

# Stem cells for myocardial infarction: an update

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REVIEW

## ABSTRACT

Cardiovascular diseases are the major cause of death in the world. Current treatments have not been able to reverse this scenario, creating the need for the development of new treatments. Cell therapies have emerged as an alternative for cardiac diseases of distinct causes in experimental animal studies and more recently in clinical trials. The recent breakthroughs in stem cell studies, molecular and cell biology, and tissue engineering have prepared the route for developing a new biomedical discipline: regenerative medicine. In the field of cardiovascular medicine, the real promise of a stem cell-based regeneration and repair lies in the promotion of myogenesis at the site of the cell graft and in the development of angiogenesis processes to achieve both structural and functional benefits. This review critically discusses the recent findings in the field of stem cells and their use in the treatment of vascular diseases. Here we analyze the sources of cells available for therapy in cardiovascular diseases. We also propose the mechanisms by which stem cells can promote angiogenesis and functional improvement. Finally we discuss the most recent results in pre-clinical experimentation and clinical trials regarding myocardial infarction.

**Keywords:** cellular therapy, stem cells, ischemia, cardiovascular, infarction

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## RESUMEN

**Células madre en infarto miocárdico: una actualización.** Las enfermedades cardiovasculares constituyen la principal causa de muerte en el mundo. Los tratamientos actuales no han sido capaces de revertir este escenario, promoviendo la necesidad de desarrollar nuevos tratamientos. Las terapias celulares han emergido como alternativas para las enfermedades cardíacas de distinto origen, tanto en estudios experimentales en animales como en ensayos clínicos más recientes. Los avances en el estudio de las células madre, la biología molecular y celular, y en la ingeniería de tejidos, han permitido el desarrollo de una nueva disciplina de la biomedicina: la medicina regenerativa. En el campo de la medicina cardiovascular, la promesa de la regeneración y reparación basadas en las células madre, se basa en la promoción de biogénesis en el sitio del implante celular y en el desarrollo de procesos de angiogénesis para obtener beneficios estructurales y funcionales. Esta revisión discute críticamente los hallazgos recientes en el campo de las células madre y su utilización en el tratamiento de enfermedades cardiovasculares. También proponemos los mecanismos a través de los cuales las células madre pueden promover la angiogénesis y el mejoramiento funcional. Finalmente, se discuten los resultados más recientes en el campo de la experimentación preclínica y los ensayos clínicos relacionados con el infarto miocárdico.

**Palabras clave:** terapia celular, células madre, isquemia, cardiovascular, infarto

## Introduction

Cardiovascular disease (CVD) has become a major health issue throughout the world, exceeding infection as the leading cause of death worldwide [1]. In the western world, particularly in the USA, CVD even exceeds the next five causes of death combined. The increasing prevalence of Heart Failure (HF) is caused by several factors including the improved survival of patients with acute coronary syndromes (nearly 40% of whom will manifest eventual HF by 7 years) and by the significant reduction in sudden cardiac death due to the use of internal cardio-defibrillators [2]. However, the factor with the greatest impact on the incidence and prevalence of HF is its association with advanced aging.

Although heart transplantation is the preferred treatment for end-stage heart failure, chronic organ shortage and complications with immunosuppression have led to a search for alternate options. Recently, cellular-based therapy has emerged as a potential new therapy for patients with advanced heart failure.

Experimental and human data suggesting progenitor cells possess the capacity to regenerate tissue and augment repair in injured organs has generated widespread interest in the basic research and clinical communities. Nowhere have these findings been more exciting than in human cardiovascular diseases, in which vasculogenesis and myocardial regeneration remain as attractive therapeutic targets.

It is now obvious that cell-based cardiac and vascular repair are feasible, both early and later in the disease process. In fact, cell therapy may offer an unparalleled opportunity for improvement to millions of individuals living with cardiovascular disease. However, many questions about the technology still remain.

This review critically discusses the recent findings in the field of stem cells and their use in the treatment of vascular diseases. Here we analyze the sources of cells available for cell therapy. We also propose the mechanisms by which stem cells can promote angiogenesis and functional improvement. Finally we

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present the most recent results in preclinical experimentation and clinical trials regarding myocardial infarction and the state of art of this therapy in Cuba.

### Myocardial infarction

Myocardial infarction (MI) is associated with dysfunction and irreversible loss of cardiomyocytes (CMCs). The heart, with a scarce number of resident stem cells and a limited capacity of CMCs to re-enter into the cardiac cycle, has an inadequate capacity to repair itself [3]. Cardiac allograft is the gold standard treatment modality, but is limited by the shortage of donors and immunosuppressive complications. There is a pressing need to develop alternative methods to manage this problem. Cell implantation has been viewed as a potential strategy for patients with coronary artery disease and heart failure. Most of the reported studies have documented improved heart function after implantation of donor cells via regeneration of heart muscle [4]. Extensive preclinical and limited clinical studies have shown the safety, efficacy, and feasibility of cell engraftment for cardiac repair [5, for review].

Regeneration of infarcted myocardium with pluripotent stem cells can be accomplished by two main approaches (Figure 1). The first technique is stem cell mobilization, which involves stimulating the expansion of specific populations of stem cells within the bone marrow, and then directing released stem cells or progenitor cells to the infarct zone without ever removing them from the patient. This form of transplantation requires developing pharmacological or genetic means to stimulate specific populations of stem cells and direct their homing to the damaged tissue. The second more feasible approach is autologous stem cell transplantation and involves isolating autologous stem cells from bone marrow or other tissue and re-injecting them directly into the myocardium or blood stream sometime after myocardial infarction.

### Cell types for transplant. Current status

Experimental studies indicate that cells derived from the bone marrow, blood, skeletal muscle and other tissues possess regenerative capacity in the heart and vasculature. These findings have raised the hope of clinically-applicable cell therapy for tissue repair in the cardio vascular system. Next we detailed some characteristics of the progenitor cells from different sources that have been used in cardiovascular regenerative therapy. Table 1 shows, in a comparative and summarized form, the advantages and disadvantages of its use in several applications.

