

"Second-generation" stem cells for cardiac repair

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an inspiring alternative to restore cardiac function after myocardial infarction. A large body of evidence has been obtained in this field but there is no conclusive data on the efficacy of these treatments. Preclinical studies and early reports in humans have been encouraging and have fostered a rapid clinical translation, but positive results have not been uniformly observed and when present, they have been modest. Several types of stem cells, manufacturing methods and delivery routes have been tested in different clinical settings but direct comparison between them is challenging and hinders further research. Despite enormous achievements, major barriers have been found and many fundamental issues remain to be resolved. A better knowledge of the molecular mechanisms implicated in cardiac development and myocardial regeneration is critically needed to overcome some of these hurdles. Genetic and pharmacological priming together with the discovery of new sources of cells have led to a "second generation" of cell products that holds an encouraging promise in cardiovascular regenerative medicine. In this report, we review recent advances in this field focusing on the new types of stem cells that are currently being tested in human beings and on the novel strategies employed to boost cell performance in order to improve cardiac function and outcomes after myocardial infarction.

Key words: Stem cells; Cardiac repair; Myocardial infarction; Heart failure; Second generation

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Core tip: Myocardial infarction and heart failure represent two of the most prevalent and fatal diseases. Stem cell therapies represent a novel approach capable of restoring the cellular loss observed in these conditions. Data from initial human studies have been encouraging but inconclusive. However, refinements in cell populations as well as new stem cell sources are currently being tested in large phase III clinical trials after showing positive results in preclinical models and early clinical reports, thus holding a promise for the achievement of a true

Abstract

Over the last years, stem cell therapy has emerged as

myocardial regeneration after myocardial infarction. We review here recent developments in this field.

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INTRODUCTION

Cardiac heart disease is a leading cause of mortality and morbidity worldwide. Coronary heart disease (CHD) is estimated to affect near 16 million people in the United States and accounts for about 1 of every 6 deaths^[1]. CHD and myocardial infarction (MI) are also a major cause of heart failure (HF), which is an important public health issue with an estimated prevalence of 5 million in the United States^[1] and 23 million worldwide^[2].

Risk factor modification, advances in medical, device-based therapies and interventional management have improved cardiovascular outcomes^[3], but even when mortality has shown a decline in most developed countries since mid 70s^[1,4-6], CHD is the leading cause of death in the United States^[7]. Its main consequence - HF - carries a poor prognosis, even worse than many cancers^[8]. Moreover, the decline in MI acute mortality achieved with primary percutaneous coronary interventions (PCI), has augmented the prevalence of HF among survivors^[9,10], because of the development of substantial scarring despite reperfusion strategies^[11].

Current treatment of systolic HF is mainly focused on counterweighting the neurohormonal activation cascade, improving contractile function and reducing the incidence of sudden cardiac death^[3]. None of these approaches is capable of restoring the cellular loss produced after MI, which is the cornerstone of the adverse ventricular remodeling process that leads to HF. Heart transplantation is a not viable alternative in most cases, and is hampered by limited donor hearts.

Since the beginning of the past decade, regenerative cardiac therapies have emerged as an option to satisfy these unmet needs^[12-14]. Until the end of the last century, the human heart was believed to be a terminally differentiated post mitotic organ, unable to be repaired after an injury such acute MI. This dogma was challenged in 2001 by the evidence of mitosis in cardiomyocytes after MI^[15]. Beyond that, and in contrast to some lower vertebrates^[16], cardiac self-repair ability is limited in human adults^[17], and unable to replace the massive cellular loss that occurs after MI, but its discovery opened up the possibility to be externally enhanced. In order to achieve this objective, two approaches can be adopted: (1) the addition of

progenitor cells for the repopulation of the damaged heart with contractile cells and vascular structures; and (2) the enhancement of cardiac self-repair ability. Most times both mechanisms are concomitant. The former, represented by stem cell therapy, has been the most extensively investigated, being tested in several studies enrolling thousands of patients worldwide and evaluating different cell types and delivery routes. Promising results were reported in preclinical studies, leading to a quick translation into clinical trials. Initial reports in human beings were encouraging and established the safety of these therapies. But their efficacy has been subject of continuous debate, since robust evidence is lacking due to inconsistency in benefits observed in clinical trials. Differences in methodologies have been invoked to explain these discrepancies. Notwithstanding, this initial preclinical and clinical data have provided important insights into the selection and manufacturing of cell products, their delivery and the mechanisms of action that drive myocardial regeneration. The initial concept of achieving cardiac repair by differentiation of stem cells into functional cardiomyocytes and vascular structures has shifted towards a paracrine paradigm, where stem cells exert their beneficial effects by promoting myocardial salvage and self-repair mechanisms.

