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STEM CELLS FOR CARDIAC REPAIR: STATE OF THE ART

Gianluigi Condorelli [1,3] and Cesare Peschle [2,4]

[1] Laboratory of Molecular Cardiology, San Raffaele Biomedical Science Park and Multimedica Hospital, Milan, [2] Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanita, Rome, Italy, [3] Institute of Molecular Medicine, University of California San Diego, [4] Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA, USA

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1. ABSTRACT

Stem cell research has generated great excitement over the last few years. In cardiovascular medicine, the possibility to cure heart failure with newly generated cardiomyocytes (CMCs) has sparked the interest of many scientists; as a result, different "ideal" cardiac stem cells were proposed for therapeutic use. We review here the literature in the cardiac stem cell field, which sometimes is "dialectic" in methodology and results.

2. CARDIOMYOCYTES: PROLIFERATING OR TERMINALLY DIFFERENTIATED CELLS?

The proliferating potential of CMCs is limited. In fact, after myocardial infarction, the contractile parenchyma is substituted by fibrotic tissue. Clinical evidence indicates that in most of the area affected by a large infarction, contractile elements are permanently lost, as demonstrated by the persistence of a Q (death) wave on the electrocardiogram (ECG) throughout lifetime. CMCs can undergo a round of proliferation during the first few days of birth, as shown in cell culture experiments of murine CMCs and in *in vivo* experiments using BrdU as a tracer. After this period, their replicating capacity is lost (1). Nevertheless, a high percentage of post-natal CMCs are binucleated while a significant percentage are tri- or tetra-nucleated (1). Nuclei of CMCs in vitro can undergo DNA synthesis and thus overcome the G1-S checkpoint under certain conditions. In fact, incubation of CMC nuclei with the cytoplasm of proliferating cells induces DNA synthesis (2). However, forcing DNA synthesis by oncogene over-expression in differentiated CMCs in vitro induces them to undergo apoptotic death; moreover, in vitro stimulation of CMCs with growth factors only, does not induce DNA synthesis (3). Thus, it seems that G2/M checkpoint and consequent cytodieresis cannot be overrun in CMCs even with the "help" of oncogenes or growth factors. More recently, alpha-myosin heavy-chain alpha-MHC promoter driven over-expression of cyclin D2, but not D1 or D3, in transgenic mice induced DNA synthesis and cell proliferation in CMCs in vivo, ultimately resulting in infarct regression (4). It therefore seems that if a proper proliferation gene is expressed during the perinatal age, such as that induced by the alpha-MHC promoter, a small subset of differentiated CMCs can continue to proliferate. In fact, cell lines of differentiated beating *and* proliferating cardiomyoblasts using cardiac-selective over-expression of Large T-antigen, driven by the atrial natriuretic factor (ANF) or alpha-MHC promoter were generated (5, 6);

using the Cre-Lox recombination system, upon Large T-antigen removal, proliferating cardiomyoblasts became beating terminally differentiated cells (7).

In adult human hearts, Piero Anversa's group demonstrated proliferating CMCs in myocardial tissue. In fact, mitotic CMCs have been described in the myocardium of end-stage heart failure (8) and in the peripheral myocardial infarction area, thus suggesting that CMCs can undergo a full cycle of mitotic division, not only DNA synthesis (9). This group also showed that cardiac-specific over-expression of bcl-2 (10), an anti-apoptotic protein, or IFG-1 (11) resulted in increased CMC proliferation in vivo.

Considering the limited potential of CMCs to undergo proliferation and the self-healing capacity of the myocardium, many groups have tried different strategies to manipulate CMC cell growth in vitro in order to use newly generated CMCs as a therapeutic tool for heart failure. We review here the current status of this field, giving a description of the type of cells used and the major findings associated with each of them.

3. EMBRYONIC CELLS

The key characteristic of embryonic cells is their pluripotency. In fact, three types of pluripotent stem cell lines have been established from mammalian embryos: embryonic carcinoma (EC), embryonic stem (ES), and embryonic germ (EG) cells. ES cells derive from the preimplantation embryo; and like EG cells, can be grown in vitro in their undifferentiated state by cultivation on feeder layers (mouse embryonic fibroblasts or STO cells) or addition of a differentiation inhibitor factor, ie, leukemia inhibitory factor (LIF), self-renewing unlimitedly with a relatively stable karyotype. Once re-implanted into a host blastocyst, they contribute to the generation of all tissues, including gonads, resulting in chimeras from which transgenic lineages with homologous recombination of specific genes can be selected.
CMCs were obtained by all three types of murine stem cells. The most widely studied are ES cells, due to a wide use of mouse homologous recombination techniques.

