Marrow Stromal Cells As "Universal Donor Cells" For Myocardial Regenerative Therapy

By

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PREFACE

The experiments leading to this thesis were all performed at the University Surgical Clinic and Laboratories of the Montreal General Hospital, McGill University Health Center and funded by a grant from the Heart and Stroke Foundation of Quebec (HSFQ) and Fonds de la Recherche en Sante du Quebec (FRSQ).

This thesis starts with an introduction (Chapter I) which includes a detailed review of the literature explaining the scientific basis for this work. It includes information that was recently published as a chapter entitled *Bone Marrow Stromal Cells in Myocardial Regeneration and the Role of Cell Signaling* (Atoui R, Chiu RCJ and Shum-Tim D) in Artificial Cells, Cell Engineering and Therapy, Prakash S (ed.) Woodhead Publisher, 2006.

The results from Chapter II were presented at the McGill Cardiovascular Research Day in January 2006, at the McGill Annual Biomedical Graduate Conference in February 2006, at the Salon National de la Recherche in Sherbrooke in March 2006 and at the Terrence Donnelly Research Day in Toronto in April 2006. In addition, these results were granted the first prize for best presentation at both the McGill Fraser Gurd Research Day in May 2006, and the Montreal Children's Hospital Annual Research Day in May 2006.
These results were also presented at the Surgical Forum of the American College of Surgeons held in Chicago in October 2006, at the Scientific Sessions of the American Heart Association Congress held in Chicago in November 2006, and at the 16th World Congress of the World Society of Cardiothoracic Surgeons in Ottawa in August 2006.

Chapter II was recently submitted as a single manuscript: *Marrow Stromal Cells as Universal Donor Cells for Myocardial Regenerative Therapy: their Unique Immune Tolerance.* (Atoui R, Asenjo JF, Duong M, Chen G, Chiu RCJ, and Shum-Tim D) in the peer-reviewed journal *The Journal of Thoracic and Cardiovascular Surgery.*

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ABSTRACT

BACKGROUND Recently rodent and porcine bone marrow stromal cells (MSCs) have been reported to be uniquely immune tolerant. In order to confirm these findings in human cells, we tested the hypothesis that human MSCs are also immune tolerant, such that they can be useful as "universal donor cells' for myocardial regenerative therapy.

METHODS Immunocompetent female rats underwent left coronary ligations (n=90). They were randomized into 3 groups. In Group I, lac-Z labeled male human MSCs were implanted into the peri-infarcted area. In Group II and III isogenic rat MSCs or culture medium were injected respectively. Echocardiography was carried out to assess cardiac function, and the specimens were examined serially for up to 8 weeks with immunohistochemistry, FISH and PCR to examine MSCs survival and differentiation.

RESULTS Human MSCs were found to survive within the rat myocardium without immunosuppression. This was confirmed by PCR and FISH test. No cellular infiltration characteristic of immune rejection was noted. Some of these cells appeared to express cardiomyocyte-specific markers such as troponin-Ic and connexin-43. Furthermore, the implanted MSCs significantly contributed to the improvement in ventricular function and attenuated LV remodeling.

CONCLUSIONS Human MSC survived within this xenogeneic environment, and contributed to the improvement in cardiac function. Our findings support the feasibility of using these cells as "universal donor cells" for xeno- or allo-geneic cell therapy, as they can be tested, prepared and stored well in advance for urgent use. Allogeneic MSCs from healthy donors may be particularly useful for severely ill or elderly patients whose own MSCs could be dysfunctional.

RÉSUMÉ

INTRODUCTION Plusieurs études ont récemment démontré la tolérance immunologique des cellules souches stromales (CSS) issues de rongeurs et de porcinés. Pour confirmer ces résultats chez les cellules humaines, l'étude actuelle évalue l'effet des CSS humaines sur la régénération du myocarde chez des rats immunocompétents et étudie la possibilité d'utiliser ces CSS comme « donatrices universelles » à la suite d'un infarctus.

MÉTHODES Après avoir créé des infarctus par ligation coronarienne, les rats (n=90) ont été divisés en 3 groupes. Dans le groupe I, des CSS humaines ont été directement injectées dans le myocarde. Dans le groupe II, des CSS de rats ont été implantées autour de la zone de l'infarctus. Le groupe III a servi de groupe contrôle. La fonction cardiaque a été évaluée par échocardiographie transthoracique et les spécimens ont été examinés à des intervalles de temps différents par immunohistochimie, hybridation in situ fluorescente (FISH) et réaction en chaîne de polymérase (PCR) pour évaluer la survie et la différenciation des CSS humaines.

RÉSULTATS Nous confirmons la survie des CSS humaines dans le myocarde du rat sans immunosuppression. Ces résultats ont été confirmés par PCR et FISH. Aucune infiltration cellulaire charactérisant un rejet immunologique a été démontrée. De plus, certaines cellules ont démontré l'expression de marqueurs spécifiques aux cardiomyocytes comme le troponine-IC et le connexin-43. Ces résultats sont également accompagnés d'une amélioration significative de la fonction cardiaque et du remodelage ventriculaire.

CONCLUSIONS Les CSS humaines ont survécu dans cet environement xénogénique et ont contribué à l'amélioration significative de la fonction cardiaque. Nos résultats soutiennent la praticabilité d'employer ces cellules comme "cellules universelles" pour la xéno- ou allo-

génique transplantation cellulaire, car elles peuvent être bien testées, préparées et stockées à l'avance pour un éventuel usage urgent. Les CSS de donneurs en bonne santé peuvent être particulièrement utiles pour les patients sévèrement malades dont les CSS sont probablement dysfonctionnelles.

Chapter I

INTRODUCTION AND BACKGROUND

Myocardial infarction remains a widespread and important cause of morbidity and mortality amongst adults, accounting for more than 15 million new cases worldwide each year[1]. The loss of cardiomyocytes that results, combined with the limited endogenous repair mechanism, sets into play the remodeling process that ultimately leads to progressive heart failure. End-stage heart failure still has a grave prognosis with an estimated 5-year mortality rate of 60%[2].

In addition to medical therapy, the management of heart failure currently includes the use of mechanical ventricular assist devices, pacing for ventricular synchronization, and other surgical techniques such as ventricular resection, and mitral valve repair. Heart transplantation has been successful, but only benefits few patients due to limited donor supply. A novel approach currently under intensive investigation is cellular transplantation which is directly aimed to overcome the problem of myocardial cell loss. We first introduced the term "cellular cardiomyoplasty" in 1995 to indicate this new therapeutic strategy consisting of replacing dead cardiomyocytes with newly functional contracting cells[3]. This method consists of transplanting cells into the infarcted area of the myocardium to 1) increase or preserve the number of cardiomyocytes , 2) to improve vascular supply, and 3) to augment the contractile function of the injured myocardium.