So far, the optimal source for stem cell therapy as well as its processing, delivery route and dosage, remains unknown and many fundamental issues need to be addressed. Ideal stem cell therapy must accomplish with several requirements including: (1) the ability to regenerate damaged myocardium; (2) the ease of obtaining, storing and delivering; (3) no tumorigenesis, immunogenicity or ongoing ethical issues; and (4) cost-effectiveness. The heterogeneous cell products employed in the early years of regenerative cardiac medicine have been replaced by more purified stem cell populations with a greater reparative potential, and *in vitro* priming of stem cells to enhance their engraftment, survival, plasticity and paracrine activity, has also been extensively investigated. All of these advances have lead to a new generation of stem cells ("second-generation" stem cells) that should overcome the hurdles found with first-generation ones. In this review we summarize recent research and novel strategies in this field, focusing on priming of "first-generation" cells and on the new cell products that are being tested for cardiac regeneration after MI.

GENETICALLY ENGINEERED SKELETAL MYOBLASTS

The first type of stem cell thought to be useful for cardiac regenerative purposes were autologous skeletal myoblasts. Their muscular phenotype and many other advantageous features including ease of isolation through muscle biopsy, rapid expansion *in vitro* and lack of ethical or immunological issues made them

an attractive option^[18]. In fact, their use in animal models^[19-21] and phase I non-randomized human trials^[22-26] described their ability to form some cardiac structures and yielded promising results regarding improvement in cardiac performance after MI.

Nevertheless, subsequent studies documented that myoblasts differentiate into skeletal myocytes instead of cardiomyocytes^[27], and the first and larger randomized controlled trial in humans, the MAGIC trial, showed no benefits on cardiac function^[28].

More worrisome is the lack of electro-mechanical coupling of these cells, that made them prone to generate ventricular arrhythmias due to their inability to express certain cardiac-specific genes codifying important proteins of the gap junctions, as N-cadherin and connexin-4^[25,28,29]. Down-regulation of these genes is induced by the transdifferentiation process^[29]. However, improved electrical coupling as well as a reduction in the arrhythmogenic potential of the transplanted cells was demonstrated by the enhancement of connexin-43 expression *via* genetic manipulation^[30-32].

Another drawback of skeletal myoblasts in their application for cardiac repair is massive apoptosis and their low survival rate when applied to the ischemic myocardium^[33]. Pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), have showed their ability to induce angiogenesis^[34,35]. Indeed, transfected skeletal myoblasts with augmented VEGF and FGF expression exhibit increased survival, promoted by an anti-inflammatory and angiogenic effect^[36-38]. Cell survival after transplantation can also be improved using myoblasts lacking the *MyoD* gene. These myoblasts induce angiogenesis *via* secretion of stromal cell-derived factor-1 (SDF-1) and placental growth factor^[39], and are less sensitive to apoptosis by up-regulation of a number of anti-apoptotic genes (*Pax3*, *Bcl-2*, *Bcl-xl*)^[40].

Despite of these advances, skeletal myoblasts seem far from being the optimal cell source, with safer and more efficacious novel cell types displacing its role in cardiac regenerative medicine.

BONE MARROW DERIVED STEM CELLS

Bone marrow derived mononuclear stem cells

Bone marrow derived mononuclear stem cells (BMMNCs) are the most extensively used type of cells for cardiac regeneration. They represent a heterogeneous mixture of mononuclear stem cells including hematopoietic and endothelial progenitors and mesenchymal stem cells. The landmark study of Orlic *et al.*^[41] was the proof-of-concept that a population of Lin⁻/c-kit⁺ BMMNCs was capable of transdifferentiating into myocytes and vascular structures and restore a large amount of the damaged myocardium after MI in a mice model. This finding generated great interest in this type of cells, spurring

an intense research and rapid clinical translation.

The exact mechanisms by which BMMNCs are thought to work remain poorly understood. Different explanations have been advocated including paracrine effects, transdifferentiation into cardiomyocytes and activation of resident cardiac stem cells^[42-44].

Many studies have shown a positive effect of BMMNCs in cardiac function after MI, both in the acute and chronic phases^[45-51], although some other trials reported a less evident or even absent benefit^[52-62]. The different clinical settings where cells have been tested, as well as methodology differences in these trials may explain, at least in part, the contradictory observed results. Five systematic reviews and meta-analyses concluded that BMMNCs transplantation after MI resulted in a better left ventricular function and remodeling with a modest but significant improvement in left ventricular ejection fraction (LVEF), ranging from 2.55% to 3.96%. No safety concerns have been observed and what is more provocative, better clinical outcomes have been documented in treated patients, although these trials did not include hard clinical endpoints as primary objectives^[63-67].