The first experiments demonstrating differentiation of beating CMCs from mES cells were done in the early nineties. Wobus et al. showed that CMCs are in the initial aggregate called embryoid bodies (EBs), which include a wide variety of other specialized cell types (12). EBs are formed by placing small drops of ES cells into plastic dishes without any fibronectin (13). Later on, EBs are dissociated and re-plated in fibronectin pre-coated dishes. CMCs are readily identifiable since right after plating they spontaneously contract.

The ultrastructural and functional characteristics of the terminally differentiated CMCs from ES cells resembles that of neonatal rodent myocytes; moreover, they contain functionally coupled gap junctions and are therefore capable of cell to cell communication.

Similarly, their electrophysiological characteristics are typical of post-natal CMCs and show pharmacological and physiological properties of specialized myocardial cells, including atrial, ventricular, Purkinje, and pacemaker cells (14). Although T tubules are not prominent, functionally these CMCs have normal contractile sensitivity to calcium, and exhibit many features of excitation-contraction coupling found in isolated fetal or neonatal CMCs (14). Terminal, unlike early, ES cell–derived CMCs are responsive to beta-adrenergic stimulation (15).

Field's group demonstrated that ES cells can be manipulated in vitro in order to improve the yield of cardiomyocyte differentiation as compared to other cell lineages: transfection of ES cells with an alpha-MHC promoter-driven aminoglycoside phosphotransferase, and further selection with G418 resulted in an almost pure CMC culture (16). G418 selected CMCs were able to form grafts in the hearts of adult mice, and functionally integrate into the host myocardium (16). Other groups have manipulated mES cells by electroporation with different cardiac-restricted promoters, generating gain-of-function phenotypes (14).

ES cells could also be used to generate myocardial tissue in vitro, not only myocytes. The group of Thomas Eschenhagen and Wolfram-H. Zimmermann reconstituted engineered heart tissue (EHT) by mixing cardiac myocytes from neonatal rats with liquid collagen type I, matrigel, and serum-containing culture medium. Contractile function of EHT in vitro was normal (17). EHT grafts could be used as a patch in vivo and function was preserved. Recently this group is focusing on the use of stem cell-derived cardiac myocytes to generate EHT.
ES-like cells were recently obtained from neonatal mouse testis; they differentiated into various types of somatic cells, including CMCs, in vitro, under conditions used to induce the differentiation of ES cells, and produced teratomas after inoculation into mice (18).

Human ES (hES) cell lines have been derived from human blastocysts (19) and human embryonic germ (EG) cell lines (20) from primordial germ cells. ES cells were generated from embryos frozen from in vitro fertilization procedures. Few cell lines were available until a few months ago, the ones available before the application of a moratorium on ES cell line derivation from human embryos from most Western Country Governments. However, Doug Melton's group set up a simplified procedure for generating ES cell lines from the inner cell masses of frozen embryos, through which it was possible to produce 17 cell lines that were made easily available to the scientific community (21). His lab also shares a manual for using these cell lines with the rest of the scientific community.

Similar to mES cells, hES cells grow in culture in an undifferentiated state on a feeder layer of mouse embryonic fibroblast (MEF) cells, but not with LIF, and when removed from the MEF feeder layer and cultivated in suspension, begin to differentiate by forming EBs. Gepstein and collaborators were able to generate spontaneously

beating CMCs from hES cells, which were dissociated into small clumps of 3 to 20 cells and grown in suspension for 7 to 10 days, allowing the formation of EBs which were then plated on gelatin-coated culture dishes: rhythmically contracting areas appeared at 4 to 22 days after plating in 8.1% of the EBs (22). Thus, hES cells differentiate in vitro into CMCs at a slower pace in comparison to mES (22).

Beating CMCs from contracting areas expressed cardiac-specific markers and could be compared to early-stage human CMCs, including electrical activity, calcium transients, and chronotropic response to adrenergic agents. Cells formed a functional syncytium with synchronous action potential propagation and pacemaker activity. More recently, Gepstein's group showed that CMCs from hES cells formed structural and electromechanical connections with cultured rat CMCs and, when transplanted in a swine model of atrioventricular block, they paced the swine hearts (23).