Since its introduction, several infarct models performed on rodents, sheep, dogs, swine or monkeys have shown that the transplantation of a wide range of stem cells and progenitor cells is possible and contributes to the improvement in the ventricular function. Notable among the donor cells are the satellite cells[3] / myoblasts[4] derived from the skeletal muscle, embryonic stem cells[5], adult marrow stem cells[6] (MSCs), dermal fibroblasts, fetal or neonatal cardiomyocytes[7], other BM-derived cells (CD34+), and proendothelial cells[8].

In this introduction, we will focus on the role of marrow stromal cells in myocardial regeneration, their effect in various experimental models of myocardial injury and we will review the most updated information regarding the cellular and molecular signaling mechanisms for these MSCs to be recruited to the injury site and then undergo "milieu-dependent" or *in situ* differentiation[9]. Furthermore, we will introduce the recent concept of MSCs immunotolerance and explore the evidence and the different mechanisms proposed for this property.

Cellular cardiomyoplasty and myocardial repair

The ideal candidate donor cell would be a cell that can relatively be easily obtained, and expanded. Once implanted, it can home to the injury site, proliferate and differentiate into morphologically and functionally normal cardiomyocyte. As mentioned above, various types of cells have been administered to an ischemic myocardium and studies by different groups have repeatedly documented the successful engraftment of these cells in adult myocardium. Furthermore, most of these studies have also shown an improvement in the ventricular function after transplantation and are summarized in several good review articles[10, 11].

One clear division of the stem cell family is between those found in the embryo, known as embryonic stem cells (ES), and those found in adult somatic tissue. Skeletal myoblasts can be isolated from adult muscle and expanded in culture[12]. In the early 1990s, our laboratory reported the first successful transplantation of satellite cells into the injured myocardium[3]. Since then, several other groups have reproduced this finding, both on animal models and since 2001, in clinical trials on humans[13]. Although it still remains controversial, their long-term clinical utility may be limited by the finding that these cells are capable to only differentiate

into mature skeletal myofibers and unable to form functional gap junctions with the host cardiomyofibers[7, 14, 15]. Fetal cardiomyocytes and ES cell-derived cardiomyocytes have also been transplanted[5, 16]. However, major ethical, moral and legal limitations as well as shortage of donors and the issue of chronic rejection hinder their clinical use. Thus in this introduction, we will focus our discussion on the marrow derived stem cells (MSCs) as the donor cells for cellular cardiomyoplasty.

As opposed to other cell types, MSCs appear to possess some unique properties. They can be harvested and handled relatively easily, multiplied in culture, and implanted without encountering immuno-rejection, as will be further discussed later. Furthermore, they have been shown to differentiate into several lineages, including the cardiomyocytic phenotype[17]. With this apparent plasticity, MSCs could be an ideal cellular source for cell therapy.

Marrow stromal cells as adult stem cells

A stem cell is generally defined as a primitive cell capable of self-renewal, and able to undergo pluripotent differentiation when exposed to the appropriate conditions.

Bone marrow stromal cells (MSCs), also called "stromal stem cells", "marrow progenitor cells", "marrow mononuclear cells", "mesenchymal stem cells" and "marrow-derived adult stem cells" essentially represent a heterogeneous population of fibroblast-like cells, which can be found in the bone marrow (BM) stroma. There is evidence that at least some "adult stem cells" isolated from muscle, skin, adipose tissue, and peripheral blood originated from the bone marrow. Furthermore, the pluripotent stem cells derived from the amniotic fluid, placenta, and umbilical cord blood shows some characteristics similar to those of MSCs.

The MSCs residing in the bone marrow were previously believed to play only supportive roles for hematopoiesis by expressing various cytokines, growth factors and adhesion molecules. Cohnheim in the 19th century first implied the presence of these cells in the blood and their possible role in wound repair[18]. Friedenstein and his group were the first in the early 1970s to better describe these MSCs in a number of species, including mice, rats, rabbits, guinea pigs, hamsters and humans, showing their differentiation potential into cells of mesenchymal lineage including chrondrocytes, osteoblasts, myocytes and adipocytes[19, 20, 21]. Because these cells appeared clonal in nature, they were initially termed colony-forming unit fibroblasts (CFU-F). Isolation of MSCs was then undertaken by Caplan who described a technique still used today by isolating the cells that adhered to the bottom of the plates when the bone marrow cells are cultured *in vitro*[22]. Furthermore, several *in vivo* and *in vitro* studies have confirmed the pluripotent potential of these cells and have observed the presence of injected MSCs in host adipose tissue, lung, cartilage, central nervous system, liver, spleen, thymus and skeletal muscle[23,24,25,26,27,28]. In the last few years, studies have also shown the capacity of these MSCs to differentiate into cells of lineages other than mesenchymal, such as hepatocytes[23], kidney, and even early astrocytes[29].

1- MSCs characteristics and subpopulations

Although MSCs pluripotent potential has been demonstrated in many studies, controversy still exists as to what proportion of these cells is truly pluripotent. Thus, although they are collectively called marrow-stromal cells, not all stromal cells are pluripotent[30]. In fact, it was reported that up to one-third of the initial adherent stromal colonies are truly polypotent[31]. Plating studies confirm the rarity of MSCs in the adult bone marrow, representing

approximately 0.01% to 0.05% of the nucleated cells, being much less abundant than their hematopoietic counterpart[32]. Nonetheless both cell types appear to contribute to myocardial repair.

The human MSCs can be cloned and expanded to greater than 1 million-fold and still retain the ability to differentiate into several mesenchymal lineages. After isolating human MSCs from over 600 patients, Pittenger and his co-workers have shown that these cells behaved as a homogenous population, and retained their multilineage potential for several passages, although not indefinitely[31].

