Stem Cells and Cardiac Disease: Where are We Going?

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Abstract: During the last 10 years we have witnessed the development of a new field in research termed Stem Cell Therapy. Classically, it was considered that cells had a limited division and differentiation ability; however, this dogma was challenged when new exciting results about cell multi/pluripotency were presented to the scientific community. It was found that cells from one adult tissue source were able to originate cells of a very different type. The possibility of transplanting these cells into damaged organs with the aim of substituting sick or dead tissue, triggered many studies to understand the plasticity of the stem cells and their potential in pathological situations. Nowadays, much more is understood about stem cells, although of course, many questions, especially about their mechanism of action, still need to be answered. Their benefit after transplantation has been shown experimentally and even clinically in some cases; however, the degree of stem cell contribution through their own differentiation into the transplanted tissue, has turned out to be generally low, and increasing evidence indicates that a trophic effect must play an important role in such a benefit. A better understanding of the paracrine mechanisms involved could be of great relevance in order to develop new therapies focused on stimulating endogenous cells. On the other hand, more sophisticated methods for cell transplantation combined with bio-engineering techniques have been devised in cardiac disease models. In this review we will try to provide a critical overview of the stem cell studies performed until now and to discuss some of the questions raised about the mechanisms that are involved in their putative reparative effect in cardiovascular diseases, and their origin.

INTRODUCTION

The presence of progenitor cells with the ability to replace senescent tissues in the different organs was reported some time ago; however, this potential was believed to be restricted to certain tissues, and only during the last few years has the existence of rare cell populations with multipotent or even pluripotent capabilities been described in most adult tissues. Such findings have been especially striking in organs like the heart and the brain, which were typically considered organs with extremely low self-renewal capacity, consisting of cardiomyocytes (CMs) and neurons classically considered cells that lose their potential to proliferate right after birth. However, although the presence of progenitors with a proliferative and differentiation capacity has been shown, unfortunately, in the case of severe diseases like myocardial infarction (MI) or stroke, their potential seems not to be sufficient to restore a damaged organ. A deeper understanding of the origin and "behavior" of these stem cells is mandatory to be able to manipulate them and induce their activation and differentiation to regenerate the damaged tissues.

Although the risk of death in patients with myocardial infarction during the acute **stage** has been significantly diminished, this has brought about an increased incidence of chronic heart problems. Drug treatments can only partially improve the patient's quality of life and cannot counteract the adverse remodeling processes that take place after acute infarction. As consequence of the ischemia, a progressive contractile dysfunction of the viable myocardium will follow which **will end up** in many cases in heart failure [1]. Taking

these aspects into account, an ideal therapy should be able to regenerate the damaged tissue providing new cells, which ideally could be applied during the first stages of the diseases with the aim of reversing the initial damage and controlling the remodeling processes initiated as a consequence of the acute ischemia.

In this article, we will review the potential of the different types of stem cells identified until now, focusing on their *in vitro* capacity to differentiate to mesoderm-derived cells like CMs and vascular cells, and therefore, their application in animal models of myocardial infarction. Finally, we will describe and discuss some of the more relevant clinical trials in the field of cardiac diseases.

STEM CELLS AND CARDIAC DISEASE

The replacement of the dead tissue in the ischemic heart by new CMs (and vascular cells) has become one of the main objectives of stem cell therapy in cardiac disease. In general, it has been demonstrated that stem cells can be manipulated *in vitro* to differentiate into different mesodermal cell types, which express tissue specific markers and in some cases, functionally behave like them. Thus, the embryonic stem cells (ESCs), which are isolated from the inner cell mass of the embryo, are the cells with the greatest differentiation potential, since it is possible to derive them to all somatic and germinal tissues. A number of studies performed with mice and human ESCs have addressed the basic signaling mechanisms involved in tissue development but also the potential to manipulate them *in vitro* and *in vivo* for tissue regeneration [2]. On the basis of these studies and knowledge about embryo development, the differentiation potential of the adult stem cells (SC) has also been broadly tested. The cardiac (and vascular) differentiation potential of embryonic

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and adult stem cells and their regeneration capability in animal models of cardiac ischemia will be discussed next.

Stem Cell-Based Experimental Studies

Embryonic Stem Cells (Escs)