BMMNCs-based therapies face many limitations: (1) homing, engraftment and maintenance of cells in the transplanted tissue^[68]; (2) limited plasticity when using adult stem cells^[69,70]; and (3) depletion and impairment of their functionality associated with advancing age and comorbidities^[71,72]. Many different strategies for cell enhancement have been studied in the last years in order to overcome these problems.

MicroRNA (miRs) are small non-coding RNAs controlling gene expression, either by inducing mRNA degradation or by blocking mRNA translation^[73]. They are involved in the maintenance of the pluripotent state of stem cells and its over-expression is able to induce such a state in somatic cells^[73,74]. They also participate in the differentiation and lineage commitment processes^[73,74]. Thus, miRNAs have been investigated as a target for stem cell enhancement. For instance, cardiovascular diseases and ageing promote up-regulation of the pro-apoptotic and anti-proliferative miR-34a in human BMMNCs. Its inhibition *in vitro* has shown to improve cell survival and their therapeutic benefit in a mice model of MI^[75]. Furthermore, BMMNCs seem to regulate the expression of miRs in cardiomyocytes *in vivo*. In a mice model, intramyocardial delivery of BMMNCs mediated a paracrine effect by releasing insulin-like growth factor 1 (IGF-1) which inhibited the expression of the pro-apoptotic miR-34, thereby exerting a cardioprotective effect^[76].

But as in the case of skeletal myoblasts, new and more refined cell products are supplanting BMMNCs. In the meanwhile, definitive conclusions about the efficacy of BMMNCs after MI are expected to be elicited by the ongoing BAMI trial (NCT01569178). This large scaled, multinational, multicenter randomized and controlled trial, will test for the first time, the effect of

intracoronary infusion of BMMNCs three to six days after acute MI with LVEF < 45% on all-cause mortality as its primary endpoint. Over 3000 patients in 11 European countries are planned to be enrolled. This trial will help to trace the future directions that should be followed with BMMNCs therapies, supporting its administration, warranting further investigations or definitively leaving apart their use to focus on new generations of cells.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also known as multipotent stromal cells, represent a subset of BMMNCs discovered more than 40 years ago^[77]. They can be found within connective tissue in other organs, from which they can be easily isolated and cultured. They have awoken a big interest in recent years because they display a number of traits that made them an attractive cell product for cardiac repair^[78]. MSCs are multipotent and have the capability to transdifferentiate into lineages of mesodermal tissues^[79,80], including cardiomyocytes^[81]. *In vivo* studies have also proved the ability of human MSCs to differentiate into cardiomyocytes in adult mice hearts^[82]. They also display a great paracrine potential, secreting growth factors that promote endogenous healing^[83].

A number of preclinical studies have shown the benefit of these cells in cardiac function after MI^[84-86]. Clinical trials have also elicited promising results^[87,88] and a small and recent randomized phase I and II placebo-controlled trial suggested that transendocardial injection of MSCs is superior to BMMNCs and placebo in reducing scar size in chronic ischemic cardiomyopathy^[89].

But similarly to BMMNCs, autologous use of MSCs is hampered by their loss of functionality associated with ageing and comorbidities^[71,72], and their heterogeneous phenotype compromises their therapeutic effect^[90]. A variety of different strategies have been developed in order to improve MSCs regenerative potential. One of the most promising is the so-called "guided cardiopoiesis" of MSCs. This term defines the process by which a stem cell is engaged towards a cardiac differentiation program while its proliferative and self-renewal capacities remain intact^[91]. This can be achieved by mimicking the cardiogenic instructive signals that drive the embryonic development of the heart^[92]. The up-regulation of certain cardiac transcription factors such as Nkx-2.5, MEF2C, FOG-2, TBX5, MESP1 and GATA-4 is responsible of the adoption of a cardiogenic phenotype in MSCs, preserving their proliferative ability before the final differentiation step towards sarcomerogenesis begins^[92,93]. The up-regulation of these cardiac transcription factors is feasible when MSCs are cultured within a cardiogenic cocktail containing transforming growth factor beta (TGF- β), bone morphogenic protein (BMP)-4, activin A, FGF-2, IGF-1, interleukin (IL)-6, factor IIa (h-alpha-trombin) and retinoic acid^[93]. Differentiation beyond this intermediate state is possible by exposing the cells to a 1% human