Unlike hematopoietic cells, MSCs are CD34- and CD45-. Although still not fully identified, some other characteristic MSCs surface markers include CD29, CD44, CD71, CD90, CD 106, CD120a, CD 124, SH2, SH3 and SH4-69. It is important to keep in mind that this is an incomplete list, and as mentioned above, some variation has been seen from laboratory to laboratory[30]. In fact there is currently considerable confusion regarding the definition and composition of such cells. For this reason, no unique phenotype has been identified that allows the reproducible isolation of MSCs with predictable lineage differentiation. The reasons behind such uncertainty lie primarily in the experimental conditions used such as the heterogeneity of culture conditions, cell separation techniques and different molecular cell markers used by various investigators. Thus, while the principle of clonal homogeneity is used by some experts to define these cells[31], others use a different combination of molecular cell markers such as c-kit+/Lin- cells[32], Sac-1+ Lin-/cKit+ cells[33], c-kit+/CD34- cells[34] etc. Furthermore, early studies suggested a common precursor between the hematopoietic and mesenchymal lineages[36], identified as CD34+, CD38-, HLA DR-. Waller and his group further subdivided the two lineages based on the CD50 marker; thus defining MSCs precursors as CD50-, CD34+ cells[37].

Because of such differences, it is often difficult to compare the findings among different studies[35]. Standardization of such classifications is of paramount importance as it will be very helpful in the further exploration of the mechanisms of MSCs differentiation.

One possible reason behind this confusion might be because probably, only fully mature cells can be characterized by a defined set of specific markers. In fact, because of their undifferentiated state, a constantly changing set of markers may be continuously defining the "labile" phenotype of MSCs.

2- Pathophysiological role of MSCs in cardiac injury

In contrast to the ES cells, whose goal is to develop a new organism, cumulative information gathered during the past several years suggests that adult stem cells participate in tissue growth and repair throughout postnatal life[38,39]. In fact, there is currently ample evidence suggesting that MSCs can be recruited from the BM to various tissues to participate in tissue repair and regeneration in response to either apoptosis or tissue injury[40,41]. In fact, progenitor stem cells have been shown to be recruited from the BM to contribute to angiogenesis in wound healing, vascularization post myocardial ischemia, and even growth of certain tumors[42].

A hypothesis driven by our laboratory to explain the role of MSCs in the bone marrow is that they serve as "reserve" cells to participate in tissue repair when needed[43]. Indeed, several studies have shown that MSCs differentiation occurs almost exclusively in organs that have been damaged. For instance, differentiation to endothelial cells, hepatocytes, and myoblasts is seen in cases of ischemia, cirrhosis and muscular dystrophy[26,44,45]. In this case, it is hypothesized that upon injury, stem cells can proliferate in vivo and are then recruited to the injured environment. There, they will differentiate in response to local cues[38,46] (see below).

Several evidence published in the last several years has confirmed this pathophysiological role of marrow-derived adult stem cells. Orlic and his group have shown that labeled MSCs can be mobilized within hours of myocardial infarction to home to the injured myocardium[47]. Furthermore, by using a coronary artery ligation model, Bittira et al. from our laboratory[38], demonstrated that in response to a myocardial injury, labeled MSCs are recruited from the bone marrow, traffic through the circulation to home to the peri-infarct area within hours to days. In the following weeks, these MSCs underwent "milieu-dependent" differentiation and expressed various phenotypes including cardiomyocytes, myofibroblasts, endothelial and smooth muscle cells. Our hypothesis is that each type of cell is somehow involved in the pathophysiological process following myocardial infarction. For instance, newly formed endothelial cells can participate in the process of angiogenesis; cardiomyocytes can functionally integrate into the myocardium; and myofibroblasts can contribute to scar maturation, which favorably alters the remodeling process.

It is important to keep in mind that this hypothesis, although appears very credible, can only explain part of the mechanism since it is clear that these cells do not always fully repair the damaged myocardium, as evidenced by the clinical consequences of a myocardial infarct. Further questions remain to be answered. For instance, it is known that immediately after birth, a low level of quiescent progenitor cells, including stem cell precursors, are released into the peripheral circulation[48]. Although it has been shown that these circulating stem cells can repopulate areas of damaged bone marrow and thymus, the exact physiological role of these cells and their fate are currently unknown.

Plasticity of adult MSCs: milieu-dependent differentiation

Plasticity describes a property that allows adult stem cells, assumed until recently to be committed to a fixed lineage, to switch to produce other specialized sets of cells appropriate to their new microenvironment[49]. To explain this, Verfaillie proposed a hypothesis in which these MSCs can proliferate and differentiate in response to local cues provided by the environment they are recruited to[46].

Stem cells have been identified in most organ tissues. The best studied so far is the hematopoietic stem cell (HSC)[47]. Several studies have shown that HSC can repopulate the hematopoietic cell pool when transplanted into lethally irradiated animals or humans[48,49]. Many studies later confirmed the differentiation potential of the MSCs with respect to the mesenchymal lineage, in particular bone and cartilage[35]. For instance, it was found that human MSCs can express genes characteristic of both the osteoblastic and adipocytic lineages, thus clearly indicating their progenitor phenotype[50]. Furthermore, it was also clear that differentiated human adventicular reticular cells can mature into adipocytes upon pharmacological myelosuppression *in vivo.* These cells are thus able to switch phenotypes among two terminal stages within the progeny of the MSCs[51]. This finding may highlight the plasticity of the bone marrow stroma and distinguishes it from the hematopoietic system in which such shifts among differentiated cells do not occur.

Historically, the connection between the bone marrow and osteogenesis was first observed in the 19th century, and later revived by Friedenstein and his group[21]. It was clear from these series of experiments that the extent of bone formation varies broadly depending on the transplantation conditions. Placement of the cells into diffusion chambers allowed the flow of

nutrients, but not the movement of host cells. The production of mesenchymal lineage following transplantation confirmed that the differentiation capacity lays with the donor MSCs[52]. This finding was later confirmed by Owen and his group on rabbit bone marrow cells[53]. Since these initial observations, more definitive evidence for the multipotential differentiation of the MSCs have been reported by other investigators showing the ability of the MSCs to repopulate several nonhematopoietic tissues, such as skeletal myoblasts[25,26], neuronal cells[27,54], cardiomyocytes[32,55], endothelial cells[32], hepatocytes[23], and lung, gut, kidney, pancreas and skin epithelia[28]. Taken together, these studies show that transplanted MSCs have different fates according to the microenvironment to which they locate. They expressed a smooth muscle phenotype in the scar which was absent from the vessels where an endothelial phenotype was displayed. In the noninfarcted myocardium, they exhibited a cardiomyocyte phenotype.

It is however important to keep in mind that most of these studies did not conclusively demonstrate that a single cell could differentiate into different cell lineages. Although some studies have shown that the involved cell populations were rich in hematopoietic stem cells[26,44], they did not identify the exact phenotype of the cell capable of differentiating.

Cardiomyocytic differentiation of MSCs

Data from a number of laboratories have shown that MSCs, once exposed to a variety of physiological or non-physiological stimuli, differentiate into cells with a cardiomyocytic phenotype exhibiting a myotube-like structure with typical sarcomeres, be positive for markers specific for cardiomyocytes, expressing multiple contractile proteins and displaying sinus node-like and ventricular cell-like action potentials[56,57,58,59,60].