The *in vitro* ESC differentiation potential towards cells belonging to the three germ layers is well described. ESCs differentiate into CMs (reviewed in [3] and [4]) by culturing in suspension as embryoid bodies followed by plating them a few days later. Although there is a high variability rate, spontaneous differentiation with beating areas can be generally detected [5]. Also, their co-culture with the primary visceral endoderm cell line END-2, gives a successful differentiation [6]. Importantly, not only specific cardiac proteins expression but also electromechanical coupling and electrophysiologic specialization can be detected [5,7,8]. Furthermore, their cardiomyogenic potential has been confirmed *in vivo* when transplanted, after *in vitro* differentiation, into ischemic hearts [9,10]. However, it seems that no specific directed cardiac differentiation occurs in the heart. Thus, when undifferentiated human ESCs are injected in the hind limb they give rise to the same proportion of cardiomyocytes as when they are injected in the heart [11]. Besides, teratoma formation has been detected in all cases. The high risk of tumor formation makes it mandatory to pre-differentiate the cells towards CMs and to develop techniques to isolate a highly purified population of CMs. Importantly, it has been shown that ESC-derived CMs are terminally differentiated cells that are functionally equivalent to CMs isolated from the heart (reviewed in [12]). Unlike mouse ESCs, human ESCs possess a certain degree of proliferation *in vitro* [13,14] and *in vivo* [9]. Thus, the cells can be expanded and differentiated in bio-reactors and genetically selected by using transgenes encoding fluorescent reporters or antibiotic-resistance genes controlled under a cardiac-specific promoter [15-18]. These strategies have been shown to produce almost pure CMs that once transplanted into the heart do not seem to form tumors; however, these genetic approaches present many obvious restrictions to their clinical application. On the other hand, interesting experiments have been performed by the groups of Terzic [19] and Murry [20], in which ESC cardiac specification was guided by specific cytokine treatment (TNF-α or Activin-A plus BMP4 combination respectively), and a pure cardiac population was obtained that, once transplanted in the infarcted heart, is able to partially regenerate the muscle with no tumor formation reported. Furthermore, these cells positively affected cardiac performance and also, when transplanted with a cocktail of pro-survival cytokines, induced a significantly greater improvement of cardiac function [20]. A caveat to these results is provided by a recent study published by Mummery's group, which shows that transplantation of human ESC derived CMs in a mouse model of MI induced an improvement in cardiac function (together with cell engraftment); however, this effect disappeared at 3 months [21]. Thus, long-term studies need to be performed in order to determine the safety and efficacy of ESCs. Finally, another major issue that needs consideration together with the tumorigenic potential is the immune rejection provoked by the ESCs. Nuclear transfer in order to obtain non-immunogeneic patient-matched ESCs, creation of hematopoietic cells lines derived from human ESCs or expression by the ESCs of recipient specific major-histocompatibility complex molecules, are some approaches that are being studied.

Bone Marrow-Derived Sc

In adult tissues, stem cells with differentiation potential have been found, albeit with a more limited than ESCs. However, the *in vitro* differentiation potential of Bone Marrow (BM)-derived cells towards CMs is not yet clear. Early studies performed by Fukuda *et al.* showed differentiation of BM-derived stromal cells towards spontaneous contractile cells with cardiac phenotype when treated with 5-Azacitidine [22]; however, subsequent studies showed that the differentiated cells also expressed skeletal myoblast markers. Moreover, other laboratories have not been able to reproduce these experiments. *In vivo*, however, although the degree of differentiation still remains controversial, many reports have shown cardiac differentiation potential from BM-derived cells. This evidence was supported by human sex-mismatched heart transplantation, where cardiac chimerism was determined in transplanted patients [23-26]. The percentage of contribution however, was very low in all these cases (0.02% to 0.07%) which raised questions about the physiological relevance of their contribution. On the other hand, a higher differentiation rate to endothelial cells was found.

In one of the first studies performed in a murine model, GFP positive BM-derived hematopoietic cells were transplanted into a lethally irradiated mouse model of acute MI. As a result of the transplantation, only a very low rate of GFP$^+$ CMs ($\approx$ 0.02%) were found in the perinfarct region (and 3.3% of endothelial cells) and importantly, it was proven that such cell plasticity could be due to a fusion phenomenon [27,28]. On the other hand, studies from Anversa's group have shown that transplantation of the BM fraction Lin$^-$/ckit^{high} in the infarcted myocardium, could contribute to nearly 50% of de novo CMs, endothelial and smooth muscle cells [29]. Unfortunately, these experiments have not been reproduced by other independent laboratories using similar or even the same models, leading to a general skepticism regarding the potential of BM cells to differentiate into functional cardiomyocytes [30,31]. Some interesting studies have been performed with heterozygous cKit mutant mice Kit^W/Kit^{W-V} [32]. When MI was provoked, the abnormality led to dilated cardiomyopathy and death from cardiac failure but failing hearts could be rescued by transplantation of wild type BM cells. Although an angiogenic positive effect of the cKit cells was demonstrated, no cKit-derived cardiomyocytes were found. Further studies are needed in order to better understand the role of the cKit$^+$ cells in cardiac regeneration. On the other hand, the existence of rare cell populations in the BM with much higher differentiation capability has recently been demonstrated. MAPCs were the first cells described with the capability to give rise to cells derived from the three germinal layers [33,34]; subsequently, other cells like the MIAMI [35], VSEL [36], SSEA1+ [37], Oct4+ [38] and SSEA1+ and SSEA3+ BM-derived clonal cells [39] have also been described. More detailed molecular studies need to be performed in order to determine whether these putative different cell types represent the same population at

different differentiation stages. It has been demonstrated that some of these populations possess cardiac potential, like the VSEL cells [40] or the ones described by the group of Losordo [39], which have been demonstrated to contribute *in vivo* to CMs (4.1% ±3.1% in the peri-infarct region) together with endothelial and smooth muscle cells (5.4%±3.3% and 5.8%±2.9% respectively), and which induced a favorable remodeling and improvement in the cardiac function of the ischemic heart. An augmentation of proliferation and preservation of the perinfarcted area at risk by up-regulation of paracrine factors involved in angiogenesis, apoptosis and proliferation, was also demonstrated. Interestingly, these cardiac cells were negative for the cKit marker. Also, the pluripotent BM-derived SC population MAPCs, was tested as a candidate for cardiac repair in acute and chronic infarct. Although cardiac differentiation was not proven due to rapid disappearance of the transplanted cells (probably due to the ischemic conditions of the tissue and immunological reaction against the non-host GFP or β-galactosidase cell markers), improvement of the cardiac function, which was not achieved in the control group transplanted with BM cells, was demonstrated [41,42]. In the acute model, a reduction of the infarct size and induction of the neoangiogenesis was shown. *In vitro* analysis showed that MAPCs could have had a trophic effect by secretion of inflammatory (monocyte chemoattractant protein-1 (MCP)-1) and angio/arteriogenic (VEGF, platelet derived growth factor (PDGF)-BB, and TGF-β1) factors, together with an anti-apoptotic protective effect of the myocardium at risk. Importantly also, differentiation towards endothelial (venous or arterial) and smooth muscle cells has been demonstrated *in vitro* and in other *in vivo* ischemia models [43,44].

