and their mitotic index was observed, and these events correlated with a decrease in the expression of $p21^{WAF1}$ and with an increase in mouse double minute 2 (MDM2)–p53 complexes [92].

It is noteworthy that most *in vivo* studies have been performed either by constitutive genetic deletion of relevant genes or by overexpressing target genes under the control of specific cardiac promoters that are transcriptionally active at the embryonic stage, when cardiomyocytes can still proliferate. Thus, it is difficult to discriminate whether cardiomyocyte proliferation in the adult heart is due to a true re-activation of cell-proliferation machinery or is the result of altered or incomplete cardiomyocyte differentiation during development. Another potential mechanism might be the expansion of the CSC pool, which might consequently increase the number of cells with an intermediate differentiation status [89,90]. Further studies are necessary and relevant molecular pathways should be manipulated in the adult myocardium that has developed in the absence of interfering signals. In this respect, inducible mouse models and postnatal RNA interference (RNAi) of relevant targets might be particularly informative.

Concluding remarks

Different approaches have been used in the attempt to repair the damaged myocardium. These include activation of the endogenous cardiac stem cells, stem-cell transplantation and induction of cardiomyocyte proliferation. Among such possible therapeutic strategies, only adult stem-cell transplantation has already reached the clinic phase and represents the most promising approach for the treatment of acute myocardial infarction. In this respect, several clinical trials for myocardial infarction based on BMSC therapy are ongoing. These studies demonstrate that stem-cell therapy of the heart is feasible and promising in terms of therapeutic benefits. In addition, the recent identification of CSCs that proliferate and differentiate in the heart giving origin to cardiomyocytes and vascular structures hold great hopes for clinical applications. However, major efforts should be performed not only to compare the therapeutic effects obtained either with BMSCs or with CSC transplantation but also to identify signals that can induce endogenous cardiac stem-cell activation in pathological conditions.

Future directions

Stem cells represent a promising tool for rebuilding the damaged heart. However, in our opinion, a step backwards to basic research is probably needed to understand the molecular mechanisms that underlie the commitment of stem cells to the cardiac fate and to enable the identification of factors that might provide signals that are essential for stem-cell survival and differentiation. Additionally, comparative studies are required to establish the best source of stem cells for myocardial repair and to clarify whether the different stem-cell populations identified in the heart are really distinct or represent differentiation stages of the same lineage. A thorough analysis of the mRNA, microRNA (miRNA) and proteomic profile of each population might help to resolve this issue.

Although experiments in animal models and clinical studies have shown that stem-cell transplantation into the infarcted heart leads to positive outcomes, accumulating evidence suggests that paracrine mechanisms can largely account for such beneficial effects and might explain why almost every stem-cell type seems to affect cardiac function positively. If paracrine factors are the key players, their identification and administration might improve the therapeutic efficacy of stem-cell transplantation (Box 2).

So far, several uncontrolled clinical studies in a small number of patients using different stem-cell types and administration procedures have been conducted. However, to drive conclusions on the real efficacy of stem-cell therapy for cardiac diseases, randomized, controlled, double-blind studies on intermediate-to-large number of patients are necessary, which should more rigidly compare cell dosage, timing and route of administration. Finally, it would also

Box 2. Outstanding questions

Despite recent progress in developing experimental strategies to repair the heart, several important questions remain to be answered:

- Why do many stem-cell populations exist in the heart? Are these populations really distinct or do they represent differentiation stages of the same lineage? Can more-specific cardiac stem-cell markers be identified?
- Is it possible to expand isolated CSCs form patients rapidly to obtain an adequate number of autologous cells for transplantation?
- Is it possible to activate the cardiogenic program in *in vitro* cultured adult stem cells that modulates signaling pathways and transcriptional events involved in embryonic cardiac development?
- Where do CSCs originate from? Do they have a common embryonal origin from precardiac mesoderm and therefore simply represent a subset of undifferentiated cardiac cells or do they derive from a different location and only subsequently migrate to the heart?
- Are CSCs more effective than BMSCs in regenerating the myocardium and in improving cardiac function?
- If BMSC-mediated improvement of cardiac function occurs through a paracrine effect, which paracrine factors are key players in this process and how do they work?
- How different are the effects of congenital versus postnatal cell-cycle machinery manipulation of cardiomyocytes?
- What would be an effective and safe strategy to deliver coding sequences and/or small interfering (si)RNA to reactivate cardiomyocyte cell cycle?

be important to evaluate morbidity and mortality, although these endpoints tightly depend on the number of patients and the duration of the follow-up.

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