platelet lysate medium^[93]. In a murine model of ischemic cardiomyopathy, the priming of MSCs with these growth factors led to a cardiopoietic phenotype that improved their therapeutic benefit (by yielding sarcomere-containing myocytes with electromechanical response, and with mitochondrial structures closer to that seen in adult cardiomyocytes)^[93]. In another rat model of MI, transplantation of pretreated MSCs with IGF-1, BMP-2 and FGF-2 led to increased tolerance to hypoxic conditions of the MSCs and augmented expression of connexin-43 and gap junctions linking MSCs to cardiomyocytes. Indeed, MSCs exerted a protective effect over cardiomyocytes against hypoxia mediated by these gap junctions, showing a superior myocardial salvage of the infarcted heart and leading to an improvement in left ventricular function^[94]. Recently, the C-CURE clinical trial (Table 1) addressed the feasibility and the safety of cardiopoietic autologous MSCs in a randomized trial involving 48 patients with chronic ischemic HF^[95]. In this phase II study, the lineage-guided therapy with primed MSCs resulted in a total improvement in LVEF of 7% over baseline, compared to 0.2% in the standard of care group. Reductions in left ventricular volumes and increases in the 6-min walk distance were also observed. No adverse events related with the cells were documented^[95]. This study has supported the initiation of two ongoing large phase III trials, CHART-1 (NCT01768702) and CHART-2, where autologous cardiopoietic MSCs will be tested in patients with chronic HF secondary to IM.

A drawback of MSCs therapy is the poor survival and the low retention rate of transplanted cells that limit their regenerative potential. *Ex vivo* genetic modification may enhance viability of the transplanted cells by over-expression of anti-apoptotic genes. MSCs transduced with vectors encoding for genes such heat shock protein (Hsp27)^[96], microRNA-1^[97], and protein kinase type 1 α ^[98] have shown increased survival and a more effective and efficient performance restoring cardiac function compared to conventional MSCs in rodent models of MI. Other pharmacological approaches to improve cell retention have also been evaluated. Thymosin β 4 is a protein implicated in cytoskeletal homeostasis that has been shown to protect MSCs against hypoxic injury and to increase cell retention in a rat model of MI^[99].

In summary, MSCs are among the most promising cells for cardiac reparative medicine. Novel approaches that prime cell functionality have shown encouraging results that have led to ongoing large-scaled randomized phase III clinical trials.

CARDIAC STEM CELLS

The classic concept stating that the adult mammalian heart is a post-mitotic organ without self-renewal capacity was challenged in the beginning of the past decade. The first evidence came out with the finding of mitosis among cardiomyocytes in human hearts after MI by Beltrami *et al.*^[15]. Shortly after, the same group

Table 1 Clinical trials evaluating new stem cells for cardiac repair following myocardial infarction

Study	n	Design	Type of cells	Delivery route	Clinical setting	Follow-up	Outcomes
Bartunek <i>et al.</i> ^[95] (C-CURE)	47	Multicenter, randomized 2:1 (cells vs standard of care)	Autologous bone marrow derived cardiopoietic MSCs	Endo-myocardial injection	Chronic ischemic heart failure (LVEF 15%-40%)	Safety 2 yr Efficacy 6 mo	Feasible and safe ↑ LVEF ↓ LVESV ↑ 6-min walk distance and improvements in QoL and NYHA
Bolli <i>et al.</i> ^[113] (SCIPIO)	23	Unicenter, randomized 2:1 (cells vs standard of care)	Autologous c-kit+/lin- CSCs	Intra-coronary infusion	Chronic ischemic heart failure (LVEF ≤ 40% four months post CABG)	12 mo	Feasible and safe ↑ LVEF ↓ Infarct size
Malliaras <i>et al.</i> ^[122] (CADUCEUS)	25	Two centers, randomized 2:1 (cells vs standard of care)	Autologous CDCs	Intra-coronary infusion	Chronic ischemic heart failure (1.5-3 mo after MI)	12 mo	Feasible and safe ↓ Infarct size ↑ Viable myocardium and regional contractility ≈ LVEF and ventricular volumes
Hare <i>et al.</i> ^[144] (POSEIDON)	30	Multicenter, randomized 1:1 (autologous vs allogeneic cells)	Three different doses of autologous or allogeneic bone marrow derived MSCs	Endo-myocardial injection	Chronic ischemic heart failure (LVEF ≤ 50%)	12 mo	Feasible and safe ≈ LVEF Autologous ↑ 6-min walk distance and QoL Allogeneic ↓ LVEDV

↑: Indicates increased; ↓: Indicates decreased; ≈: Indicates no change; MI: Myocardial infarction; MSCs: Mesenchymal stem cells; LVEF: Left ventricular ejection fraction; LVESV: Left ventricular end-systolic volume; QoL: Quality of life; CSCs: Cardiac stem cells; CABG: Coronary artery by-pass graft; CDCs: Cardiosphere-derived cells; LVEDV: Left ventricular end-systolic volume.

described the existence of a population of multipotent and clonogenic cells that expressed the tyrosine kinase receptor in their surface (c-kit⁺) and that were able to differentiate into the three cardiac lineages (cardiomyocytes, smooth muscle cells and endothelial cells)^[100]. Since then, strong evidence supported the concept that turnover in the adult cardiomyocyte population is provided by cardiac stem cells (CSCs)^[17,101], but the magnitude of this turnover and the exact underlying mechanisms remain unknown^[102]. CSCs are rapidly activated after myocardial injuries or physiological stimuli^[103-105]. Recently, Ellison *et al.*^[106] have demonstrated in a murine model that some of the new cardiomyocytes generated after a myocardial injury are c-kit⁺ CSCs descendants. Nevertheless, this finding does not rule out other concomitant mechanisms that could be implicated in myocardial regeneration, and a recent report by van Berlo *et al.*^[107] suggests that the new cardiomyocytes generated from CSCs are functionally insignificant. In fact, it is evident that self-renewal capacity of the adult human heart is unable to restore the large amount of cellular loss after MI.