Adipose-Derived Sc (Adsc)

The adipose tissue, like the BM, contains a population of cells that has extensive self-renewal capacity, its expansion being not only due to mature adipocyte hypertrophy but also to the presence of precursor cells in the stroma-vascular fraction (SVF). Planat-Benard *et al* showed that this particular cell fraction was able to rescue lethally irradiated mice as consequence of reconstitution of the major hematopoietic lineages [45]. Furthermore, it was shown that the ADSCs could differentiate not only into hematopoietic cells but also into mesenchymal cell types (osteoblasts and adipocytes), and most importantly, into vascular endothelium [46] and cardiac-like cells [47]. Thus, when fresh SVF are cultured in methylcellulose, they become organized forming contractile clumps which contain cardiac cells with ventricle- and atrial-like phenotype. Importantly, electrophysiological studies performed on early cultures revealed a pacemaker activity of the cells. Moreover, stimulation by adrenergic or cholinergic agonists in more mature differentiated cells was detected. Similarly, cultured cells could give rise to endothelial cells whose beneficial effect has been demonstrated *in vivo* by their contribution to neoangiogenesis in hindlimb ischemia mice models [48] and by their ability to secrete angiogenic and antiapoptotic factors [49]. On the other hand, ADSC cardiac differentiation has been recently demonstrated when transplanted in acutely infarcted mice [50], and an improvement in the cardiac function has been proven after CD29+ SVF cell transplantation [51]. Importantly, ADSCs also induced an improvement in the cardiac function associated

with an increased vasculature in an acute/reperfusion ischemia model in pig [52]. More basic studies are required to better characterize these cell populations which, due to their differentiation potential and simple and innocuous isolation technique, appears to offer a potential clinically useful source of cells for therapeutic transplantation in the heart.

Resident Cardiac Stem Cells

The existence of cardiac progenitor cells (CPCs) in the adult heart was first reported by Anversa's group [53]. Cells were shown to be distributed in small clusters in the interstices adjacent to the cardiomyocytes and were isolated and characterized by a Sca-1⁻cKit⁺ phenotype. These cells possess self-renewing, clonogenic and multipotent abilities with potential to differentiate towards cardiomyocytes, endothelial and smooth muscle cells. Importantly, an increase of the CPC pool in the heart after acute myocardial infarction was reported [54] and an improvement in cardiac function after their transplantation in rat ischemic heart muscle was also demonstrated [53]. Recently, their ability to transverse the vessel barrier to engraft into the ischemic heart after intracoronary injection has also been shown [55]. A population of Sca-1⁺cKit⁻ cardiac progenitor cells which, after stimulation *in vitro* with 5-Azacytidine, express cardiac markers has also been described [56], although no spontaneous beating cells could be detected. Importantly, when these cells were intravenously transplanted in previously infarcted and reperfused mice, they could home to the injured heart and differentiate towards cardiomyocytes, 50% of the differentiated cells being consequence of fusion events [56]. An independent laboratory in Japan was also able to isolate this Sca1⁺ population and to differentiate it *in vitro* towards cardiac cells (surprisingly with oxytocin but not with 5-azacytidine) and also *in vivo*, finding Sca1⁺ derived endothelial and smooth muscle cells [57,58]. Furthermore, a Sca-1⁺cKit^{LOW} cardiac side population defined by the expression of the transport protein Abcg-2, could be differentiated towards cardiomyocytes upon co-culture with rat cardiomyocytes [59,60]. Other studies have shown the ability of some cells derived from the murine and human heart to form clusters *in vitro* when cultured in suspension (named "cardiospheres") [61]. These clusters contain clonally derived cells which organize in a core composed by proliferating c-kit-positive cells and a surrounding layer of spontaneously differentiated cells that express markers characteristic of cardiac, endothelial and mesenchymal cells. Moreover, their transplantation into immunosuppressed infarcted mice improved their cardiac function. Also, proliferation and differentiation of these cells has been reported [62]. Finally, another population of cardiac stem cells has been recently described in rodent and human embryo, newborn and adult right atrium hearts which is characterized by the expression of the LIM-homeodomain transcription factor islet-1 [63]. Their self-renewal and CM differentiation potential has been demonstrated; unlike the other cell populations, these cells do not express cKit or Sca1 receptors. It will be interesting to know whether such a population can be stimulated in the adult heart and what role they can play in the ischemic heart.

Although many studies have focused on the potential of SC to differentiate into CMs, in order to regenerate the heart tissue, it is equally important to restore the vascular net,

which will supply the new repopulated tissue with oxygen and nutrients. Importantly, most of the SC previously described (ESCs, SVF and several populations of the BM like the EPCs (Endothelial progenitor cells) also possess the capacity to differentiate into vascular structures.