#### Embryonic stem cells

Human embryonic stem cells (hESCs) are being rapidly produced from chromosomally euploid, aneuploid, and mutant human embryos that are available from *in vitro* fertilization clinics treating patients for infertility or pre-implantation genetic diagnosis (Figure 2). The hESCs can be formed and maintained on human somatic cells in humanized serum-free culture conditions and for several passages in cell-free culture systems. They may be grown indefinitely *in vitro* while maintaining their original karyotype and epigenetic status, but this need to be confirmed from time to time in

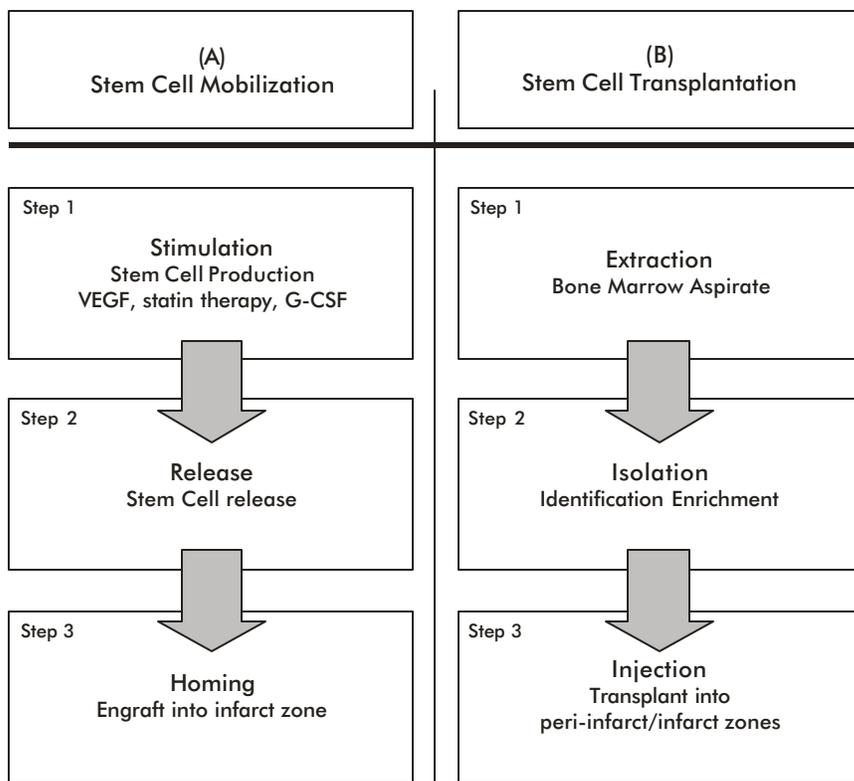


Figure 1. Two potential methods for utilization of autologous stem cell. In intrinsic stem cell mobilization (A), cells expansion and release are induced in the native bone marrow by VEGF, statin therapy or G-CSF. The released cells then home to and engraft into the infarct zone. In extrinsic stem cell transplantation (B), stem cells are extracted from the bone marrow and injected into the peri-infarct or infarct zones of the myocardium. Specific stem cells can be identified and enriched before injection using fluorescence-activated cell sorting (FACS).

Table 1. Substrates for cell-based therapies for the heart

Cell type	Source	Advantages	Disadvantages	Applications
Skeletal myoblasts	Autologous skeletal muscle biopsy	Ease of isolation and culture scalability; Genetic engineering; Autologous source (no rejection); Ischemia resistant	Inability to establish E-C; Potential arrhythmias; Uncontrolled proliferation	MI HF Cardiomyopathy
Fetal/Neonatal cardiomyocytes	Allogenic fetal/neonatal heart	Ability to establish E-C; High proliferative rate; Genetic engineering	Controversial ethical/moral issues; Donor availability; Poor long-term survivability; Immune rejection; Sensitive to ischemic injury	MI HF Cardiomyopathy Long QT disorders
Embryonic stem cells	Blastocyst (inner mass)	Pluripotent; Ability to establish E-C; Genetic engineering	Donor availability; Large-scale expansion difficult; Allogenic immune rejection; Dependence on murine feeder layers; Insufficient characterization	MI HF Cardiomyopathy Long QT disorders Tissue engineering Organogenesis
Bone marrow stromal cells (mesenchymal)	Bone marrow	Multipotent; Ease of isolation and culture; Autologous source (no rejection); Genetic engineering	Insufficient characterization; Scalability	MI HF Cardiomyopathy Tissue engineering Organogenesis
Endothelial progenitor cells	Bone marrow Peripheral blood Chord blood	Ease of isolation and culture scalability; Autologous source (no rejection); Genetic engineering	Insufficient characterization	CAD HF Blood vessel repair Vascular protheses Tissue engineering

CAD, coronary artery disease, E-C, electromechanical coupling; EPC, endothelial progenitor cell; MI, myocardial infarction; HF, heart failure.

long-term cultures. All three major embryonic lineages are produced in differentiating flat attachment cultures and unattached embryoid bodies. Cell progenitors of interest can be identified by markers, expression of reporter genes, and characteristic morphology; and the cells thereafter enriched for progenitor types and further culture to more mature cell types [6].

Wobus *et al.* in the early nineties were the first to demonstrate the capability of mouse ESC to differentiate into beating CMCs [7]. CMCs were observed in the embryoid bodies, along with a wide range of other specialized cell types [8]. The terminally differentiated CMCs have cell to cell communication capacity due to the presence of functionally coupled gap junctions. They possess pharmacological and physiologic properties of specialized myocardial cells, including ventricular, atrial, pacemaker and Purkinje cells, and also have typical postnatal CMC electrophysiological characteristics. The CMCs have normal contractile sensitivity to calcium, and many features of excitation-contraction (E-C) coupling seen in isolated fetal or neonatal CMCs [8]. ESCs-derived CMCs were able to functionally integrate into the myocardium of adult mice [9]. Gepstein's group demonstrated that CMCs derived from hESC could form structural and electromechanical connections with cultured rat CMCs. These cells were also able to pace swine hearts following transplantation into a swine model of atrioventricular block [10].

CMC differentiation is governed by a complex process of extracellular signaling, by multiple intracellular signal-transduction pathways, and by activation of multiple transcription factors that activate a repertoire of cardiac-specific genes. CMC differentiation of mouse ESC involves a complex interplay of defined endogenous factors, including Transforming Growth Factor- $\beta$ 1 [11], bone morphogenic proteins [12], fibroblast growth factors [13], nitric oxide [14], etc. The induction of mouse ESC into CMCs has been reported using retinoic acid [15], ascorbic acid [16], dynorphin B [17], 5-azacytidine [18], and oxytocin [19].