Stem cell therapy with CSCs may offer some advantages over other extra-cardiac cells, because they are believed to be more prone to differentiate towards cardiac lineages. Most research has focused on transplanting CSCs in the infarcted area and various preclinical studies using this approach in different animal models have demonstrated the ability of CSCs to alleviate left ventricular dysfunction in both acute and chronic MI^[100,108-112].

These promising results warranted translation into

clinical research and led to the conduction of the first phase I clinical trial using c-kit⁺ CSCs, the SCIPIO trial^[113] (Table 1). This study reported that isolation and expansion of CSCs from cardiac tissue, harvested during coronary artery by-pass graft surgery, was feasible. Intracoronary injection of these cells in patients with left ventricular dysfunction (LVEF < 40%) after surgical revascularization improved LVEF by 12.3% compared with baseline, reduced scar size and was associated with improvements in quality of life and New York Heart Association functional class. These are preliminary data of the first eight patients enrolled in the study, which is still ongoing.

However the number of CSCs in adult hearts is small, and albeit their *ex vivo* isolation and expansion are feasible, their availability represents a major challenge. In 2004, Messina *et al.*^[114] first described the *in vitro* formation of self-adherent multicellular spherical clusters from human surgical cardiac biopsies in culture, termed cardiospheres (CSs). These CSs were clonogenic and able to yield the three cardiac lineages *in vitro* and *in vivo* when transplanted in an animal model of MI, resulting in improvements in fractional shortening^[114]. CSs have a core of c-kit⁺ cells surrounded by progenitors in distinct stages of the differentiation process towards the three major cardiac lineages. They can also be obtained through percutaneous endomyocardial biopsy and used as source for cardiospheres-derived cells (CDCs), a mixed cell population that is clonogenic, express surface markers typical of stem cells (c-kit⁺, CD105⁺), negligible hematopoietic markers, and that can be expanded on fibronectin^[115]. This approach, described by Marbán *et al.*^[115], has some advantages, *i.e.*,

the larger number of cells that can be obtained and the shorter time of manufacturing, in comparison to other methods to produce CSCs^[111,116,117]. In animal models of MI, human CDCs have been shown to promote cardiac regeneration and to improve LVEF^[115,118,119]. The same group conducted a direct comparison between CDCs and other extracardiac stem cells including BMMNCs, MSCs and adipose derived stem cells (ADSCs) in order to address their potency for myocardial repair. *In vitro*, CDCs showed a greater myogenic differentiation potency and higher angiogenic and paracrine potential. *In vivo*, injection of CDCs in infarcted mice provided the greatest functional benefit^[120].

This preclinical evidence led to the first phase I clinical trial using CDCs, the CADUCEUS trial (Table 1). This study randomized 25 patients with LVEF 25%-45%, two to four weeks after MI, to receive intracoronary autologous CDCs obtained from endomyocardial biopsies or standard of care. The primary safety endpoint was the proportion of patients who died due to ventricular tachycardia, ventricular fibrillation, or sudden unexpected death, or had MI after cell infusion, new cardiac tumor formation on MRI, or a major adverse cardiac event (MACE: Composite of death and hospital admission for HF or non-fatal recurrent MI). Preliminary efficacy endpoints on MRI parameters were also analyzed. Autologous CDCs treatment was safe and associated with reductions in scar size, increased viable myocardium and regional contractility. Nonetheless, ventricular volumes and LVEF did not differ between groups^[121,122], casting doubts about the true regenerative effect of CDCs.

Notwithstanding, both SCIPPIO and CADUCEUS trials included a small number of patients and there are some issues concerning their results, such as the low expression of cardiac markers in CSCs in the former, and the lack of benefit in terms of LVEF in the latter. Moreover, and although in both studies a true regeneration of damaged myocardium was invoked, the real underlying mechanism of action of these cells remains unproved.

Similarly to other extra-cardiac cell types, engraftment and retention rates of the transplanted CSCs and CDCs within the harsh environment of the infarcted myocardium are low, regardless of the administration route^[112,123]. Although direct differentiation of cardiac progenitor cells in cardiac lineages has been observed, this observation of low CSCs retention rates into the myocardium further supports the hypothesis of a paracrine effect, as another pathway responsible of the positive effect of the cells^[124].