Skeletal Myoblasts and Satellite Cells

Although some initial studies suggested that skeletal myoblasts could differentiate towards CMs, their lack of cardiac differentiation potential has been clearly demonstrated. Recently, however, the existence in the skeletal muscle (Sk) of a non-satellite cell population (termed SPOC cells) has been shown, which can differentiate to spontaneously beating cells with cardiac features [64]. Furthermore, another population, which unlike SPOC cells, is phenotypically characterized by the expression of the CD34 marker, has also been identified. Importantly, when the Sk-CD34+ cells were transplanted in the ischemic heart, they induced an improvement in the function [65]. It would be interesting to determine their putative presence in the human skeletal muscle, which has not been described yet. On the other hand, referring to the skeletal myoblasts, despite their lack of cardiac differentiation potential, many studies have been performed in both small and large animals models of MI, showing also an improvement in cardiac function after their transplantation (Reviewed in [66] and [67]). These beneficial effects could be due, at least partially, to a cell paracrine effect, by secretion of factors that can induce the activation of the angiogenesis processes and modulation of the composition of the extracellular matrix. Besides, skeletal myoblasts present high resistance to hypoxia, which give them an advantage over other types of cells which, when transplanted, quickly disappear due to the low oxygen environment. Moreover, the fact that myoblasts are progenitor cells already committed to muscle differentiation with low proliferation rate avoids the tumorigeneic risk that other cells may have. On the other hand, the main limitation of skeletal myoblasts is their inability to electromechanically couple with the surrounding CMs, which it has been argued, could increase the risk of arrhythmias.

Conclusions from the *In Vivo* Experimental Results

From all the extensive *in vivo* data published until now, there are some conclusions and important points that need to be discussed: (1) the low levels of cell engraftment that have been found in the vast majority of the animal experiments performed; (2) the low degree of cell differentiation *in vivo* and (3) the near lack of correlation between the type of cells transplanted and the functional effect observed.

Cell retention and survival are one of the main technical limitations that stem cell therapy presents nowadays. In the cardiac ischemic tissue, the hypoxic environment together with the inflammation - a process typical of the MI acute stage also provoked by the needle injection during the cell transplantation - significantly diminish the number of engrafted cells which in many cases end up disappearing a few weeks after the transplant. However, despite their limited engraftment, their positive effect in the sick heart has been demonstrated in many studies. Initially, this benefit was attributed to the differentiation potential of the stem cells to cardiac or vascular cell types, but it is clear now that the per-

centage of cells that truly differentiate is very low and cannot be fully responsible for the positive effect. Besides, several cell types with very different tissue-origin and differentiation potential have been shown to induce similar benefits. Therefore, it has been hypothesized that a paracrine effect might be, at least partially, the cause of this effect. Cytokines secreted by the transplanted cells could promote angiogenesis, cell proliferation and survival, or extracellular matrix composition changes and might even attract/activate endothelial or cardiac progenitor cells present in the organism (Fig. **1**). This hypothesis has been proven by injection of conditioned media recovered from cultured SC, which can also produce some benefit in the injected hearts [68,69]. On the other hand, direct treatment with single specific cytokines has also been tested; however, the improvement induced has been significantly lower than with the transplanted cells, probably due to the instability of the cytokine and also because the paracrine cell effect must be consequence of a combination of several factors rather than only one. On these grounds, several bio-engineering approaches have been proposed and tested in order to improve the degree of cell engraftment and survival. Transplantation of stem cells embedded in matrigel or collagen matrixes have been shown to improve the level of engraftment and hence, the cardiac function [70]. Synthetic membranes where cells can be grown in monolayer and be applied as a patch, have also been found to produce significant benefit [71] and more sophisticated techniques, such as 3D scaffolds where cells can integrate, are also being tested [72]. Finally, several experiments have shown a greater positive effect when genetically modified cells expressing some anti-apoptotic factors like Akt or Bcl2 are transplanted [69,73]. Thus, the combination of stem cell therapy with other scientific fields like the bio-engineering and gene therapy could greatly improve the regenerative potential of these cells.

Clinical Trials and Therapeutic Perspectives

Although more basic studies are needed in order to understand "SC behavior" and also the mechanisms involved in cardiac repair, a number of early phase clinical as well as randomized trials have been performed. Based on the encouraging experimental results and their putative feasibility and safety, skeletal myoblasts and bone marrow derived SC (Hematopoietic SC (HSC) and Mesenchymal SC (MSC)) have been tested. Also, their autologous application which avoids the need for immune-suppression has been an important factor in their choice. More recently, ADSCs have been introduced in the clinical arena.

Clinical Trials Using Skeletal Myoblasts

The first clinical trial with skeletal myoblasts was started in 2000 on a series of 10 patients with severe ischemic heart failure (LVEF≤35%) [74,75] (See Table **1**). This group showed an increase in the LVEF during the first year (24.3% ± 4% to 31% ± 4.1%; p=0.001) which remained stable in time over 6 years of follow-up [76]. However, tachycardia episodes were detected in 5 of these patients, requiring the implantation of a defibrillator. Even with this, 3 of the patients still suffered arrhythmic storms, which aroused some concerns about the safety of myoblast transplantation. Regarding this issue, *in vitro* studies showed that neonatal car-
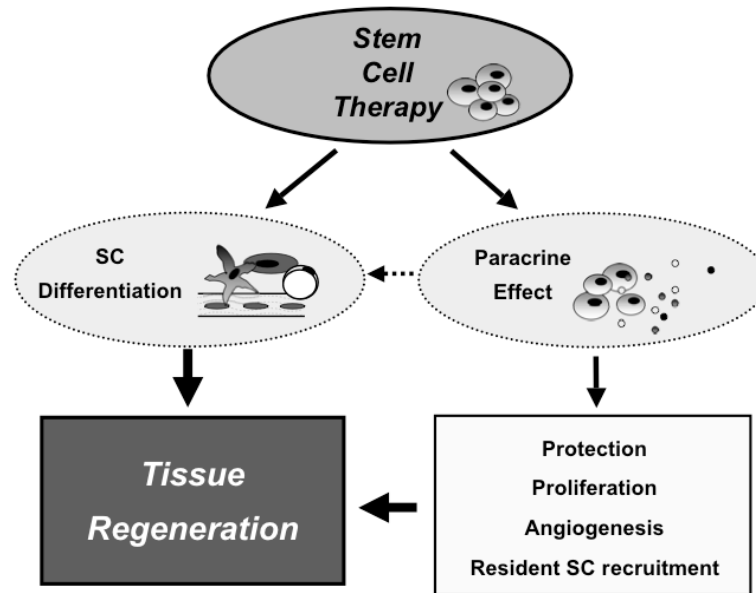
Fig. (1). therapeutic potential: Mechanisms. SC transplantation could induce repair of sick tissue (i) by differentiating itself into the required tissue cell type or (ii) by secretion of cytokines responsible to trigger several mechanisms involved in anti-apoptotic signaling, cell proliferation, angiogeneic processes or recruitment of resident stem cells able to reconstitute the damaged tissue. To better understand the key factors involved in such processes would greatly help to improve the beneficial effect of the transplanted cells.