During *in vitro* differentiation, CMCs within the embryoid bodies express cardiac-specific genes, proteins, ion channels, receptors, and signal transduction pathways in a developmental pattern that closely resembles the developmental pattern of early *in vivo* cardiomyogenesis [8]. Studies have suggested that the early-differentiated CMC are typical of primary myocardium and subsequently differentiate to atrial, ventricle, Purkinje, and pacemaker-like CMC [20]. Human ESC derived CMCs show the expected molecular, structural, electrophysiological, and contractile properties of nascent myocardium [21]. Given the undeniably inefficient cardiogenesis within the embryoid bodies, it might be worthwhile to devise strategies for enriching ESC-derived CMCs from spontaneously differentiating embryoid bodies. In this direction, the most successful approach so far has been genetic selection using a selection marker driven by a cardiac-specific promoter [22].

Several issues must be addressed before hESCs can be used for cardiac regenerative therapy. First, the culture conditions need to be optimized including the correct cytokine mix to increase CMC differentiation from hESCs. Second, ESCs rejection following

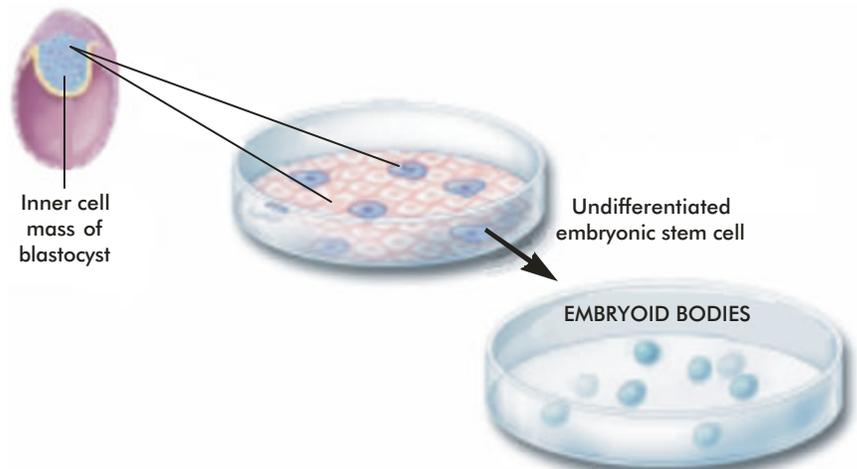


Figure 2. Embryonic stem cells.

transplantation needs to be blocked. A decreased expression of the molecules of the Major Histocompatibility Complex (MHC) would improve immunologic tolerance, as ESCs express MHC I, with low or absent MHC II expression [23]. Also, hESCs grown on mouse feeder layer cells express an immunogenic nonhuman sialic acid Neu5Gc which would need to be eliminated, preferably starting with fresh hESCs that have never been exposed to animal products using human serum with human feeder layers [24]. In addition, an increase in the efficiency of CMC differentiation from hESCs is necessary to determine the extent of maturity of the CMCs in terms of E-C coupling.

The propensity of embryonic stem cells for multilineage differentiation carries, however, the liability of neoplastic growth, impeding therapeutic application. Behfar *et al.* suppressed the tumorigenic threat associated with embryonic stem cell transplantation by cardiac-restricted transgenic expression of the reprogramming cytokine TNF- $\alpha$ , enhancing the cardiogenic competence of recipient heart. The *in vivo* aptitude of TNF- $\alpha$  to promote cardiac differentiation was recapitulated in embryoid bodies *in vitro*. The pro-cardiogenic action was mediated by secreted cardio-inductive signals. Resolved TNF- $\alpha$ -induced endoderm-derived factors, combined in a cocktail, secured guided differentiation of embryonic stem cells in monolayers produce cardiac progenitors termed cardiopoietic cells. This predetermined population yielded functional CMC progeny, characterized by a down-regulation of oncogenic markers, up-regulation, and nuclear translocation of cardiac transcription factors. Recruited cardiopoietic cells delivered in infarcted hearts generated CMCs that proliferated into scar tissue, integrating with host myocardium for tumor-free repair [25].

### Bone marrow hematopoietic stem cells

Hematopoietic Stem Cells (HSCs) isolated from the mononuclear component of rat bone marrow (BM) have been shown to significantly regenerate the infarcted myocardium. The cells were isolated using negative and positive selection [26]. Orlic *et al.* de-

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monstrated that injection of c-kit+/lin- (BMSCs) into the rat myocardium generated smooth muscle, endothelial and cardiomyocytic cells, regenerating 60-70% of ischemia damaged tissue [26]. Quaini *et al.* also confirmed the therapeutic potential of BMSCs in cardiac repair in humans. The type of stem cell differentiation was determined using tissue specific markers and the Y chromosome was used as a marker of cell origin. Markers of smooth muscle, endothelium or cardiomyocytes were expressed in more than 20% of the cells with a Y chromosome, which would indicate BMSCs have the capacity to generate all types of cardiac cells [27].

Results using genetically modified mice with the cre-lox recombination system have dramatically challenged the concept of differentiation of BM cells into CMCs, strongly indicating that fusion between cells, as opposed to differentiation, may account for the results from studies dealing with myocardium regeneration potential of BMSCs [28]. In another kind of experiments to prove fusion mechanism, green fluorescent protein (GFP)-labelled hematopoietic stem/progenitor cells were intravenously injected into newborn cyan fluorescent protein (CFP)-expressing recipients that may fully potentiate the developmental plasticity of stem cells. Transplantation of mouse BM lineage antigen-negative (Lin-) cells resulted in the generation of the cells that displayed CMC-specific antigenic profiles and contractile function when transplanted into syngeneic newborn recipients. Confocal microscopy successfully separated GFP and CFP, and revealed that donor GFP+ CMCs co-expressed host-derived CFP. On the other hand, the same authors injected human cord blood derived HSCs into neonatal immune-deficient mice. Fluorescence *in situ* hybridization analysis of recipient cardiac tissues demonstrated that human and murine chromosomes were co-localized in the same CMCs, indicating that cell fusion occurred between human hematopoietic progeny and mouse CMCs. Both results provide compelling evidence that hematopoietic stem/progenitor cells contribute to the postnatal generation of CMCs through cell fusion, not through transdifferentiation [29].

A different study demonstrated a purified population of hematopoietic stem and progenitor cells, as well as unfractionated bone marrow cells, engrafted within the infarcted myocardium. The engraftment was hematopoietic and transient, whereas a few bone marrow-derived CMCs were located outside the infarcted myocardium, which were derived via cell fusion [30].

Human cord blood CD34+ progenitor cells, in particular the KDR+/CD34+ fraction, were able to induce angiogenesis in ischemic damaged heart tissue of non obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, yet very few CMCs stained positive for human nuclei [29]. *In vivo*, these cells have an anti-apoptotic effect on CMCs and they also synthesize growth factors. Therefore, the cardiac therapeutic potential of BM cells may be dependent on endothelial cell generation and growth factor synthesis. Following this observation, it was more recently shown that the number of circulating KDR+/CD34+ progenitor cells inversely correlate with cardiovascular events in man [31]. But the use of HSCs has not

always been successful. In some cases, post-infarction transplantation of genetically labeled hematopoietic stem cells in mice did not result in CMC transdifferentiation [32, 33].