There are a number of issues to solve for the clinical application of hES cells in cardiac diseases characterized by heart failure. First, the yield of CMC production has to be dramatically improved. It is therefore fundamental to work on the "ideal" culture conditions, including the right cytokine mix for increasing the CMC differentiation percentage of hES cells. Secondly, immune rejection has to be blocked: in fact, upon differentiation, ES cells express molecules of the major histocompatibility complex (MHC), in particular MHC I, while MHC II expression levels are low or absent (24). Thus, decreasing the expression of MHC I could improve immunologic tolerance. Other considerations must also be taken into account. In fact, it was demonstrated that nonhuman sialic acid Neu5Gc, against which many humans have circulating antibodies, is incorporated in hES cells, which are grown on mouse feeder layer cells. Complete elimination of Neu5Gc would be likely to require using human serum with human feeder layers, ideally starting with fresh hESCs that have never been exposed to animal products (25).
The other important issue is to what extent these cells can be considered mature CMCs in terms of excitation-contraction coupling. This question cannot be accurately answered at the moment since the differentiation procedure from hES cells is not efficient; a low yield does not help address this critical question.

4. HEMATOPOIETIC STEM AND PROGENITOR CELLS (HSCS/HPCS)

A great deal of excitement shook the scientific community when it was shown that a population enriched for HSCs significantly repaired the infarcted myocardium. Stem cells were isolated from the mononuclear component of rat bone marrow (BM) using both negative (Lin^- cells: obtained by immunodepleting cells expressing markers of differentiated hematopoietic lineages, including CD34, CD45, CD20, CD45RO, and CD8) and positive ($c\text{-}kit^+$ cells: immunopositive for c-kit, the receptor for Stem Cell Factor, SCF) selection (26); cells used in these experiments were of male rats and thus could be identified for the Y chromosome. Thus, according to Orlic et al., $c\text{-}kit^+$/lin- bone marrow stem cell (BMSC) injection into the myocardium of rats undergoing ischemia could repair 60-70% of the damaged tissue by originating smooth muscle, endothelial and cardiomyocytic cells (26). Anversa's group has also shown that injection of SCF and granulocyte colony stimulating factor (G-CSF) mobilize BMSCs and dramatically improve cardiac function after myocardial infarction (MI) (27). Further proof of the therapeutic potential of BMSCs was shown by Quaini et al., who analyzed heart biopsies of female organ donors in male recipients a few weeks after heart transplantation. Y chromosome (Y-Chr) was used as a marker of cell origin while tissue-specific markers were used to determine the type of stem cell differentiation (28). It was shown that the number of Y-Chr containing cells expressing markers of SM, endothelium or CMCs was very high (more than 20%), thus implying that BMSCs were able to generate all types of myocardial cells. Following this very encouraging report, a number of groups tried to reproduce these data; however, numbers differed dramatically compared to the initial report, with newly generated CMCs of well below 1% using BMSC, following transplantation. Laflamme et al noted that the mean percentage of CMCs arising from the host was estimated to be 0.04% with a median of 0.016%, and Y-positive CMCs were associated with regions of acute rejection, suggesting such chimerism involves an injury event (29). Differences in tissue histology detection techniques were advocated to account for more than two log differences in Y-Chr positive CMCs between the two studies. In another study, hearts of female subjects who had undergone sex-mismatched bone marrow transplantation (BMT) were recovered at autopsy and analyzed for the presence of Y chromosome-positive CMCs. Four female gender-matched BMT subjects served as controls. Fluorescence in situ hybridization (FISH) for the Y chromosome was performed on paraffin-embedded sections to identify cells of BM origin with concomitant immunofluorescent labeling for alpha-sarcomeric actin to identify CMCs. The mean percentage of Y chromosome-positive CMCs in patients with sex-mismatched BMT was 0.23+/-0.06% (30). In another study, autopsy samples from male control patients who had received a female donor heart were compared with samples from patients who developed myocardial infarction after transplantation. Results suggested that myocardial infarction enhances the invasion of extracardiac progenitor cells and their regeneration of endothelial cells. However, a significant differentiation into CMCs as a physiological mechanism of post-ischaemic regeneration did not occur in transplanted patients (31). More recently, it was shown that G-CSF acts directly on CMCs and promotes their survival after myocardial infarction, activating through a specific receptor, the Jak/Stat pathway. This effect, not mobilization of BMSCs, seems to be primarily responsible for its therapeutic potential following myocardial infarction (32).