diomyocytes that were co-cultured with skeletal myoblasts suffered a decrease in the conduction velocity together with arrhythmic contractions [77]. Interestingly, this effect was cell-dose dependent and was detected as long as co-cultures contained more than 20% of myoblasts but never when the percentage was lower than 5%. Also, *in vivo* experiments in rats showed the same cell-dose dependency [78]. In patients, although some cases of arrhythmias have been reported, overall, an improvement in the cardiac function together with an increase in the viability and perfusion has been found in most of the clinical trials published so far [79] [76] [80-85]. Some studies like that published by Gavira *et al*. have been successful with no cardiac arrhythmia reported after one-year follow-up [80]. In this study, cells were cultured in autologous serum instead of bovine serum, which might be associated with lower inflammation. Echocardiography results showed an increase in the ejection fraction from $35.5\% \pm 2.3\%$ to $55.1\% \pm 8.2\%$ (p<0.01) together with an improvement in the regional wall contractility (wall motion index from 3.02 ± 0.17 to 1.36 ± 0.14 (p<0.0001). PET (positron emission tomography) analysis also detected an increase in viabilty and perfusion levels. Importantly, other studies, tested a wide range of cell doses, from 4×10^5 to 5×10^7 [82] or from $1-300 \times 10^6$ cell-dose [81] and although a few cases of arrhythmia were reported, overall, an improvement in the cardiac function together with an increase in the viability and perfusion was determined. The study published by Siminiak *et al* including 10 patients reported an increase from 35.2% to 42.0% in LVEF 4 months after transplant which was maintained during the first year of follow-up. Dib *et al* (30 patients) showed an increase in the ejection fraction of 7% (from 28% to 35%; p<0.02) during the first year and 8% after the second year (p<0.01) together with an improvement in tissue viability. Tachycardia episodes were detected in 3 of the patients. Interestingly, cells were deliv-

ered percutaneously, so the feasibility and safety of this route of transplantation were confirmed [86]. A second study was also performed by the group led by Serruys [79] proving also the safety of the method and documenting an increase in the global ejection fraction. Although all these data are quite suggestive, the small number of patients included in these studies as well as the lack of placebo groups make it impossible to draw any definitive conclusion regarding the beneficial effect of this approach. Very importantly, a randomized, double-blind, placebo-controlled dose-ranging trial (MAGIC) has recently been finished including 92 patients from different hospitals [87]. The study was performed in patients with a LVEF between 15 and 35%, a history of acute MI with residual akinesia and clinical indication for CABG (coronary artery bypass graft). Importantly, no significant differences in survival and first ventricular arrhythmia were detected among the three groups at 1 and 6 months. Furthermore, two doses of cells (400×10^6 and 800×10^6) were tested. It was demonstrated that administration of the high cell dose significantly reduced the end-systolic and end-diastolic volumes which translated into an increase of 3% in the LVEF compared with the placebo group (p=0.04). Despite some limitations in this trial like the low number of patients, the lack of long term follow-up and the functional quantification by echocardiography rather than magnetic resonance imaging (MRI), this study has helped to clarify the safety of skeletal myoblast treatment and suggest the relative efficiency of the treatment, opening new perspectives for treatment of MI provided that phase III trials confirm the results.

Bone Marrow Derived-Sc Clinical Trials

Bone marrow has been the most common source of SC tested in human clinical trials (See Table **2**). The first trials performed determined the safety and feasibility of SC trans-