### Bone marrow mesenchymal stem cells

Most attempts for cardiomyoplasty have considered the bone marrow as the source of the repair stem cells, assuming that the hematopoietic stem cells can do the work. However, bone marrow is also the residence of other progenitor cells, including mesenchymal stem cells (MSCs) (Figure 3). Since 1995 it has been known that under *in vitro* conditions, MSCs differentiate into cells exhibiting features of CMC. This pioneer work was followed by many preclinical studies that revealed that *ex vivo* expanded, bone marrow-derived MSCs may represent another option for cardiac regeneration.

Under proper stimulation, MSCs can be induced to differentiate into adipocytes, osteoblasts, chondrocytes, tenocytes, myocytes, and hematopoietic-supporting stroma [34,35]. Furthermore, MSCs may also give rise to other lineages such as endothelial, kidney, and neural, revealing a high degree of plasticity [36,37]. MSCs that are isolated from several human sources, including bone marrow and peripheral and umbilical cord blood, exhibit a high *ex vivo* expansion capacity. This property has been used to assess the biologic characteristics of MSCs [38] to perform transfection with viral vectors [39] and to initiate studies toward the use of MSCs in clinical strategies [40].

Data from different laboratories have shown that MSCs, once exposed to a variety of physiologic or

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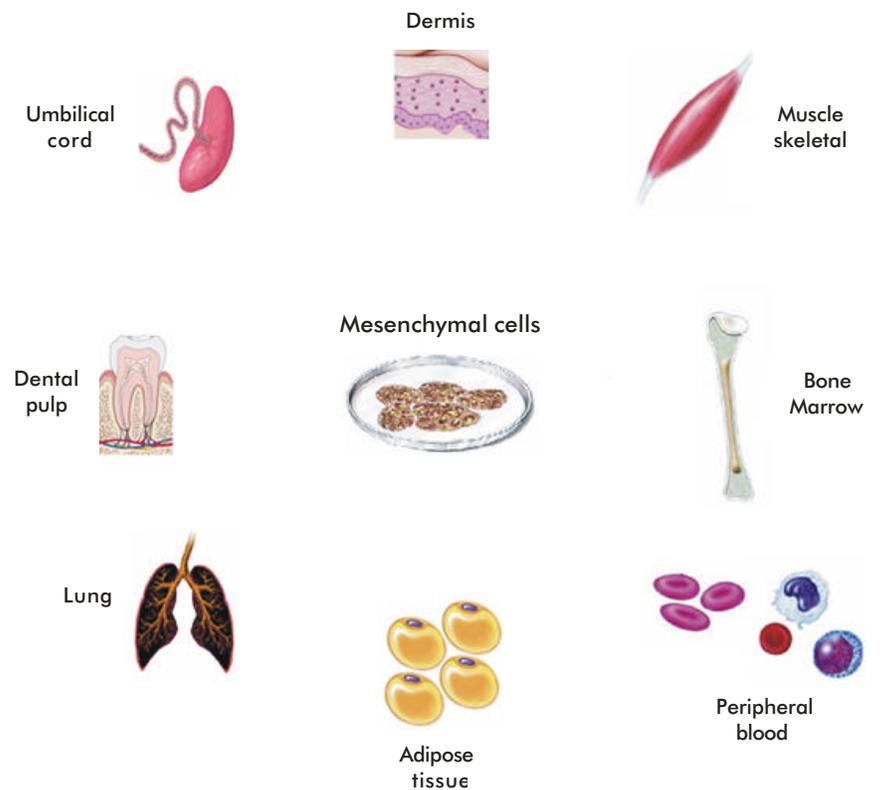


Figure 3. Different sources of adult mesenchymal stem cells.

non-physiologic stimuli, differentiate into cells displaying several features of CMCs-like cells [see 41 for a review]. Under these conditions, *ex vivo* differentiated MSCs exhibit a myotube-like structure and a time-dependent competence to synchronously beat. In turn, electron microscopic analysis revealed a CMCs-like ultrastructure including typical sarcomeres, a centrally positioned nucleus, and atrial granules. These cells show several functional features of a developing CMC including the production of peptides and the expression of multiple structural and contractile proteins. They also display, at least, sinus node-like and ventricular cell-like action potentials [42, 43].

By co-culturing human MSCs with human CMCs, it was demonstrated that the stem cells acquired a CMCs-like phenotype, characterized by the expression of myosin heavy chain, beta-actin, and troponin T. Thus, it seems that direct cell-to-cell contact is obligatory in relaying "cardiac environmental or microenvironmental" signals for MSC differentiation into a cardiomyogenic lineage [44]. Furthermore, human MSCs exhibit cell-to-cell coupling to each other and to ventricular myocytes via specific gap junctions [45].

Some reports have shown that MSCs differentiate not only into CMCs, but also into vascular smooth muscle cells/pericytes (vSMC/PC) progenitors and endothelial cells. These cell types are involved in the development of vascular systems, including angiogenic sprouting and vessel enlargement. Previous data have shown that *de novo* formation of vSMC/PC occurs after the differentiation of perivascular mesenchymal cells, in a platelet-derived growth factor B (PDGF-B)-dependent process [46]. The mechanisms underlying MSC differentiation to cardiovascular cells, and subsequent improvement in neovascularization and cardiac function, involve the paracrine secretion of growth factors by MSCs [47, 48].

In general, bone marrow cells are considered to have the greatest potential as a source for CMCs. The two main stem cell populations, the MSCs and the HSCs, have been reported to differentiate into CMCs; however, it has not been determined which population is a better source for CMCs. Yamada's group showed that CD133-positive brown adipose tissue derived cells (BATDCs) induce BMCs into CMCs, not through cell fusion, but through bivalent cation mediated cell-to-cell contact when cocultured. Moreover, BMCs induced by BATDCs are able to act as CMC replenition in an *in vivo* infarction model. Finally, it is demonstrated that CD45(-) CD31(-) CD105(+) non-hematopoietic cells, when cocultured with BATDCs, generated more than 20 times the number of CMCs compared with *lin*(-)*c-kit*(+) HSCs. Taken together, the authors suggest that CD133-positive BATDCs are a useful tool as CMC inducers, as well as a source of CMCs and that the non-hematopoietic fraction in BM is also a major source for CMCs [49].