1- *In-vitro* studies

Wakitani and his group were the first to show that a hypomethylating agent, 5-azacytidine, can convert rat MSCs to multinucleated myotubes that contracted when exposed to acetylcholine and stained positively for skeletal muscle-specific myosin[59]. Another landmark study by Makino et al. established a cardiomyocytic cell lineage after treating MSCs with 5-azacytidine, expressing cardiomyocyte-specific genes, with evidence of ventricular like action potentials[56] and expressing β-adrenergic and muscarinic receptors[61]. However, 5-azacytidine is known to be toxic *in vivo*.

In order to determine the nature of the possible *in vivo* signals involved, Tomita and his group used a co-culture system and found that when the labeled-MSCs were co-cultured with cardiomyocytes, only with direct cell-to-cell contact could they induce cardiomyocytic differentiation. Separating the two populations with a filter shield, hence allowing the passage of macromolecules but preventing direct cell-to-cell contact, failed to induce such differentiation[62]. These results are consistent with the hypothesis, described above, that cell-to-cell contact may play a crucial role in the milieu dependent differentiation of MSCs, relaying cardiac environmental signals. Furthermore, other studies have shown the presence of specific gap junctions allowing direct cell-to-cell contact between the implanted human MSCs and ventricular myocytes[63], as well as the remarkable cellular and molecular similarity between "true" cardiac cells in culture and the cardiomyocytic-like cell that differentiated from MSCs[64].

2- Animal *in-vivo* studies

To confirm the *in-vitro* studies mentioned above, many laboratories, including ours looked at the potential of the MSCs to differentiate *in vivo* into functional cardiomyocytes.

Tomita and his coworkers were the first to report the differentiation of rat MSC into myogenic cells expressing cardiac-specific genes[65]. After creating ischemic rat myocardium, 5-azacytidine-pretreated MSCs were observed in the transplanted area but not in the control scars. Furthermore, a higher degree of angiogenesis, a smaller transmural scar as well as an improved ventricular function was observed in the transplanted group. It should be noted that bone marrow, as opposed to a purified population of MSCs was used in this study.

In our laboratory, we explored the hypothesis that MSCs, when implanted, will choose to express a specific phenotype based on the principle of milieu-dependent differentiation[66]. If this is the case, we would not need to pre-treat the MSCs with 5-azacytidine to induce the cardiomyocytic phenotype. In our experiment, we implanted labeled MSCs near the peri-infarct area in rats. It was noted that the stem cells surrounded by scar tissue appear to differentiate into fibroblasts, whereas those in direct contact with native cardiomyocytes expressed phenotypic molecular markers specific to cardiomyocytes such as connexin 43 and troponin I-C[66]. This finding supported our hypothesis that these cells received signals from neighboring cells to express phenotypes specific to their microenvironment. Thus, depending on the surrounding milieu, our cells appear to have differentiated into cardiomyocytes, fibroblasts, endothelial cells or adipocytes. From an evolution point of view, one can postulate that by obtaining such signals from the surrounding neighborhood, MSCs may avoid undergoing heterotopic differentiation[43].

It is of interest to note that if one injects 5-azacytidine pre-treated MSCs into the scar tissue of an injured myocardium, cardiomyogenic differentiation of these cells can still occur within the scar. One can thus suggest that such a pre-treatment *in vitro* can alter MSCs gene expression such that these cells will no longer respond to microenvironmental signals, but rather undergo lineage-specific differentiation[58, 65].

In another series of experiments, Wang et al. in our laboratory injected male rats with labeled rat MSCs and showed that these cells will develop into cells morphologically similar to cardiomyocytes, exhibiting organized contractile fibers[6]. This view appeared consistent with our subsequent study[67], whereby MSCs were injected directly into the coronary arteries of an ischemic rat myocardium. These cells were subsequently found to migrate out of the coronary vasculature and differentiate into cells of multiple lineages, depending on their microenvironment. These studies further supported the hypothesis that the fate of the implanted MSCs is defined by its cardiac microenvironment, thus consolidated the concept of "milieu-dependent" differentiation, a term that was originally suggested by Edelman in relation to embryogenesis[9].

These findings were subsequently confirmed by many laboratories around the world. Pittenger and his group used a swine myocardial infarction model and demonstrated the differentiation of MSCs toward a myogenic lineage with expression of α-actinin, troponin-T and tropomyosin[68] resulting in an improved overall left ventricular function. Furthermore, Orlic et al. injected labeled Lin-/ckit+ cells from male mice into an ischemic female mice myocardium[32] and found newly formed Y-containing myocytes occupying up to 2/3 of the infarct area. Similar findings were obtained by Toma et al. using adult mice[17], and by Davani and his group[69]. In a swine model, investigators have used MR fluoroscopy to identify target

sites on the myocardium in order to guide their injections. In these studies, not only MSCs were shown to engraft and express several cardiac markers, but a significant improvement in the ventricular function was also noted accompanied with a reduction in wall thinning[68,70]. Moreover, Kawada et al. transplanted labeled MSCs into the BM of a mouse ischemic model that was treated with G-CSF. They were then able to demonstrate the presence of labeled cells in the ischemic myocardium, suggesting that most of the labeled cardiomyocytes originated from the implanted MSCs[71].

Other studies focusing on gender mismatched human heart transplants, have found a wide difference in the estimate of the levels of Y chromosome-containing cells ranging from 0.04% to 18%[72,73]. The discrepancies in the amount of chimerism among different groups are most likely due to technical differences. Although these results are still controversial[74], they highlighted the repair function of extracardiac stem cells and their potential of regenerating the injured myocardium.

In addition, recent studies evaluating the effect of MSCs on myocardial perfusion have also shown the ability of these cells to enhance neovascularization. Implanted MSCs were shown to express von Willebrand factor (vWF), vascular endothelial growth factor (VEGF) and other proteins indication ongoing angiogenesis[32,75]. Kinnaird et al. recently showed that MSCs secreted a variety of angiogenic cytokines such as fibroblast growth factor (FGF), VEGF, Insulin-like growth factor (IGF), hepatocyte growth factor (HGF), matrix metalloproteinases (MMP), platelet-derived growth factor (PDGF), IL-1, angiopoetin, TGF-β, TNF-α and many others, most of which are upregulated following a myocardial infarction and probably contribute to stimulating neovascularization following a myocardial infarction[76,77]. Among all these factors, VEGF seems to be the key regulatory cytokine orchestrating endogenous

neovascularization by modulating stem cell migration and proliferation[76]. Not only it stimulates the development of microvessels, but it also contributes to endothelial cell survival through VEGF-mediated phosphorylation of protein kinase B and nitric oxide synthase proteins[77].