Table 1. Most relevant SkM transplantation Clinical Trials in CMI

Study (Year)	Cell Type	Number of Patients (Treated/co)	Cell-Dose (%cd56+ Cells) (x 10⁶)	Delivery Time/ Route	Follow-up Time (Months)	%Lvef Increase (Basal vs. Treated) (p in Treated Group)	Lvef Imaging Assessment	Other Functional Outcomes
Non-Randomized Controlled Trials								
Menasche *et al.* **(75)** 2003 Hagege *et al.* **(76)** 2006	SkM	10/0	780-1060 (86)	3-228m/ TEp w/o CABG	10.9 52	8 (*) (10.9m) 4 (NS) (52m)	Echo	↑Regional wall motion (at 10.9m); ↑Global LVEF (at 10.9m).
Smits *et al.* **(85)** 2003	SkM	5/0	90-310 (25-85D+)	24-132m/ TEc	3, 6	5 (**) (3m); 9 (NS) (6m)	LV angiography, Echo, MRI	↑Regional wall motion; ↑Global LVEF.
Ince *et al.* **(84)** 2004	SkM	6/6	60-360	NP TEc (EMM)	12	8 (*) Co: -3	Echo	↑Global LVEF.
Gavira *et al.* **(80)** 2006	SkM	12/14	190±120 (65.6)	3-168m TEp + CABG	3, 12	8 (**) (3m), 9 (**) (12m) Co: 3 (NS) (12m)	Echo	↑Regional wall motion; ↑Global LVEF; ↑Tissue Viability; ↑Perfusion.
Siminiak *et al.* **(82)** 2004	SkM	10/0	0.4-50 (65.4D+)	4-108m/ TEp w/o CABG	4, 12	7 (*) (4m), 7 (*) (12m)	Echo	↑Regional wall motion; ↑Global LVEF.
Chachques *et al.* **(83)** 2004	SkM	20/0	300±20 (78±5D+)	NP/ TEp w/o CABG	9, 19	24 (*)	Echo, SPECT	↑Regional wall motion; ↑Global LVEF; ↑Tissue Viability.
Dib *et al.* **(81)** 2005	SkM	30/0	2.2-300 (42-98)	NP/ TEp w/o CABG or LVAD	12, 24	7 (*) (12m), 8 (*) (24m)	Echo, SPECT, MRI	↑Regional wall motion; ↑Global LVEF; ↑Tissue Viability; ↓ESV; ↓EDV.
Siminiak *et al.* **(86)** 2005	SkM	9/0	17-106 (65)	5-96m/ TC	2.5	3-8 in 6/9 patients (Not statistically analyzed)	Echo	Symptoms improved.
Biagini *et al.* **(79)** 2006	SkM	10/0	217±111 (64±27D+)	28-140m/ TEc	1, 3, 6, 12	6 (**) (12m)	Echo, TDI	↑Regional wall motion; ↓ESV; ≈EDV.
Randomized Controlled Trials								
MAGIC/ Menasche *et al.* **(87)** 2008	SkM	30HD /34 33LD /34	800 (HD) 400 (LD) (89)	NP TEp + CABG	6	0.8 (NS) (HD)	Echo	≈Global LVEF; ↓ESV (HD).

Abbreviations: SkM: Skeletal Myoblasts; Co: Control group; D+: desmin positive; NP: Not Provided; TEp: Transepicardial; TEc: Transendocardial; TC: Transcoronary; NS: Not

plantation, showing also a functional improvement [88-93]. However, these trials included a limited number of patients, and this, along with the lack of randomization and double-blinded performance, meant that the reliability of the results could not be tested. Moreover, the significant heterogeneity in the type of cell populations used (BM-Mononuclear cells (BM-MNC), sorted BM fractions, *in vitro* cultured cells (MSCs) or G-CSF mobilized cells), the delivery route (endo-cardial catheter-based or epicardial surgical-based or percutaneous catheter-based intracoronary injec tion) and the tim-

ing of transplantation all limited the conclusions of these studies.

The first randomized trial, the BOOST I trial (BOne marrow transfer to enhance ST-elevation infarct regeneration) was performed in 60 patients with acute MI. Thirty of the patients received $2.5\pm0.9 \times 10^9$ unfractionated BM-MNCs by intracoronary delivery ~6 days after occlusion. Although a control group that did not receive cells was included, no bone marrow aspiration or sham infusion was performed in

Table 2. Most Relevant BM Transplantation Clinical Trials in AMI

Study (Year)	Cell Type	Number Of Patients (Treated/co)	Cell-Dose (%cd34+ Cells) (x 10^6)	Sham?	Delivery Time/ Route	Follow-up Time (Months)	%Lvef Increase (Basal vs. Treated) (p in Treated Group)	Lvef Imaging Assesment	Other Functional Outcomes
Non-Randomized Controlled Trials									
Strauer *et al.* **(93)** 2002	BMC	20 (10/10)	9-28 (0.39)	No	5-9d+/IC	3	4 vs 5; (NS)	LV angiography	↓ESV; ≈EDV; ↑Perfusion; ↑Regional wall motion; ↓Infarct Size.
TOPCARE-AMI / Assmus *et al.* **(92)** 2002, Britten *et al.* **(91)** 2003, Schachinger *et al.* **(88)** 2004.	BMC/CPC	30 (19/11)	240(7.4)	No	4d+/IC	4, 12	2.5 vs 8.5; (**)	LV angiography, Echo	↓ESV; ≈EDV; ↑Regional wall motion; ↓Infarct Size.
Fernandez-Aviles *et al.* **(89)** 2004	BMC	33 (20/13)	37-119	No	8-19d+/IC	6	5.8; (**)	Cardiac MRI	↓ESV; ≈EDV; ↑Thickness of infarct wall.
Bartunek *et al.* **(98)** 2005	CD133+	35 (19/16)	NP (12.6)	No	10.2-13d+/IC	4	4.3 vs 7.1; (*)	SPECT, Echo	≈EDV; ↑Perfusion.
Randomized Controlled Trials									
BOOST I / Wollert *et al.* **(96)** 2004 Schaefer **(95)** 2006	BMC	60 (30/30)	2460 (9.5)	No	6d+/IC	6, 18	0.7 vs 6.7; (**) (6m) 3.1 vs 5.9; (NS) (18m)	MRI	↓Infarct Size.
Chen *et al.* **(97)** 2004	MSC	69 (34/35)	4800-6000	Yes	18d+/IC	6	6 vs 8; (*)	LV angiography	↓ESV; ↑Perfusion; ↑Regional wall motion.
Jannsens *et al.* **(99)** 2006	BMC	66	304 (2.8)	Yes	24h+/IC	4	2.2 vs 3.3 ; (NS)	MRI	↑Perfusion; ↓Infarct Size; ↑Tissue Viability.
ASTAMI/ Lunde *et al.* **(102)** 2006, Lunde *et al.* **(103)** 2007	BMC	97 (47/50)	68 (0.7)	No	4-8d+/IC	6	6.7 vs 8; (NS)	SPECT, MRI, Echo	≈ESV; ≈EDV; ≈Infarct Size; ≈Perfusion.