In order to overcome the limited survival and retention of the transplanted cells, different strategies have been tested in animal models. Mohsin *et al.*^[125] used genetic manipulation to deliver the pro-survival gene Pim-1 kinase into human CSCs, resulting in a superior cellular engraftment and differentiation, that led to a superior reparative potential compared with conventional CSCs in an immuno-compromised

mice model of MI. Interestingly, no oncogenic issues were observed because telomere lengthening induced by Pim-1 overexpression was transitory^[126]. Another option for priming CSCs and make them less prone to apoptosis under hypoxic conditions is the preconditioning with cytoprotective pharmacological agents. For example, treatment of CSCs with cobalt protoporphyrin reduces oxidative stress-induced apoptosis, by up-regulation of heme oxygenase 1, COX-2, and anti-apoptotic proteins (BCL2, BCL2-A1, and MCL-1) and increased phosphorylation of NRF2^[127]. Hypoxic protection can also be achieved by means of β -O-linkage of N-acetylglucosamine to certain pathway proteins. Increases in this pro-survival signaling system by thiamet-G significantly improved CDCs survival after hypoxic injury^[128]. In addition, exposure of CSCs to H₂O₂ for 2 d before transplantation stimulates neoangiogenesis in the peri-infarct area following ischemic-reperfusion injury and could be a viable therapeutic option to prevent HF^[129]. CDCs engraftment and differentiation can also be enhanced by controlled release of FGF in ischemic myocardium in a pig model of MI, resulting in a significant functional improvement^[130].

Finally, previous reports suggest that one of the mechanisms by which MSCs promote cardiac repair is the stimulation of endogenous CSCs *via* paracrine effects^[131]. This finding raised the question whether combining both MSCs and CSCs could amplify the response to cell therapy. This hypothesis has been recently addressed by Williams *et al.*^[132], they found that intramyocardial delivery of human CSCs and bone marrow MSCs in a swine model of MI resulted in a 2-fold greater scar reduction and a 7-fold enhancement of engraftment of stem cells compared with each cell therapy alone, leading to improvements in hemodynamics and LVEF.

The discovery of CSC has revolutionized regenerative medicine. As they are derived from the target organ, they are supposed to be more committed with a cardiac fate, and the understanding of the molecular mechanisms that steer their mode of action is providing new insights that extend application of cell-based therapies. Early clinical experience with CSCs/CDCs warrants further investigation in larger phase II studies that will address the unresolved issues of SCIPPIO and CADUCEUS. At present, three clinical trials are evaluating the effect of CSCs and CSs in myocardial regeneration after MI: the RECONSTRUCT (NCT01496209), the ALLSTAR (NCT01458405) and the ALCADIA trials.

ALLOGENEIC STEM CELL THERAPY FOR CARDIAC REPAIR

Allogeneic stem cell therapy may offer many advantages in cardiac regenerative medicine. Firstly, allogeneic cells could be used as a scalable and reproducible cell product readily available "off-the-shelf"

that could be administered in the setting of primary PCI avoiding delays inherent to harvest and culture of autologous stem cells. Secondly, this type of cells can be obtained from young and healthy donors, thereby avoiding the aforementioned impairment in autologous stem cells functionality observed with advancing age and comorbidities.

Most research in allogeneic cardiac regeneration has been done with MSCs due to their immunoprivileged profile and immunosuppressive properties. MSCs lack expression of major histocompatibility class II antigens^[133], down-regulate T cells response through direct contact and secretion of anti-inflammatory cytokines^[134] and significantly affect the ability of dendritic cells to prime T-cell responses^[135]. These findings raised interest in MSCs for allogeneic stem cell transplantation. Nonetheless, recent data have challenged this assumption suggesting that under certain inflammatory environment and during their differentiation process, MSCs can switch their immune phenotype towards an immune-enhancing pattern, limiting their long term survival and benefits^[136,137]. However, and as pointed out before, autologous stem cell therapy faces the same problem, and if the advocated paracrine effect is responsible of stem cell regenerative capacity, survival and maintenance of cells in the transplanted heart could not be indispensable^[138].