### Local cardiac stem cells

Until recently, the heart was considered terminally differentiated without any significant regenerative potential [50]. This dogmatic view was first challenged

by Piero Anversa and coworkers who reported the existence of small proliferating cells in the heart, expressing myocyte specific markers [51]. In addition, these cells were up-regulated under pathological cardiac conditions [52].

At least three cardiac progenitor cell (CPC) pools residing in postnatal hearts have been reported in different species. These cells are distinguished by different expression of marker proteins. The three populations include the *c-kit*+ cells [53], cells from the side population [54], and the so-called cardioblasts expressing *islet-1* [55]. See reference [56] for a recent overview of resident CPCs found in the heart.

The adult human heart contains small populations of endogenous committed cardiac stem cells or multipotent cardiac progenitor cells, identified by their cell-surface expression of *c-kit* (the receptor for stem cell factor), P-glycoprotein (a member of the multidrug resistance protein family), and Sca-1 (stem cell antigen 1, a mouse hematopoietic stem cell marker) or a Sca-1-like protein [57]. These cells do not express cardiac specific genes under normal conditions, however, in the presence of the DNA demethylating agent 5'-azacytidine they are able to differentiate via a process that is partly dependent on a receptor for bone morphogenetic proteins [58].

In other set of experiments, when cultured cardiac side population cells (CSPs) from neonatal rat hearts were treated with oxytocin or trichostatin A, some CSPs expressed cardiac-specific genes and proteins and showed spontaneous beating. When green fluorescent protein-positive CSPs were intravenously infused into adult rats, many more (approximately 12-fold) CSPs were migrated and homed in injured heart than in normal heart. CSPs in injured heart differentiated into CMCs (4.4%), endothelial cells (6.7%), or smooth muscle cells (29%) of total CSP-derived cells, respectively [59].

For some authors, cardiac stem cells represent a logical source to exploit in cardiac regeneration therapy because, unlike other adult stem cells, they are likely to be intrinsically programmed to generate cardiac tissue *in vitro* and to increase cardiac tissue viability *in vitro* [57]. These CPCs can differentiate into all the constituting cells of the heart [53, 60], thus making them theoretically capable of repairing injuries in the heart.

Remarkably, in animal studies, administration of *in vitro* expanded CPCs substantially improved cardiac performance. However, CPCs do not *in natura* have the capacity to heal larger structural injuries as e. g. myocardial infarctions show poor functional regeneration. It has been compellingly demonstrated that the CPC pool can differentiate into at least cardiac myocytes, endothelial and vascular smooth muscle cells that are functionally integrated in the heart. Scientific data suggest that CPCs are able to differentiate into functional myocytes and vessels integrating into the diseased myocardium [60-63].

### Skeletal myoblasts

Skeletal myoblasts are an endogenous population of muscle cells characterized by regenerative capacity. These cells can be autologously obtained from a small biopsy, expanded in culture and injected back into the hearts of patients who suffered from a myocardial

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infarction. Myoblasts have a high resistance to ischemia. This property seems to be a major advantage in the hypoxic and hypo-vascularized environment of a myocardial scar [64]. Although transdifferentiation of skeletal myoblasts into CMCs does not occur in this setting [65], several studies in rodent and porcine infarction models reported improved cardiac performance after myogenic cell transplantation into the heart [66-68]. Interestingly, stem cells residing in the skeletal muscle are reported to differentiate into CMCs [69]. "Side population" cells isolated from the skeletal muscle express cardiac specific proteins together with cadherin and connexin 43 at the junctions with neighbouring cells when co-cultured with neonatal CMCs. Apparently beating of the cells was necessary for transdifferentiation to occur.

The crucial difference between skeletal myoblasts and those chimeric CMCs that were shown to be generated in transplanted hearts [70] is that engrafted myoblast tubes do not develop connexin 43 and gap junctions resulting in electrical coupling to the surrounding myocardium [71]. Intracellular recordings of grafted myoblasts in infarcted rat myocardium showed a contractile activity of newly formed myotubes, which was fully independent of neighbouring CMCs [72]. This phenomenon could lead to arrhythmias after myoblast transplantation [73].

Indirect effects were utilized to explain the observed positive effects of myoblast transplantation since the engrafted skeletal muscle seems not to increase the inotropism of recipient hearts with an active contraction mechanism and because skeletal myoblasts fail to transdifferentiate into CMCs [71]. Paracrine mechanisms as the secretion of hepatocyte growth factor (HGF) [74] could explain the achievement of a better left ventricular function. HGF has cardioprotective as well as antifibrotic effects and the HGF-receptor c-Met is also expressed in ischemic myocardium [75].

### Endothelial progenitor cells

Several lines of evidence point to the existence and persistence of a stem cell named "hemangioblast" characterized by a hematopoietic and endothelial forming potential [76]. These cells express antigens shared by hematopoietic and endothelial cells, such as CD34 [77], vascular endothelial growth factor (VEGF) receptor 2 (FLK-1/KDR) [78] or CD133 [79]. Endothelial committed cells, named endothelial progenitor cells (EPCs), derive from the hemangioblast and can be expanded *ex vivo* from bone marrow-derived- or blood-borne mononuclear cells [80]. They express antigens shared by hematopoietic stem cells together with endothelial specific ones [81]. In addition the ability for uptake of diacylated low density lipoprotein has also been utilized as a marker of this cell type.

It has been shown that BM-derived EPCs are involved in reendothelialization of vascular lesions and that statin treatment can accelerate this process by enhancing the circulating pool of EPCs and the recruitment of bone BM-derived endothelial cells into the injured vessel wall [82]. Therefore, the positive contribution of EPCs to adult neovascularization has been considered to be potentially useful in attenuating

myocardial ischemia in coronary artery disease [81]. Moreover, since EPCs, as well as endothelial cells, have been shown to transdifferentiate into CMCs [83], it is possible to hypothesize that EPCs may contribute to myocardial regeneration.

Since it is unknown whether transdifferentiation of EPCs into CMCs occurs *in vivo* in humans, it remains unclear if the application of these cells can also lead to regeneration of myocardium or if the observed positive effects are depending solely on "improved revascularization".

### Preclinical experiments

It has been demonstrated that intramyocardial injection of human STRO-1-selected precursors in an athymic rat model of acute myocardial infarction results in induction of vascular network formation and arteriogenesis coupled with global functional cardiac recovery. Immunoselection with monoclonal antibodies against STRO-1 and vascular-cell-adhesion molecule 1 (VCAM1/CD106) prior to expansion results in a 1000-fold enrichment of mesenchymal precursors compared to standard isolation techniques [84].