In a recent study, Tang et al. highlighted the paracrine action of the engrafted MSCs in the ischemic myocardium and the resulting stimulation of neovascularization[77]. They also showed that the release of bFGF, VEGF and SDF-1 not only leads to efficient vascular regeneration but also attenuates the apoptotic pathway by downregulating the prosurvival protein Bax. Finally, it was shown that local injection of MSCs-derived conditioned media alone containing several arteriogenic cytokines can enhance collateral perfusion in a murine model of hindlimb ischemia, hence highlighting the important role of paracrine signaling[76].

3- Trans-differentiation vs. fusion

The studies reviewed above support the idea of MSCs differentiation. However, this concept has been challenged recently with the demonstration of cell fusion whereby a new cell is derived from the fusion of the implanted cell and a native host cell. Several *in vitro* and *in vivo* studies published in the last few years[78,79,80] showed that cell fusion can be responsible for a certain percentage of phenotypic changes observed following transplantation. Terada and his group demonstrated the presence of polyploidy DNA content when female BM cells were co-cultured with male ES cells[78]. Although still highly controversial, it is important to keep in mind that the frequency of this phenomenon varied widely among different studies and can not by itself explain all the significant regeneration observed in previous studies. In any case,

future studies must examine this mechanism with rigor in order to better understand the mechanisms of cellular transplantation.

Furthermore, in the last several years, compelling evidence has emerged showing the potential of cardiomyocytes to reenter the cell cycle and undergo mitosis[81,82]. To add more to this confusion, the concept of resident cardiac stem cells was recently introduced as well[83,84,85]. It has recently been shown that, in the regions adjacent to the infarcts, 4% of myocyte nuclei expressed the Ki-67 cell proliferation marker[77]. Despite their ability of giving rise to endothelial cells, smooth muscle cells and functional cardiomyocytes[67], their physiological role appears so far minimal.

4- Improvement in ventricular function

Most of these previous studies have noted an improvement in the ventricular function in the transplanted group. It is of interest to note that even the experiments that did not show extensive myocytic differentiation did nonetheless show an improvement in the global functioning of the heart. How can these cells, without apparent connection to the native myocardium contribute to the improvement in ventricular function remains perplexing. A number of mechanisms have been proposed[86] and include contraction of the implanted cells, changes in the extracellular matrix by an autocrine mechanism, with an improvement in the elastic property of the transplanted region, thus limiting the remodeling process; and an enhancement of the angiogenesis in order to rescue hibernating myocardium.

Cell signaling and mechanisms of differentiation

A number of studies analyzed the signaling mechanisms involving stem cells' regulation and proliferation. Most of these reports focused on HSC signaling. However, as we will see, and perhaps not surprisingly, many of the factors and pathways involved have also been shown to be implicated in MSCs differentiation.

A number of studies have tried to analyze the endogenous and environmental factors that are involved in the regulation of stem cells, including inflammatory cytokines, growth factors, surface receptors, proteases, transcription factors, telomerase activities, hypoxia-responding proteins and stem cell-matrix interaction. Furthermore, Lapidot and Petit even suggested the existence of a dynamic situation in which there is a constant turnover, proliferation, migration and homing of stem cells as part of their developmental program, a process that may even be linked to the dynamic interaction between osteoblasts/osteoclasts in BM remodeling[48]. Interestingly, G-CSF stimulation induced both MSCs mobilization and osteoclast-mediated BM resorption[95].

1- Stem cell mobilization

Several previous studies focusing on knock-out embryos revealed the critical roles of SDF-1α, a member of the CXC chemokine family that was shown to bind to its 7-transmembrane-spanning G protein-coupled receptor CXCR4. Its constitutive expression in various tissues as well as its highly conserved amino acid sequences between different species highlights its important biological role, namely in cardiogenesis, stem cell hematopoiesis, vasculogenesis, and cerebral development[48]. These studies confirmed the role of SDF-1 as the key regulator of

stem cell trafficking between the BM and the peripheral circulation. In fact, Peled et al. have demonstrated in a series of studies that SDF-1/CXCR4 interactions not only tightly regulate stem cells homing but are also involved in transendothelial migration by mobilizing progenitor stem cells and activation of major integrins such as LFA-1, VLA-4 and VLA-5 mediating cell-to-cell and cell-to-matrix interactions in response to tissue stress or injury [96,97,98].

The mobilization process whereby stem cells are released from the BM was first documented in 1970s and has been shown to be induced both in animals and humans by a wide number of molecules, including cytokines such as G-CSF, GM-CSF, interleukin IL-7, IL-3, IL-12, stem cell factor (SCF) and flt-3 ligand; chemokines like IL-8, Mip-1α, Groβ, or SDF-1 and a variety of chemotherapeutic agents. For instance, IL-8, which is secreted in response to SDF-1 stimulation, is believed to stimulate stem cell mobilization by activating MMP-9 and the integrin LFA-1[99,100,101,102]. Similarly, it was found that both SDF-1 and steel factor act cooperatively to attract progenitor stem cells from the bone marow[35]. Furthermore, Sweeney et al. recently found that sulfated polysaccharides can increase the levels of SDF-1 which can ultimately result in an up regulation of the MSCs mobilization[103]. In this study, they demonstrated that these polysaccharides compete for SDF-1 binding to the BM endothelium. Furthermore, Yamaguchi et al. confirmed the hypothesis that locally administered SDF-1 can, by augmenting the levels of endothelial progenitor cells to the site of ischemia, enhance the efficacy of neovascularization after systemic EPC transplantation[104].

Although the exact mechanism of mobilization remains not fully understood, it is believed to be a multi-step process whereby a key process involves the disruption of the adhesion interactions between the stem cells and the BM when stimulated by specific signals such as an ischemic injury or stress. In fact, Papayannopoulou et al. demonstrated the critical role of

VLA-4 in the mobilization process[105] and several previous studies have shown the role of proteolytic enzymes such as elastases, peptidases, cathepsins G, MMP-2 and MMP-9 in inactivating SDF-1 by cleaving part of its N-terminus. Furthermore, a recent study showed that the increase in the level of cathepsin G and elastase correlate with stem cell mobilzation[106]. In accordance with these studies, Petit and her group demonstrated the proteolytic degradation of SDF-1 by elastase induced by G-CSF, accompanied by a gradual increase in CXCR4 expression on bone marrow cells[107]. Studies by Moore et al. confirmed further the critical role of MMP-2 and MMP-9, as well as their natural tissue inhibitors (TIMP) in allowing SDF-1 and G-CSF mediated stem cell mobilization[108]. The importance of these metalloproteinases is also highlighted by their role in maintaining low levels of surface CXCR4 to keep the stem cells in the circulation[109]. Furthermore, Heissig and his group[110] showed that the activation of MMP-9 is followed by the release of SCF into the circulation, which is essential for SDF-1 mediated stem cell mobilization and proliferation.