Table 2. Contd….

Study (Year)	Cell Type	Number Of Patients (Treated/co)	Cell-Dose (%cd34+ Cells) (x 10^6)	Sham?	Delivery Time/ Route	Follow-up Time (Months)	%Lvef Increase (Basal vs. Treated) (p in Treated Group)	Lvef Imaging Assesment	Other Functional Outcomes
REPAIR-AMI/ Schachinger *et al.* **(101)** 2006 Erbs *et al.* **(100)** 2007	BMC	204 (101/103)	236 (3.6)	Yes	3-6d+/IC	4, 12	3 vs 5.5; (*)	LV angiography, MRI	≈ESV; ≈EDV; ≈Infarct Size.
Meluzin *et al.* **(105)** 2006 Meluzin *et al* **(104)** 2007	BMC	66 (22/22+22)	100 (HD) 10 (LD)	No	6-10d+/IC	3, 6, 12	3 vs 6; (NS) (3m) 0 vs 7; (*) (6m) (HD) 0 vs 7; (**) (12m) (HD)	SPECT, Echo	↓ESV; ≈EDV; ↑Perfusion.

Abbreviations: BMC: Bone Marrow Cells. CPC: Blood derived-Circulating Progenitor Cells. Co: Control group. NP: Not Provided. HD: High cell Dose. IC: Intracoronary. NS: Not Significant. *: P<0.05. **: P<0.01. LV: Left Ventricular. Echo: Echocardiography. MRI: Magnetic Resonance Imaging. SPECT: Single Photon Emission Tomography. ESV: End Systolic Volume. EDV: End Diastolic Volume.

the control group. After 6 months, an improvement in the ejection fraction (50.0% to 56.7% in treated *vs.* 51.3% to 52.0% in controls) with no significant changes in the LV-end-diastolic volumes or reduction of the infarct size, was demonstrated; Follow up of these patients showed that such improvement was transitory and by 18 months there was no statistical differences in the global LV ejection fraction between the control and the treated groups (3.1 percentage points in the control group *vs.* 5.9 in the treated group) [94-96].

Close in time, another similar study performed with MSCs in 69 patients showed an improvement in the global LVEF (from 48±10 to 54±5 in the control group and from 49±9 to 67±3 in the treated group; p=0.01), together with a reduction of the end-systolic and diastolic volumes and an improved contractility index, wall motion and velocity and increased tissue viability. Unfortunately, the functional studies were performed at 6 months and no long-term follow-up was performed [97]. In another clinical trial, CD133+ selected BM-MNC were transferred to a series of 14 patients. Global LVEF, regional wall motion and tissue perfusion increased in the treated group after 4 months. However, secondary effects like restenosis or *de novo* lesions developed in 6 of the 14 patients, provoking some concerns among the scientific community [98]. Importantly, in 2006, the group of Janssen *et al.* published the results of the first randomized, double-blinded, placebo-controlled trial [99]. In this study, intracoronary transplantation of BM-MNC was performed in 33 patients with AMI, 24h after reperfusion (34 were included as placebo). A reduction of the infarct size and a better recovery of the regional systolic function were detected after 4 months, but no significant functional improvement or a significant improvement of myocardial perfusion and metabolism indexes were detected. During the same year, the results of another two studies performed with a larger number of patients were also published. Contradictory results were obtained; whereas in the REPAIR-AMI trial (Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction), an improvement of the ejection fraction together with a reduction of the infarct size and a restoration of the microvascular function of the infarct-

related artery [100] 4 months after transplantation were seen [101], no differences were found in the ASTAMI trial (Autologous Stem Cell Transplantation in Acute Myocardial Infarction) [102] despite an improvement in exercise time and heart rate responses to exercise [103]. In both studies, the method of cell delivery (intracoronary) and the SC source used (BM-MNC) were similar. It is possible, however, that the timing of cell-delivery, the method of cell isolation and/or the degree of severity on the patients condition could vary between these trials, explaining the contradictory results. Also, a slightly smaller number of cells (2-4 times fewer) was injected in the ASTAMI trial. Along similar lines, the last two studies published by Meluzin *et al.* [104,105] showed that the benefit of BM-MNC transplantation in acute MI can be cell-dose dependent. Three months after transplantation, a significant functional improvement was detected only in the group transplanted with the higher cell-dose (10^8 cells *vs.* the lower cell-dose 10^7 cells) with a 5% vs. a 3% LVEF increase (p<0.05 *vs.* control group).

A very interesting meta-analysis [106] of 10 controlled clinical trials performed with BM-MNC in patients with recent acute MI (≤14 days) has recently been published. The statistical results suggest that intracoronary cell therapy following percutaneous coronary intervention for acute MI can provide statistically and clinically relevant benefits for cardiac function and remodeling. Moreover, it has also suggested a dose-response association with such benefits.