Tang *et al.* transplanted MSCs in a rat myocardial infarct heart with reperfusion model. Measurement of hemodynamics 4 weeks after transplantation *in vivo* showed left ventricular function to be significantly greater in MSCs than in the control group. Semi-quantitative histomorphometric examinations showed a significantly lower infarct size and a greater left ventricle wall thickness in the MSC-treated group compared to the control group. Immunofluorescence demonstrated that transplanted MSCs were positive for cardiac troponin T, suggesting that a small number of transplanted MSCs can differentiate into CMCs. Other MSCs were positive for CD31 and smooth muscle actin. Hematoxylin-Eosine staining showed marked augmentation of neovascularization in the MSC group. Semi-quantitative analysis demonstrated that capillary density was significantly higher in the MSC group than in the control group [85].

Genetically modified MSCs can improve cardiac functioning when implanted in different models of chronic ischemia. Huang *et al.* transplanted MSCs infected with adenovirus containing angiogenin gene (MSC-AdAng) or null adenovirus (MSC-AdNull) into a porcine chronic ischemic model. After 4 weeks, MSCs were observed within the implanted area in both cell transplantation groups. Angiogenin protein levels were significantly greater in the MSC (AdAng) group than in control group and were associated with greater neovessel formation. MSC transplantation decreased scar size and increased scar thickness. A synergistic effect of MSC and angiogenin was observed in the MSC(AdAng) group because myocardial perfusion and cardiac function increased significantly in this group compared with control [86].

Bhakta *et al.* studied the safety and feasibility of autologous bone marrow MNCs intracoronary therapy in a porcine model of chronic myocardial ischemia. After euthanasia, troponin I levels, gross inspection and histopathology did not reveal evidence of myocardial infarction. Labeled cells were observed in perivascular structures in myocardium at the injection

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site in all animals and in the spleen from one animal. Localization of labeled cells in bone marrow aspirates suggested a role for bone marrow engraftment and repopulation as a possible mechanism for progenitor cell localization in myocardium [87].

MSCs can also prolong the survival of transplanted hearts in a rat allograft model, after intravenous administration. MSCs have recently been shown to have profound immunomodulatory effects both *in vitro* and *in vivo*. In a model where Wistar rat MSCs were infused via the tail vein in recipient Fisher 344 rats previously transplanted with hearts from inbred Wistar rats, the survival of the allografts was markedly prolonged by administration of MSCs compared with the controls. Real-time PCR showed a shift in the Th1/Th2 balance toward Th2. By Mixed Lymphocyte Reaction and Cell-Mediated Lympholysis assays, untreated control rats showed greater alloreactivity than did MSC-treated rats. These results indicated that MSCs suppressed allogeneic T-cell responses and possibly, induced allograft tolerance through changing the Th1/Th2 balance [88].

Improvement of cardiac function has also been observed after transplantation of pluripotent ESCs, but the risk of teratoma generation is still present. Nelson *et al.* transplanted low numbers of two murine ESC lines, respectively engineered to express a beta-galactosidase gene from either a constitutive (elongation factor) or a cardiac-specific (alpha-myosin heavy chain) promoter, into infarcted mouse myocardium. Although ESC-derived tumors formed within the pericardial space in 21% of injected hearts, lacZ histochemistry revealed that engraftment of ESC was restricted to the ischemic myocardium. Echocardiographic monitoring of ESC-injected hearts that did not form tumors revealed functional improvements by 4 weeks postinfarction, including significant increases in ejection fraction, circumferential fiber shortening velocity, and peak mitral blood flow velocity. These experiments indicated that the infarcted myocardial environment can support engraftment and cardiomyogenic differentiation of pluripotent ESCs, concomitant with partial functional recovery [89].

The hypothesis that stem cells exert their biological actions on infarcted or ischemic areas through paracrine signals becomes more attractive, since it has been demonstrated in several animal models. Noiseux *et al.* reported that intramyocardial injection of bone marrow-derived MSCs overexpressing Akt (MSC-Akt) decreased infarct size at 3 days and restored early cardiac function, with a low rate of differentiation or fusion of MSC and CMCs. The authors concluded that early paracrine mechanisms mediated by MSC are responsible for enhancing the survival of existing myocytes and that Akt could alter the secretion of various cytokines and growth factors [90]. The same group demonstrated that conditioned medium from hypoxic Akt-MSCs markedly inhibits hypoxia-induced apoptosis and triggers vigorous spontaneous contraction of adult rat CMCs *in vitro*. When injected into infarcted hearts, the Akt-MSC conditioned medium significantly limits infarct size and improves ventricular function relative to controls. Support to the paracrine hypothesis is provided by data showing that several genes, coding for factors (VEGF, FGF-2,

HGF, IGF-I, and TB4) that are potential mediators of the effects exerted by the Akt-MSC conditioned medium, are significantly up-regulated in the Akt-MSCs, particularly in response to hypoxia [91].

Xu *et al.* also demonstrated that cultured BMSCs release VEGF, basic fibroblast growth factor (bFGF), stromal cell-derived factor-1 (SDF-1) and insulin growth factor-1 to culture medium and that hypoxia-induced cell apoptosis was diminished in CMCs co-cultured with BMSCs. Moreover, conditioned medium from cultured BMSCs also induced up-regulation of Bcl-2 antiapoptotic protein in CMCs [92].

While virtually all studies of cell transfer into damaged cardiac tissues reported functional benefits, especially in small animal models, the mechanism of these effects remains unresolved. Initial reports suggesting transdifferentiation of cells delivered to target tissues into the organ host cells have been followed by reports of cell fusion and by claims of no meaningful cell-cell transformation, leading to a major controversy. The evidence for transdifferentiation *in vivo* is contradictory with some studies finding evidence for phenotypic switches following injections of cells into the heart or other organs [83, 93] while others cannot demonstrate this phenomenon [32, 33]. The reasons for these discrepancies are manifold and include both technical as well as poorly understood biological issues.

In contrast to a highly controversial transdifferentiation, cell fusion is a well-recognized phenomenon, even if the reports differ as to the frequency with which this event occurs. The existence of cell fusion has been clearly demonstrated in several organs [30, 94] although its functional significance has not been determined.

Cell integration, another plausible mechanism of cell transfer benefit, refers to the ability of certain cell types to integrate into the cardiac syncytium without transdifferentiating into the cardiac muscle in the process improving cardiac performance. For example, electromechanical coupling between the transplanted skeletal myoblasts and the myocardium [95] would contribute to this functional integration, although its existence has been challenged [72].