There is a large body of preclinical work supporting the safety and the efficacy of allogeneic MSCs therapy^[84,139-142]. In a large animal model of MI in pigs, Amado *et al.*^[139] demonstrated that allogeneic MSCs injections in the necrotic area were feasible with no evidence of rejection and provided near-normalization of cardiac function and Quevedo *et al.*^[84] refrained these results in a swine model of chronic ischemic cardiomyopathy, where allogeneic MSCs showed to be able to differentiate in the three cardiac lineages and to improve LVEF. These findings supported the first-in-man clinical trial using allogeneic bone marrow-derived MSCs by Hare *et al.*^[143]. They conducted a randomized, double-blinded, placebo-controlled phase I trial in which 53 patients with reperfused MI were allocated to intravenous administration of allogeneic human MSCs or placebo. The study met its primary safety objective with no evidence of tumor formation or immunogenicity. It also suggested a greater benefit in cardiac function in larger MIs and improved outcomes regarding arrhythmias and pulmonary performance^[143]. The same group has recently conducted a head-to-head comparison between autologous and allogeneic MSCs in a phase I / II randomized pilot study involving 30 patients with chronic ischemic left ventricular dysfunction secondary to MI, the POSEIDON trial^[144] (Table 1). In this case, cell therapy was delivered by transendocardial injection. Both therapies were safe, with no or negligible allogeneic sensitization in the allogeneic group and no evidence of ectopic tissue formation. Both types of cells reduced infarct

size as measured by early enhancement defects in multidetector computed tomography, but no significant improvements in ejection fraction were documented. Intriguingly, allogeneic MSCs reduced LV end-diastolic volumes while autologous did not. Furthermore, improvements in quality of life, NYHA and 6-min walk test were more evident with autologous therapy. However, the small number of patients and the absence of a placebo group make these observations difficult to interpret. An interesting finding was the greater benefit regarding LV volumes and LVEF obtained with low-dose concentrations of MSCs vs high doses (20 million vs 200 million), which is consistent with preclinical data^[145] and warrants further investigation of the optimum dose before undertaking large clinical trials.

Mesenchymal precursor cells (MPCs) represent an immature subpopulation of bone marrow-derived cells that express the Stro3⁺ marker^[146]. MPCs are multipotent cells with an extensive proliferative and differentiation capacity as well as a great paracrine potential that outperforms the one of MSCs^[147,148]. They also display an immunoprivileged phenotype, appropriate for allogeneic transplantation. Previous animal studies have provided evidence on the positive effect of these cells in the setting of acute MI when delivered intramyocardially^[145,148,149] or by intracoronary infusion^[150]. Houtgraaf *et al.*^[150] have recently reported the benefit of the intracoronary infusion of different doses of allogeneic MPCs, when delivered few minutes after reperfusion in a sheep model of MI. The therapy was safe and provided reductions in infarct size *via* cardiomyocyte salvation and proliferation, and induced angiogenesis. It was also suggested that the smaller size of MPCs, compared to MSCs, prevents microvascular obstruction due to cellular aggregation observed in previous studies. Similarly to the findings of the POSEIDON trial, the benefit was more evident in the low-dose group (< 75 million vs 200 million), an observation which may be related to the better coronary flow observed after infusion with lower number of cells.

Recently, Penn *et al.*^[151] have tested a different bone marrow-derived cell product, named MultiStem, in 19 patients with acute MI in an allogeneic setting and using a novel delivery approach. Three different doses of cells were injected through a microneedle in the culprit vessel adventitia two to five days after primary PCI. The procedure was safe and LVEF was increased when compared to registry controls.

Another milestone in the field of allogeneic cell therapy is being carried out in the CAREMI clinical trial. This study represents the first-in-man experience with allogeneic CSCs in the acute setting of CHD. It is a phase I / II clinical trial that has been designed with a first dose-escalation phase and a second part with a randomized and controlled design. In this trial, 55 patients with acute reperfused MI and LVEF < 45% will be treated with intracoronary infusion of human

allogeneic CSCs, being the primary objective the rate of death from any cause at 30 d (dose-escalation phase) and MACE and magnetic resonance parameters at 12 mo (randomized phase).

In summary, allogeneic cell therapy represent a novel approach in cardiac regeneration that could overcome some of the strong barriers found by autologous cell products. Before large scaled trials are performed, some concerning issues as immunogenicity and rejection must be thoroughly examined.

OTHER SOURCES OF STEM CELLS

In contrast to adult stem cells, embryonic stem cells (ESCs) are pluripotent, *i.e.*, they are capable of differentiate into cells of the three germ layers. ESCs isolated from blastocysts demonstrate a great potential to generate functional cardiomyocytes and their transplantation into infarcted hearts in animal models have been evaluated showing improvements in LV function^[152,153]. Despite these findings, ESCs-based therapies face strong barriers that go beyond biological aspects, such as immune rejection or tumor formation. Ethical issues also remain a major limitation to the widespread use of these cells. So far, no clinical trial has tested ESCs in humans and is unlikely to be done.