Finally, Flores et al.[111] recently highlighted the role of telomerase Tert and telomere length as critical determinants in the mobilization and proliferation of epidermal stem cells for their niches.

2- BM: niche for HSC

Engraftment of the stem cells in the bone marrow can be seen as the end of a complex series of events in which circulating HSC are first recruited by the BM vasculature followed by their transendothelial migration into the extravascular hematopoietic cords of the marrow[112]. Once in the BM, the regulation of HSC proliferation and differentiation occurs by a complex interplay of cells, growth factors, adhesion molecules and other signals, not yet fully

understood. This is then followed by lodgment whereby cells selectively migrate to a suitable niche in the extravascular compartment of the BM.

Previous studies suggested key roles for P-selectin[113], E-selectin[114], the β1 integrin very late antigen-4 (VLA-4)[115], SDF-1 and CXCR4[116], in the homing of HSC to the bone marrow. Thus, to home to the BM, stem cells must first roll on E and P sleectins, which are expressed on the BM vascular cells. After their adhesion to the vessel wall via the major integrins (VLA-4 ,-5 and LFA-1) and their vascular ligands (VCAM-1 and ICAM-1), they extravasate into the hematopoietic compartment. Almost each of these previous steps has been shown to be activated by SDF-1[117].

Furthermore, recent data demonstrate that flt-3 ligand plays an important role in the proliferation of HSC in tightly regulating the actions of VLA-4 and VLA-5[118]. Also, Driessen and coworkers recently confirmed the transmembrane isoform of SCF as important in the lodgment of HSC in their niche[119].

In another recent study, Mohle and his group showed that other non-peptide mediators such as cysteinyl-leukotriene receptor CysLT1 similarly stimulate HSC migration[120]. Also, Netelenbos et al. introduced the role of proteoglycans such as heparin and dermatan sulfate in HSC homing by showing their attachment to SDF-1[121]. The glycosaminoglycan hyaluronic acid (HA) recently found to be synthesized by HSC, was also found to have a key role on their migration and engraftment[122]. Hence, it seems that the processes of mobilization and homing are "mirror images" involving the same molecules in "opposite directions"[123]. Thus, by activating adhesion molecules, SDF-1 plays an important role in homing of the stem cells and engraftment in the BM. On the other hand, desensitization of the SDF-1/CXCR4 pathway is required for the successful mobilization of the stem cells from the BM.

Other studies have focused on the impact of cytokine exposure on the homing mechanism. Ahmed et al.[124] reported that cytokine-activated CD34+ cells (with IL-3, IL-6 and SCF) showed irreversible impaired homing ability, possibly through the induction of pro-apoptotic genes such as Fas/CD95 [125]. Furthermore, Zheng et al. reported significant upregulation in the concentrations of homing-related signals such as CD49, CD54, CXCR4, MMP-4 and MMP-2 when stem cells were shortly exposed to SCF[126].

Although these findings can give us some insight into the mechanism of stem cell mobilization, it is clear that many more studies are needed to fully understand this complex event that involves the interplay between several adhesion molecules, chemo-cytokines, proteolytic enzymes and the BM. Clinically, this is very relevant since it is possible that the manipulation of SDF-1/CXCR-4 interactions, as well as the simultaneous infusion of stromal cells with the hematopoietic component could improve the outcome of human BM transplantations[127]. In fact, several studies are currently underway taking advantage of the MSCs in autologous and allogeneic transplantation[48].

3- Interactions between MSCs and HSC

As we described previously, the interactions between MSCs, HSC as well as other mediators in the BM are important in the homing and proliferation process. In fact, several studies have identified numerous receptors on MSCs important for cell adhesion with HSC and the rest of the extracellular matrix such as ICAM, VCAM, platelet endothelial cell adhesion molecule PECAM, L-and P-selectins. It is also likely that the adhesive interactions that occur between HSC and MSCs help not only in the homing and engraftment of the HSCs in the BM, but are also involved in the proliferation and differentiation process of progenitor cells[112]. For instance,

several studies have identified a number of growth factors, expressed in MSCs cultures which are associated with hematopoietic support such as IL-6, IL-11, LIF, CSF, G-CSF, GM-CSF and SCF[128]. Furthermore, Calvi and his group recently highlighted the role of osteoblasts, present within the endosteal region, as key cellular elements in influencing stem cell differentiation through Notch activation[90]. Further pathways involving both MSCs and HSC have been shown to have an important impact on stem cell differentiation and proliferation such as the Wnt signaling pathway[129] as well as the bone morphogenic protein receptor type 1A activation of specific osteoblastic cells[130].

Homing of the MSCs to the infarcted site

One of the most intriguing properties of MSCs is their ability to home to sites of inflammation or tissue damage. Although the steps responsible for this migration have yet to be fully elucidated, it entails a 2-step process whereby stem cells first bind to their adhesive complexes around the injury zone, followed by local chemotaxis to the site of engraftment[131]. This phenomenon has been demonstrated in various settings including infarcted hearts[132], cerebral ischemia[133], and bone fractures[134]. In fact, Saito et al. from our laboratory were the first to demonstrate that MSCs administered intravenously engraft within the infarcted myocardium, whereas those injected in healthy rats, home to the bone marrow[132]. In another study, Sorger et al.[135] showed the remarkable specificity with which MSCs can home to infarcted regions. Although the specific factors responsible for this migration have not yet been defined clearly, further complexity is added by a recent finding suggesting that expansion of murine MSCs in culture may actually diminish their homing ability[136].