Another potential mechanism of the observed beneficial effects is the possibility that cell transfer can stimulate proliferation and/or differentiation of endogenous progenitors, based on a paracrine effect of transplanted cells (*i.e.* release of various chemokines and cytokines that can affect endogenous cell differentiation, myocardial remodeling and function) [96-98]. It has been shown that endothelium-derived growth factors affect the development of apparently unrelated organs and cells, and a paracrine effect has also been demonstrated for other cell types. Finally, the mechanical effects of cell transfer (*i.e.*, strengthening of the scar by the presence of a new tissue mass) may but itself favorably alter left ventricle geometry and improve cardiac function.

## Clinical trials

Based on the urgent need for alternative therapies, the promising results obtained with animal models have been quickly transferred to the clinical field, where numerous small pilot studies using different cell types

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are already ongoing and/or have reported promising results. Nevertheless, the lack of randomization, the variability and small size of the treated cohorts and the use of mixed populations of cells have often clouded the significance and prevented a mechanistic interpretation of the results.

There are various modalities for cell delivery in cardiac stem cell therapy. Cells may be delivered by means of direct surgical injection, intracoronary infusion, retrograde venous infusion, transendocardial injection, and peripheral infusion. Each technique has its own peculiarities, and so the choice of the modality should be based on the particular clinical scenario [for a review see 99].

The first clinical trial using cell therapy to treat heart disease was initiated by Philippe Menasche and colleagues in 2000. In this trial, an average of  $870 \times 10^6$  autologous skeletal myoblasts were injected into non-revascularizable, scarred left ventricle as an adjunct to coronary artery bypass grafting. The initial outcome was encouraging; however, no control group was included in the study and each patient received adjunctive revascularization at the time of cell transplantation. Four of ten patients experienced ventricular tachycardia requiring internal cardio-defibrillator implantation. None of the patients experienced fatal arrhythmia [100].

To date, the safety and feasibility of myoblast and bone marrow cell therapy has been evaluated in a number of clinical studies with cells delivered either surgically as an adjunct to coronary artery bypass grafting or percutaneously as an adjunct to reperfusion or as a stand-alone treatment. Percutaneous delivery has been either endoventricular or intracoronary. Most recently, transvenous cell delivery has been begun clinically [reviewed in 101].

The rationale for using a mixture of "repair cells" instead of a single cell type lies in the following foundations. First, bone marrow derived mononuclear cells represent an important source of endothelial progenitors [102]. Clinical data have shown that the implantation of mononuclear cells to myocardial infarction patients [103], as well as to patients with ischemic limbs [104], was effective in promoting therapeutic angiogenesis. Second, MSCs have the capability to differentiate into CMC-like cells. Third, MSCs produce angiogenic growth factors like basic fibroblast growth factor, vascular endothelial growth factor, and stem-cell homing factor [105, 106]. Therefore, the co-transplantation of MSCs and MNCs may result in the enhancement of both cardiomyogenesis and angiogenesis. Myocardial co-transplantation of MSCs with other cells is not without precedent; results have shown that intramyocardial transplantation of MSCs either with fetal CMCs or bone marrow cells resulted in a marked increase in myocardial regeneration. The latter was probably due to triggering cellular and molecular events associated with neocardiomyogenesis, neoangiogenesis, and/or nerve sprouting and atrial sympathetic hyperinnervation [107].

In 2004 it was carried out the Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) trial [108]. The researchers performed a percutaneous coronary intervention after acute

myocardial infarction. It was a randomized trial, where 30 patients received approximately  $2.5 \times 10^9$  unfractionated bone marrow cells and 30 received no infusion. Left ventricle ejection fraction (LVEF) was assessed by magnetic resonance imaging. At 6 months LVEF was 6% greater in BMC group than in control group. At 18 months no significant difference in LVEF was found between the 2 groups, suggesting that the main effect was an acceleration of recovery [109].

Janssens *et al.* also performed a percutaneous coronary intervention after acute myocardial infarction. They transplanted 33 patients with  $3 \times 10^8$  Ficoll-separated BMC and 34 received placebo infusion. They did not detect an improvement in global ventricular function at 4 months in the BMC group as compared with the control group, although infarct size was reduced and regional wall motion was improved in the BMC group [110].

The Transplantation of Progenitor Cells and Recovery of LV Function in Patients with Chronic Ischemic Heart Disease (TOPCARE-CHD) trial performed by Assmus *et al.* evaluated the effects of BMC or progenitor cells derived from circulating blood (CPC) in patients with chronic ventricular dysfunction. In this randomized, crossover trial, the absolute change in LVEF was significantly greater among patients receiving BMC than among those receiving CPC. The groups received the other type of cell in the next phase of the trial, but the result was independent of the order in which the cells were given, suggesting that the BMC effect is somewhat specific. Although the benefit observed after BMC infusion was modest (an increase in LVEF by 2.9 percentage points), it is remarkable that any benefit was seen in these patients, who were studied on average more than 6 years after infarction and who were already receiving optimal medical care. The TOPCARE-CHD trial suggests that BMC can have effects beyond simple acceleration of healing after infarction [111].

In the ASTAMI trial, Lunde *et al.* injected approximately  $7 \times 10^7$  Ficoll-separated BMC in 47 patients after acute myocardial infarction, while 50 patients received no infusion. At 6 months there was no significant difference in LVEF between the 2 groups [112].

In the largest study of cardiac cell therapy to date, Schächinger *et al.* report the results of the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction

REPAIR-AMI trial, a multicenter trial of the intracoronary infusion of BMC after successful percutaneous coronary intervention for acute myocardial infarction. At 4 months, the absolute improvement in LVEF, measured by angiography, was greater among patients treated with BMC than among those given placebo. This double-blind and fully controlled trial provides the best evidence yet for beneficial effects of BMC after acute myocardial infarction. Ultimately, the validation of cardiac cell therapy will require demonstration of benefit with regard to clinical outcomes - as was the case with reperfusion. Studies performed to date have not been designed or powered to evaluate clinical outcomes. Nevertheless, it is encouraging that the REPAIR-AMI investigators found the rate of adverse clinical events to be significantly lower at 1

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year among patients receiving BMC than among those receiving placebo. Given the relatively small number of events, this result will require replication in larger cohorts [113].