Finally, various other trials have been performed with mobilized cells after G-CSF treatment. The first clinical trial performed (Front-Integrated Revascularization and Stem cell Liberation In Evolving Acute Myocardial Infarction: FIRSTLINE-AMI) [107] [108] suggested an improvement of cardiac function, but a placebo-controlled clinical trial (REgenerate VItal Myocardium by Vigorous Activation of bone marrow stem cells: REVIVAL II and STEM cells in Myocardial Infarction: STEMMI) did not confirm such a benefit [109-111]. It could be argued that the late application of the G-CSF in the REVIVAL II study (5 days after reperfusion *vs.* 90 min) could be the cause of the different results in these studies. There is growing evidence that G-CSF could have a

more cardioprotective than cell-mediated effect [112], and therefore, the time of injection could be a critical factor for its positive effect. However, in the last randomized, double-blind, placebo-controlled trial performed, the STEMMI trial, G-SCF was applied also at an earlier time-point (12h after reperfusion), with a similar pattern to the FIRSTLINE-AMI trial, although a functional benefit could not be detected. LVEF improved similarly in the control and treated groups measured both by MRI (8.5 *vs.* 8.0; *p*=0.9) and echocardiography (5.7 *vs.* 3.7; *p*=0.7). Importantly, no clinical adverse effects were detected.

In conclusion, performing multicenter randomized trials with long-term follow-up is mandatory in order to demonstrate the efficacy of BM-MNC transplantation and to establish, for the greatest efficacy, the ideal cell type, cell-dose and surgical procedure (delivery route, transplantation time-point, etc.).

FUTURE STUDIES

The scientific community and the media have hailed Stem Cell therapy as a therapeutic alternative for conventional medicine based on pharmacologic treatment. The idea of replacing damaged tissues or dysfunctional cells - sometimes due to genetic diseases- opened a new perspective on traditional medicine. However, although our basic knowledge about these recently discovered cells is growing, there are many questions and technical limitations that need to be answered or solved in order to better apply these cells as an effective treatment. Both basic research and clinical trials suggest that SC transplantation has a positive effect in certain patients with myocardial infarction. However, it is still very important to investigate how to augment the stability of the cells once transplanted and also to understand the molecular mechanisms involved in such benefits. Due to the low engraftment and permanence of the transplanted cells in the heart, and the fact that they still resulted in functional improvement, it has been proposed that the factors and cytokines released by the transplanted cells could trigger several mechanisms, such as angiogenesis, proliferation and protection, which could contribute to tissue regeneration. Although other mechanisms cannot be discarded, a better understanding of the paracrine effect, identifying the key molecules and pathways responsible for such improvement, would enormously help to direct the therapy in a more specific and efficient way. Also, the differentiation pathways and molecular mechanisms at the protein, transcriptional and epigenetic levels are being studied intensely, in order to find ways to manipulate the differentiation of these cells towards the required cell type. This last issue is an important subject if we take into account the fact that even if the cells present multi- or pluri-potent capabilities, once they are transplanted in the sick tissue it is probable that they will not receive the proper signaling for it. One possible alternative to this limitation could be to transplant *in vitro* pre-differentiated cells.

In summary, we could conclude from all the results reviewed until now that stem cell application in cardiovascular disease presents great potential and that we can be optimistic about its therapeutic potential. None the less, many studies need to be performed in order to understand their origin and behavior and to be able to apply them in routine clinical practice.

ABBREVIATIONS LIST

ADSCs	=	pose Derived Stem Cells
ASTAMI	=	Autologous Stem Cell Transplantation in cute Myocardial Infarction
BM	=	Bone Marrow
BM-MNC	=	Bone Marrow Mononuclear Cells
BMP4	=	Bone Morphogenetic protein 4
BOOST	=	Bone Marrow transfer to enhance ST-elevation infarct regeneration
CABG	=	Coronary Artery Bypass Graft
CMs	=	Cardiomyocytes
CPCs	=	Cardiac Progenitor Cells
3D	=	Three Dimensions
EPCs	=	Endothelial Progenitor Cells
FIRST-INE-AMI	=	Front-Integrated revascularization and tem ell Liberation In Evolving Acute Myo cardial nfarction
G-CSF	=	Granulocyte-Colony Stimulating Factor
GFP	=	Green Fluorescent Protein
HSC	=	Hematopoietic Stem Cells
LVEF	=	Left Ventricular Ejection Fraction
MAGIC	=	Myoblast Autologous Grafting in Ischemic Cardiomyopathy
MAPCs	=	Multipotent Adult Progenitor Cells
MCP-1	=	Monocyte Chemoattractant Protein-1
MI	=	Myocardial Infarction
MIAMI	=	Marrow-Isolated Adult Multilineage Inducible cells
MRI	=	Magnetic Resonance Imaging
MSC	=	Mesenchymal Stem Cells
Oct4	=	Octamer-4
TNF-α	=	Tumor Necrosis Factor - α
PDGF-BB	=	Platelet-derived Growth Factor-BB
PET	=	Positron Emission Tomography
REPAIR-AMI	=	Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction
REVIVAL	=	Regenerate Vital Myocardium by Vigorous Activation of bone marrow stem cells

Sca1	=	Stem Cell Antigen-1
Sk	=	Skeletal muscle
SPOC	=	Skeletal Precursors Of Cardiomyocytes
SSEA	=	Stage Specific Embryonic Antigen
STEMMI	=	STEM cells in Myocardial Infarction
SVF	=	Stromal Vascular Fraction
TGF-β1	=	Transforming Growth Factor -β1
VEGF	=	Vascular Endothelial Growth Factor
VSEL	=	Very Small Embryonic Like cells

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