As we saw previously, SDF-1 and its receptor CXCR4 are required for stem cells to home to the BM. Their role in coronary artery disease is less clear. Previous studies have shown the expression of SDF-1 in atherosclerotic plaques, its upregulation in the heart early after MI as well as the increase in neovascularization following its exogenous expression[131]. Askari et al. further reinforced the role of SDF-1 in stem cell homing in a study whereby cardiac fibroblasts expressing SDF1 were transplanted into the infarcted regions of rat hearts[137]. After using G-CSF to mobilize stem cells, a significant homing of c-kit cells to the injured area as well as an improved cardiac function was found in treated animals. Orlic and his group have also demonstrated the upregulation of MSCs homing and differentiation with the use of G-CSF[47]. In this study, a 250-fold increase in the levels of Lin-/c-kit+ cells as well as an improvement in the ventricular function were found in rats that were pretreated with G-CSF and SCF. A similar finding was obtained when granulocyte-macrophage stimulating factor (GM-CSF) was used[138]. Although the exact mechanism is yet to be understood, Harada et al. recently showed that this G-CSF-mediated stem cell mobilization and improvement in cardiac function occur through the activation of the Jak/Stat pathway in the cardiomyocytes, hence inducing a number of antiapoptotic proteins and angiogenic factors[139].

Other than SDF-1, SCF is also involved in the regulation of stem cells migration by binding to its tyrosine kinase receptor, c-kit, which is expressed on a variety of stem cell lines[1,140]. This is confirmed by further studies showing the role of SCF in the induction of the expression of CXCR4 on human CD34+ cells resulting in an increase in their migration in response to SDF1[116]. A wide variety of chemokines have actually been shown to modulate such migration; however the largest response was seen with α and β SDF1[141]. Furthermore, it is important to realize that although SDF-1 is required in stem cell mobilization to the injured site, it is not

singularly sufficient, hence reflecting the need for additional factors. In fact, patients with acute coronary syndromes have elevated levels of many factors other than SDF-1 including MMP-2, MMP-9, ICAM, and VCAM[131].

As we saw previously, cell-to-cell interactions as well as other environmental factors involving a combination of paracrine growth factors promote stem cell migration and differentiation. Eghbali-Webb has recently reviewed the role of cardiac fibroblasts in regulating myocardial regeneration by the release of various soluble factors within the extracellular matrix such as VEGF, FGF, TGF-β1, PDGF, and MMPs, highlighting the coordinated cell-to-cell and cell-to-environment interactions[142]. It is also possible that the hypoxia following an ischemic insult can enhance the expression of some adhesion molecules and thus facilitates MSCs migration. For instance, the increase in MMP-9 level following the use of mobilizing agents such as SDF-1, VEGF and G-CSF or after a myocardial infarction, leads to an up-regulation of soluble kit, which ultimately results in an increase in MSCs mobilization and proliferation[143,144].

It is of interest to note that MacDonald et al. in our laboratory have observed that MSCs which can migrate to the acutely injured site can lose this ability in chronic scar tissue, when the inflammatory mediators have probably subsided[145].

The clinical application of modulating the SF1-CXCR4 axis to improve stem cell homing using monoclonal antibodies or antagonists against CXCR4 remains to be determined[144]. Furthermore, strategies to improve the engraftment of BM stem cells into the ischemic areas by the local administration of SDF-1 remains to be fully investigated. Finally, it was proposed that in order for the implanted stem cells to survive in the ischemic myocardium, one must also control the different factors that influence apoptosis, including cytokines and growth factors

(such as HGF, GATA-4), expression of apoptosis-regulating genes (such as Fas, p53 and caspases) mitochondrial dysfunction, telomerase activities and hypoxia-responding proteins (such as hypoxia-inducible factor HIF-1 and erythropoietin)[146].

Therapeutic use of MSCs

The recognition of the broad growth and differentiation potential of MSCs and their relative ease of handling has opened the door to several clinical applications.

In the hematological field, clinical studies have progressed farthest in the use of human MSC in repopulating the BM stroma after myeloablative therapy, in conjunction with the reconstitution of the hematopoietic system with BM transplantation[128].

Furthermore, the ability of MSCs to proliferate makes it an excellent target for retroviral gene therapy [147]. In several studies, it was shown that stromal cells can be efficiently transduced with a variety of growth factors and hematological factors such as VEGF, VWF, factor VIII or XI [35,148,149,150]. Schwartz et al. were able to engineer MSCs expressing L-DOPA when implanted into the brain in a rat model of Parkinson's disease[151].

In the last few years, > 10 clinical trials around the world[152,153,154,155] have been completed and several are ongoing to assess the effect of autologous BM cells transplantation after acute myocardial infarction. With the exception of one[156], all others showed encouraging results, with a significant increase in the ejection fraction and myocardial viability and a decrease in end-systolic LV volumes. It is of interest to note that in most of these trials, a heterogenous fraction of BM mononuclear cells was used, containing B and T lymphocytes, myeloid cells, endothelial cells and a low number of hematopoietic and MSCs. However, some of these trials involved purified fractions of cells such as CD34+ or CD133+ progenitors, as well as skeletal

myoblasts. Furthermore, different delivery techniques were used, concomitant with medical therapy, angioplasty or coronary artery bypass surgery. In a recent randomized trial, Chen et al. injected MSCs directly into the coronaries of patients post myocardial infarctions[157]. It was found that treated patients had a decrease in the proportion of hypokinetic and akinetic segments, as well as a significant improvement in the ventricular function and wall motion.

All of the studies mentioned above are limited by their small sample size and nonrandomized design. However, a large randomized controlled clinical trial has now been reported[75 new]. In this study, 60 patients underwent percutaneous coronary intervention with stent implantation for acute myocardial infarction. They were randomized into 2 groups, either receiving or not autologous BM-MNCs. All patients received standard pharmacological treatment. After 6 months, the authors reported a significant increase in the ejection fraction in the cell-treated patients. However, the left ventricular diastolic volume was not different between groups, indicating a lack of improvement of ventricular remodeling during follow-up. No adverse events have been reported so far in this study.

Finally in addition to all these trials mentioned earlier, MSCs and allogeneic bone marrow transplantation have also been used, although with limited success so far, in various mitochondrial defects and inborn metabolic diseases[158].

1- Adverse events

To date, publications regarding adverse events in experimental studies have been relatively rare. However, the development of microinfarction has been reported when infusing MSCs directly into a dog's coronary artery[78]. Adverse calcification has also been shown to be a rare problem a in a rat model[79] and is probably due to the differentiated state of the MSCs used.

In phase I trial using skeletal myoblasts, ventricular arrhythmias developed, probably due to the lack of gap junctions between transplanted myoblasts and host cardiomyocytes and to the difference between the action potential of the two cell types[68].

In contrast, arrhythmias were not per se a problem with clinical trials using BM-MNC. The major concern with these trials may be the development of angiogenic neoplasias since endothelial-progenitor cells can contribute to tumor neovascularisation[67]. However, this adverse event has not been yet reported in clinical trials.