Jackson and collaborators used a genetic approach with transgenic animals to show that BMSCs can undergo CMC differentiation in vivo (33). They transplanted highly enriched HSCs, the so-called side population (SP) cells, into lethally irradiated mice whose hearts were subsequently rendered ischemic by coronary

artery occlusion for 60 minutes followed by reperfusion. BM cells from Rosa26 transgenic mice, which express lacZ were injected into the peri-infarctual area of the immunocompromised mice undergoing myocardial infarction. Donor-derived CMCs were found primarily in the peri-infarct region at a prevalence of around 0.02% and were identified by expression of lacZ and alpha-actinin, and lack of expression of CD45. Donor-derived endothelial cells were identified by expression of lacZ and Flt-1, an endothelial marker shown to be absent on SP cells. Endothelial engraftment was found at a prevalence of around 3.3%, primarily in small vessels adjacent to the infarct. This paper suggested that the cardiomyogenic potential of BMSCs was limited.

Our group reported that embryonic endothelial cells of the aorta-gonad-mesonephros (AGM) region, either freshly isolated from embryonic vessels or established as homogeneous cells in culture, differentiate into CMCs and express cardiac markers when co-cultured with neonatal rat CMCs (34).

The excitement produced by BM cell "transdifferentiation" into CMCs was hampered when genetic models were used to prove this hypothesis. Taking advantage of the cre-lox recombination system, it was suggested that fusion between cells instead of differentiation could be a major reason of confusion in interpreting experimental data using BMSCs for cardiac repair. *Cre* is a DNA recombinase that cleaves the DNA at specific palindromic sequences, called *lox* sites, with high efficiency. Mice carrying the *cre* gene under the ubiquitous cytomegalovirus (CMV) promoter were crossed with mice having an allele in which the beta-galactosidase gene is arranged in a way that once it is cleaved at the lox site by cre it becomes active and the cells turn blue. BM cells from mice containing Cre under a universal promoter were injected into mice containing cre-activatable, lox-containing beta-gal gene. Neurons, hepatocytes and CMCs turned blue, although at low frequency, suggesting that beta-gal activation derived from fusion between cells of different genetic origin, not "transdifferentiation"(35).

Using similar approaches, two other groups seriously challenged the concept of "transdifferentiation" of BM cells into CMCs. Murry et al. used mice in which the beta-gal gene was under the alpha-MHC promoter and thus cells turned blue only when the differentiation lineage was activated (36). They found no blue cells after MI in alpha-MHC beta-gal mice. Balsam et al used a model of parasymbiosis between a mouse expressing green fluorescent protein (GFP) under a universal promoter and another with a wild-type genome. They induced an infarct in the wild type (WT) mouse and determined whether GFP+ BM cells from the "donor" mouse migrated to the peri-infarct area of the WT mouse, since ischemic tissues produce stem cell chemo-attractants (37). They found no GFP-CMCs, while the only GFP-positive cells were of hematopoietic lineage. Thus, these two manuscripts represent a major blow to the theory of BMSC "transdifferentiation" into CMCs.

Another report showed that unfractionated bone marrow cells and a purified population of hematopoietic stem and progenitor cells efficiently engraft within the infarcted myocardium. Engraftment was transient, however, and hematopoietic in nature. In contrast, bone marrow-derived cardiomyocytes were observed outside the infarcted myocardium at a low frequency and were derived exclusively through cell fusion (38).

However, BM cells also contain endothelial progenitor cells; in fact, the CD34+ fraction of BM mononuclear cells (BM-MNCs) can be induced to differentiate into endothelial cells in vivo and in vitro, and experimental evidence in murine models of acute ischemic damage suggested that infusion of BM-MNCs or expanded endothelial (End) cells improves myocardial perfusion and viability through angiogenesis (39). Similarly, human cord blood CD34+ progenitor cells could give rise to angiogenesis in the ischemic myocardium of NOD/SCID mice, while few CMCs were detected staining positive for human nuclei (40). Moreover, these cells synthesize growth factors and have an anti-apoptotic effect on CMCs in vivo. Thus, the therapeutic potential of these cells can be dependent on growth factor production and endothelial cell generation.