Given these constraints, Takahashi and Yamanaka reported in 2006 the feasibility of generating induced pluripotent stem cells (iPSCs) from mouse adult fibroblasts by the transduction of four transcription factors that led to a phenotype similar to that of ESCs^[154]. This achievement could prevent the immunogenicity issues inherent to allogeneic ESCs application, but do not avoid oncogenic risks^[155]. On top of this, current reprogramming of somatic cells is a low efficient process that should be refined before translation to clinical trials. Therefore, nowadays, iPSCs represent a new source of cells that holds a great promise for reparative medicine but is not ready for their application in human beings yet.

ADSCs represent a population of stem cells located in the adipose tissue that are able to differentiate into multiple cell lineages including cardiomyocytes and vascular cells^[156]. They offer two major advantages over some previously mentioned types of cells: firstly, the easy and repeatable access that makes it possible to harvest large amounts of adipose tissue by a minimally invasive method and, secondly, their increased proliferative potential in culture^[157]. Preclinical reports have documented that administration of ADSCs after MI improves cardiac function^[158,159], in a range similar to BMMNCs^[159]. They have also been tested in 2 clinical trials, the APOLLO^[160] and the PRECISE trials^[161]. The APOLLO trial was a small randomized, double-blind, placebo controlled, phase I / II study designed to assess the safety and the feasibility of intracoronary infusion of ADSCs in patients with large ST-segment elevation acute MIs. The study proved the feasibility and the safety of both liposuction and ADSCs

intracoronary infusion after MI, and showed a trend towards improved cardiac function and reductions in scar formation^[160]. The PRECISE trial reported that intramyocardial injection of ADSCs in patients with refractory angina not amenable for revascularization ("no-option" patients) improved exercise capacity and myocardial perfusion, reduced scar size and preserved maximal oxygen consumption^[161]. However, the discovery of CSCs and new developments with MSCs and allogeneic therapies have somehow waned the initial interest in ADSCs.

STEMLESS APPROACHES

To conclude, stemless therapies to restore cardiac function after MI haven also been proposed. As outlined above, if stem cell benefits are mediated by a paracrine effect and by activation of self-repair mechanisms, exogenous administration of the cytokines and growth factors implicated in the regeneration process could theoretically replace cell transplantation with a readily and "off-the-self" available product, similarly to other current biological treatments. Nadal-Ginard *et al.*^[102] advocate this cell-free therapies. They have reported that intracoronary administration of IGF-1 and hepatocyte growth factor after coronary reperfusion reduced cardiac remodeling, induced myocardial regeneration and improved ventricular function in a pig model of MI. This was achieved by activation of c-kit⁺ endogenous CSCs, which expanded and generated new cardiomyocytes and microvessels^[162,163]. Other acellular approaches have tried to promote stem cell recruitment by the infarcted myocardium. Modulation of the CXC-chemokine receptor 4 and SDF-1 axis *via* gene therapy has shown to exert beneficial effects in preclinical studies and in human phase I trials^[164,165].

In spite of that, molecular, cellular and myocardial tissue regeneration mechanisms are highly complex and driven by the interplay of several factors, not yet completely understood. Therefore, it seems unlikely that the simple administration of one or two growth factors could be capable of inducing a full cardiac regeneration. Nevertheless, further research in this field is warranted in order to shed light on these mechanisms and to elucidate if stemless approaches are a feasible option in the future.

CONCLUSION

Regenerative therapies represent a novel paradigm in cardiovascular medicine, and have grown up over infancy and into adolescence. Translation of basic research and animal studies into the clinical scenario has never been so quick, driven by the enormous enthusiasm raised by the possibility of achieving heart regeneration. This initial euphoria has been dampened by some contradictory results and by the modest benefits observed in cardiac function, leading

to skepticism in a part of the scientific community. But these pioneering results should not be interpreted as the definite evidence in favor or against cell-based reparative therapies. Instead, they have established their safety and have shown the strong barriers that should be overcome, providing important insights about the source of cells to be used as well as about manufacturing processes and delivery routes. With all this background, the state-of-the-art in the field of stem cell therapy can be summarized as follows: (1) The molecular mechanisms that rule myocardial repair are highly intricate and still remain poorly understood. It is unlikely that the simple administration of "first-generation" stem cells or growth factors to the failing heart will be able to achieve a complete restoration of cardiac function; (2) As a result of a better knowledge of the molecular and genetic mechanisms that induce progenitor cell proliferation and differentiation into cardiac structures, a new generation of cell products ("second-generation" stem cells) are now being evaluated in large clinical trials after promising and encouraging results in phase I and II studies; and (3) Combining cellular, molecular and genetic basic research with preclinical studies and well-designed clinical trials, together with more collaborative research networks and with the definitive help of bioengineering, will be the keys for the definitive development of cardiac regenerative medicine.

Keeping all this in mind, the future of stem cell-based therapies is more promising than ever, and the goal of a true regeneration or repair of the damaged myocardium stands awaiting in the years to come.

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