In this moment just in the United States, there are more than 100 clinical trials of stem cell therapy, recruiting patients with different medical conditions, such as coronary artery disease, myocardial infarction, ischemic cardiomyopathy, coronary occlusion, coronary arteriosclerosis, dilated cardiomyopathy, etc. The trials are designed to prove safety and efficacy in improving cardiac function of different kind of stem cells, for example: aldehyde dehydrogenase bright stem cells; autologous bone marrow mononuclear stem cells, AC133-positive autologous marrow-derived stem cells; adipose-derived stem cells; bone marrow-derived mesenchymal stem cells; CD34-positive stem cells, etc.

### Cuban experience in the field

The employment of the regenerative medicine, and especially the use of stem cells in Cuba, is at initial stages. There are several research projects, mainly related to the application of hematopoietic stem cells in the treatment of lower limb critical ischemia and obliterant atherosclerosis. Certain degree of experimentation also exists regarding the use of stem cells in patients with diabetic foot lesions and in patients with myocardial injury. From a methodological point of view, some researchers work in the comparison of protocols for bone marrow derived stem cell mobilization, using granulocyte colony stimulating factors, and in the characterization of the cellular fractions obtained from the bone marrow.

Among the institutions that stand out in the experimentation and application of regenerative medicine are the Institute of Haematology and Immunology, the Enrique Cabrera Hospital, Center of Medical and Surgical Investigations (CIMEQ) and the William Soler Pediatric Hospital, among others.

On February 24th 2004, the first implant of adult autologous stem cells in a patient with lower limb critical ischemia was carried out in our country. The patient had a very favorable evolution and it was possible to avoid the affected inferior member's amputation. This result was considered a landmark in the Cuban science and it opened new perspectives for the treatment of this serious vascular complication [114]. Starting from that date, the regenerative cellular therapy continued being used in patients with lower limbs critical ischemia. November 26th 2004, the same protocol was carried out in Hospital CIMEQ and later on this treatment continued being carried out by the Haematology and Angiology services in patients with peripheral angiopathies.

Of April 23th to 27th 2007, it took place in Havana, the I Congress of the Latin American Chapter of the International Society of Vascular Specialists "ANGIOCARIBE 2007". On day 25th representatives of the cooperative group formed by specialists of the Institute of Haematology and Immunology, Enrique Cabrera, William Soler, Julio Trigo and Abel Santamaría Hospitals exposed their experiences on the application in Cuba of the auto-transplant of adult stem cells for the treatment of vascular affections.

Dr. Heriberto Artaza, from Enrique Cabrera Hospital, exposed the most outstanding results in the treatment of 30 patients with lower limb critical ischemia which received the implant of bone marrow mononuclear stem cells, mobilized to the peripheral blood by treatment with granulocyte colony stimulating factor. With this clinical procedure 67% (14/21) of the patients with previous indication of a major amputation, were rescued from amputation.

Dr. Antonio Jesús Díaz Díaz from the Abel Santamaría Hospital presented the experience of the cooperative group about the treatment of 22 patients with severe lower limb ischemia (state IIb of Fontaine's classification) with peripheral blood autologous mononuclear cells. The study evidenced remarkable improvements in clinical and hemodynamical variables.

Finally, Dra. Ángela Blanco, from Enrique Cabrera Hospital commented the preliminary results in a small number of cases of the perilesional implantation of autologous bone marrow mononuclear cells in the treatment of lesions of the diabetic foot. In this case, the therapeutic answer was valued as satisfactory.

It is necessary to highlight that, in general, these studies were carried out in a reduced number of patients, and they didn't have a group of patients treated only with conventional therapies, which are the real controls of the assay. Therefore, in spite of the results reached until now, it is imposed to design new clinical trials that allow evaluating, with experimental accuracy, the security and effectiveness of the stem cells transplant in different affections.

Recently, the implant of autologous stem cells was described in 12 patients with unilateral severe lower limb ischemia. Purification of stem cells was carried out by two different methodologies: the use of a cell separator and the centrifugation in a density gradient in Ficoll reagent. The results showed significant improvements in the rest pain and in the time of free of pain walking after the implant. The ankle-brachial index and the oxygen saturation also increased gradually after the treatment. Comparing the two purification methodologies, the authors didn't observe statistically significant differences. In both cases appropriate quantities of haematopoietic cells were obtained, compared to those reported previously in the literature. Specially, the Ficoll gradient method showed to be a simple and effective procedure, which is of great importance from an economic and technical point of view [115]. Until now, this is the only article published in international magazines that describes the clinical investigations carried out in Cuba regarding the use of the stem cells in the cardiovascular field.

### Conclusions

Cellular-based therapy has emerged as a potential new therapy for patients with advanced heart failure or peripheral vascular diseases. Cell therapy using fetal and neonatal CMCs, embryonic stem cell-derived CMCs, smooth muscle cells, fibroblasts, skeletal myoblasts and more recently bone marrow stem cells have been explored in preclinical and clinical studies. To date, a number of patients with myocardial infarction have been reported to show improved cardiac performance after receiving cell therapy. However, some ba-

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sic questions remain to be answered before cellular transplant can be adopted on a wider scale. Who should be considered for stem cell therapy? What are the optimal cell types and cell numbers for injection? What is the most appropriate cell delivery method? Can injections be repeated? What are the underlying mechanisms in the observed improvements? What are the long-term outcome and possible complications?

Cellular regeneration is a rapidly growing field, promising fascinating new therapeutic approaches. However, additional research is needed to examine a wide array of problems, for example, the identification of universal stem cell markers; the optimization of delivery routes or devices and stem cell mobilization protocols; the comparison of the effectiveness of cellular transplantation versus growth factor administration; the evaluation of the long term effects of stem cell therapy; etc.

Experimental evidence attests to the proangiogenic, vasculogenic and tissue reparative capabilities of a broad range of progenitor cells derived from the bone marrow, circulation and a number of other tissues *in vivo*. Studies demonstrating the most apparent therapeutic success are those implicated in revasculariza-

tion and repair of acute or chronically ischemic issues in the heart and the peripheral vascular system. Numerous small clinical trials have yielded promising preliminary results without clear evidence of superiority for a specific cell type or clinical disease entity as the most suitable target for cell therapy.

For future clinical trials, it must take into account that patients should be treated with cells only as part of randomized, controlled trials and only after they understand that neither the efficacy nor the long-term risks of cellular therapy are established. Researchers must simultaneously, continue to support basic and translational research that can help guide clinical investigation. Future trials should be powered to examine clinical end points and patients should be followed over the long term and for both beneficial and adverse effects. Recent randomized studies of cell therapy for heart disease represent a milestone in this rapidly developing field while serving as a reminder that many important clinical and fundamental questions have yet to be addressed. The ultimate success of this strategy is likely to depend on continued and effective coordination of rigorous basic and clinical investigations.

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