5. MESENCHYMAL STEM AND PROGENITOR CELLS

Mesenchymal stem and progenitor cells (MSCs/(MPCs) are clonogenic non-hematopoietic stem/ progenitor cells present in the BM, and are able to differentiate into multiple mesoderm-type cell lineages, for example, osteoblasts, chondrocytes and endothelial-cells (41). They adhere to plastic in vitro and expand in tissue culture with a finite lifespan of 15–50 cell doublings. A relatively elusive adherent stem-cell population referred to as multipotent adult progenitor cells (MAPCs) can be isolated by in vitro growth of BM cells in growth medium containing specific growth factors (e.g., epidermal growth factor and platelet-derived growth factor) for several months (41).

MAPCs have the ability to form classical endodermal, mesodermal, and ectodermally derived cell types such as hepatocytes, endothelial cells, and neurons in vitro (41).

A cardiomyogenic cell line from murine BM stromal cells was originally generated by Makino et al (42). Stromal cells were immortalized, and treatment with 5-azacytidine induced spontaneously beating cells and switching of the CMC differentiation programme. Subsequently, human mesenchymal stem cells (hMSCs) from adult BM were shown to undergo myogenic differentiation once transplanted into the adult murine myocardium (43). Rat mesenchymal stem cells were genetically engineered using ex vivo retroviral transduction to over-express the prosurvival gene Akt1, showing that mesenchymal stem cells genetically enhanced with Akt1 can repair infarcted myocardium, prevent remodeling and nearly normalize cardiac performance (44).Clonally

purified nonhematopoietic mesenchymal stem cells (MSCs) with cardiomyogenic potential (CMG) cells, engineered with enhanced green fluorescent protein (EGFP) under a CMC promoter were transplanted directly into BM of lethally irradiated mice; MI was induced and mice treated with G-CSF. EGFP(+) actinin(+) cells were observed in the ischemic myocardium, indicating that CMG cells had been mobilized and differentiated into CMCs, suggesting that the origin of the vast majority of BM-derived CMCs is MSCs (45).

More recently, human BM-derived multipotent stem cells (hBMSCs) which do not belong to any previously described BM-derived stem cell population were isolated and expanded in vitro, generating in co-culture conditions, not only CMCs but also cells of all 3 germ layers (46). Intramyocardial transplantation of hBMSCs after myocardial infarction resulted in engraftment of transplanted cells, which exhibited colocalization with markers of CMC, EC, and smooth muscle cell (SMC) identity, consistent with differentiation of hBMSCs into multiple lineages in vivo. Furthermore, upregulation of paracrine factors including angiogenic cytokines, anti-apoptotic factors and proliferation of host ECs and CMCs, were observed in the hBMSC-transplanted hearts (46).

6. LOCAL CARDIAC STEM CELLS

A growing number of groups have described the existence of resident cardiac "stem" cells able to replicate and differentiate in CMCs.

"Side population" or SP is a term used to describe a population of cells in BM, muscle, and skin which exclude Hoechst dye, giving a characteristic appearance in fluorescence-activated cell sorting (FACS) (47). SP cells have limited capacity to differentiate into striated skeletal myoblasts; similar, very rare cells were identified in the heart (48).
A population of cells showing the characteristics of SP and expressing stem cell antigen-1 (sca-1+ but kit-), were identified in the adult myocardium by M. D. Schneider's group (49). Under normal conditions, these cells do not express cardiac specific genes nor Nkx2.5, but differentiate in vitro in response to 5'-azacytidine. This process was shown to be partly dependent on Bmpr1a, a receptor for bone morphogenetic proteins. Using the Cre/Lox technology, it was shown that differentiation and fusion contributed equally to newly generated CMCs when these cells were administered in vivo after myocardial infarction. Interestingly, these cells "homed" spontaneously to the injured myocardium even if injected in the tail vein (49).

Anversa and colleagues demonstrated the existence of another distinct resident population of cardiac stem cells (50). These cells are similar to BMSCs described by the same group: they are negative for blood lineage markers CD34, CD45, CD20, CD45RO, and CD8 (Lin^-) and positive for c-kit ($c\text{-}kit^+$), the receptor for SCF. The cells can propagate indefinitely in vitro and can be induced to differentiate into endothelium, smooth muscle and CMCs at will. Moreover, according to Beltrami et al. (50), they significantly contribute to myocardial repair after infarction in vivo.

The group of Giulio Cossu isolated undifferentiated cells that grow as self-adherent clusters (that they have termed "cardiospheres") from subcultures of postnatal atrial or ventricular human biopsy specimens and from murine hearts. These cells are clonogenic, express stem and endothelial progenitor cell antigens/markers, and appear to have the properties of adult cardiac stem cells. They are capable of long-term self-renewal, and can differentiate in vitro and after ectopic (dorsal subcutaneous connective tissue) or orthotopic (myocardial infarction) transplantation in SCID beige mice to yield the major specialized cell types of the heart: myocytes (ie, cells demonstrating contractile activity and/or showing cardiomyocyte markers) and vascular cells (ie, cells with endothelial or smooth muscle markers) (51).

More recently, Sylvia Evans' group showed that a subpopulation of cells in the anterior pharynx expresses the homeobox gene *islet-1* (*isl1*), which is lost during CMC terminal differentiation (52). Ken Chien's group further showed that these $isl1^+$ cells can still be found in the outflow tract, the atria, and the right ventricle, which develop from the so-called "secondary heart field", of mature hearts of newborn rodents and humans where they remain undifferentiated (53). The cells do not express stem cell antigen 1 (Sca-1), CD31, or c-kit, though they do express Nkx2-5 and GATA4. A cardiac mesenchymal feeder layer allows renewal of the isolated progenitor cells with maintenance of their capability to adopt a fully differentiated cardiomyocyte phenotype. Moreover, co-culture studies with neonatal myocytes indicate that isl1+ cells efficiently convert to a mature cardiac phenotype with stable expression of myocytic markers (25%) in the absence of cell fusion, intact Ca2+-cycling, and the generation of action potentials, thus they represent cardiomyoblasts. Chien's group also used the genetic tool of the cre-lox system to show that these cells differentiate in vitro and in vivo. Whether these cells exist in adulthood is not known yet.

7. HOW FAR ARE WE FROM A RATIONAL USE OF STEM CELLS IN THE THERAPY OF HEART FAILURE?

The answer is not easy. In fact, there are a number of issues that remain difficult to answer at present. First, the dilemma of which stem cell is better for therapy has not been solved yet. It has not been determined which among the different potential cardiac stem cells have the best chance to be used for a large-scale production of differentiated CMCs. A corollary question is that it has to be determined whether ES cells are better than adult cardiac stem cells. It is true, though, that CMCs can be more readily and undisputedly obtained from mES or hES cells. If then hES cells are THE source to be used, therapeutic application is still far ahead. In fact, 1) factors significantly improving CMC differentiation have to be identified; 2) E-C coupling of newly formed CMCs from ES cells has to be that of adult cells to avoid arrhythmias;

4) in order to be transplanted, the immunogenic potential of ES cells has to be decreased; 5) myocardial tissue has to be engineered from ES cells in vitro and its physiological properties in vivo have to be assessed; and 6) methods of CMC delivery and assessment of myocardial function after cell transplantation have to be improved.

Regarding the potential clinical use of BMSCs, clinical trials were performed in patients with ischemic heart diseases. One of these trials is called TOPCARE-AMI, an acronym for Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (54-56). In this trial, patients with reperfused acute myocardial infarction (AMI) were randomly assigned to receive intracoronary infusion of either bone marrow-derived cells (BMC) or blood-derived circulating progenitor cells (CPC) into the infarct artery, a few days after AMI. One-year results showed that 1) intracoronary infusion of progenitor cells (either BMC or CPC) is safe and feasible in patients after AMI successfully revascularized by stent implantation 2) there were favorable effects on LV remodelling 3) significant arrhythmias were absent (56). Similar results were obtained in randomised controlled clinical trials in which intracoronary transfer of autologous bone-marrow-cells promoted improvement of left-ventricular systolic function in patients after AMI (57, 58). However, the improvement was not dramatic. This fact suggests that the mechanisms underlying the hemodynamic improvements shown in these clinical trials might involve not only an improved myocardial perfusion, but also a protective effect from cytokines produced by BMCs on cardiomyocytes, not an increment in the number of contractile elements (CMCs).

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Send correspondence to: Gianluigi Condorelli, MD, PhD, Institute of Molecular Medicine, University of California San Diego, 9500 Gilman Drive, BSB 5022, 1, La Jolla, CA, 92093-064, Tel: 858-534 3347, Fax: 858-534 1626, E-mail: gcondorelli@ucsd.edu

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