One recent trial[76] has been stopped because of the unacceptable rate of stent restenosis that accompanied the improvement in ventricular function. The authors hypothesized that this event might be due to the differentiation of progenitor cells into smooth muscle cells within the stented segment.

Unique immunotolerant properties

Another fascinating aspect of stem cell therapy involves the recent findings that MSCs may have a unique immunological capacity to induce tolerance in immunocompetent allotransplants or even xenotransplant recipients[132,161]. These properties may limit the ability of the recipient immune system to recognize and reject allogeneic or gene-modified MSC following transplantation.

Immunologically, MSCs share cell surface markers with thymic epithelium. The express adhesion molecules involved in T-cell interaction including VCAM-1, ICAM-1 and LFA-3[162]. In addition, MSCs express intermediate levels of major histocompatibility complex (MHC) class I molecules, negligibly low level of MHC class II and Fas ligand and no costimulatory molecules such as B7-1, B7-2, CD-40 or CD40 ligand[163,31]. The presence of these cell surface

markers, along with the findings that MSC are customary residents of the bone marrow microenvironment, suggests that MSCs may play an important role in the immunoregulation provided by the bone marrow microenvironment.

Although the exact underlying immunological mechanism is not yet well understood, the "Danger Model" theory of Matzinger [168] was invoked in our laboratory to try to explain the unexpected findings. In addition to the action of veto cells and to the proposed role of tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan degradation in MSC-mediated immunosuppression[169], Pittenger and his group recently reported that human MSCs can secrete PGE2, hence altering the cytokine secretion profile of dendritic cells, naïve and cytotoxic T lymphocytes, and NK cells[170], namely by inhibiting TNFα and IFN-γ and by stimulating IL-10 secretion to modulate the immune cell response. By doing so, they inhibit the maturation and migration of various antigen-presenting cells and alter the expression of several receptors necessary for antigen capture and processing[170]. Furthermore, by increasing IL-4 secretion, they accelerate a shift from a majority of proinflammatory Th1 cells toward an increase in anti-inflammatory TH2 cells[170].

1- *In-vitro* studies

Data supporting the contention that MSCs avoid allogeneic response has come from a large body of *in-vitro* experiments involving co-culture or mixed lymphocyte reactions (MLR). Evidence from these studies on human, baboon and murine MSCs indicate that the use of mismatched MSCs does not provoke a proliferative T cell response in allogeneic MLR, thus suggestive of an immunosuppressive role for MSCs [161,162,167,171].

This suppression of mixed lymphocyte reaction *in vitro* between MHC-mismatched stimulator and responder cells by MSCs appears to arise from both contact-dependent[172] and soluble factors[173] including hepatocyte growth factor (HGF) and transforming-growth factor β1 (TGB-β1)[171]. This immunosuppressive effect is retained even when co-stimulatory signals are added to the culture to upregulate the expression of MHC classII [163] or when T-cells are re-challenged by the same MSCs[172]. These actions have been shown to be partially mediated through the generation of $CD8^+$ regulatory cells and by inhibiting the formation of cytotoxic T lymphocytes[173].

Other experimental settings suggest that even when precultured with interferon-γ to fully express MHC class II or in the presence of CD-28 mediated costimulation, MSCs still escaped recognition by alloreactive T cells pointing to their potential role as unique nonprofessional antigen-presenting cells[161,163,174].

2- *In-vivo* studies

MSCs isolated from humans and other mammalian species including baboon, canine, caprine and rodents do not elicit a proliferative response from allogeneic lymphocytes[162].

The major limit to any solid organ graft survival is T cell recognition by the recipient of alloantigens, such as MHC antigens. There are 2 mechanisms mediating this powerful response: "direct" recognition, involved recognition by recipient $CD8^+$ or $CD4^+$ T cells of donor MHC class I or II molecules, and "indirect" mechanism involving recognition of peptides from the allogeneic tissue. Recipient antigen presenting cells (APC) such as dendritic cells (DC) process alloantigen into peptides and present these to naïve T cells on self-MHC molecules. There is supporting evidence from both *in-vitro* and *in-vivo* studies that show that MSCs avoid normal

alloresponses. Koc et al. showed no evidence of alloreactive T cells and no incidence of graft versus host disease when allogeneic MSCs were infused into patients with Hurler's syndrome or metachromatic leukodystrophy[175].

Horwitz and colleagues reported that donor MSCs contributed to bone remodeling after allogeneic stem cell transplantation in 3 children with osteogenesis imperfecta[176]. This is supported by data from Bartholomew et al. who showed that *in-vivo* administration of allogeneic MSCs prolonged third party skin graft survival in immunocompetent baboons[162]. Furthermore, other groups have reported that it can also prevent the rejection of allogeneic B16 mouse melanoma cells in immunocompetent C3H mice, and attenuate graft-versus-host disease in mice and humans[169,177]. Human MSCs have been observed to successfully engraft in brains of albino rats[178] as well as in utero in sheep[164], even after the fetuses became immunocompetent.

In our laboratory, Saito et al.[132] transplanted labeled mice MSCs into adult fully immunocompetent rats, thus producing stable cardiac chimeras for at least 12 weeks without any immunosuppression and with no evidence of rejection. Furthermore, Macdonald et al from our group have shown that not only stable chimeras are formed in which MSCs retained their ability to be recruited to the injured myocardium, but that the overall ventricular function is improved[165]. These findings were once again replicated by Luo et al from our lab who confirmed the survival of pig MSCs into fully immunocompetent rat myocardium for up to 6 months after transplantation (personal communication). This is confirmed by recent findings showing the successful engraftment of allogeneic MSCs into infarcted rat myocardium for as long as 6 months[179].

More recent work by Aggarwal and Pittenger supported the feasibility of MSCs transplantation showing that MSCs altered the phenotypes of specific immune cell subtypes thereby creating a tolerogenic environment[170].

It is of interest to note that LeBlanc et al. have shown that not only undifferentiated but also differentiated MSCs have immunosuppressive effects in third-party mixed lymphocyte cultures which appear to be alloantigen independent[161].

Proposed mechanisms of immunotolerance

All these observations support the concept that MSCs may be immunoprivileged and have unique immunotolerant properties with clearly defined therapeutic implications.

Although these data show that successful use of allogeneic MSCs in regenerative therapy is possible, such approaches are unlikely to be broadly acceptable until it is understood why these MSCs aren't rejected. This question has been the subject of intense studies and three candidate inter-related mechanisms are emerging[180]. MSCs appear to evade rejection by 1) being hypoimmunogenic; 2) modulating T cell phenotype and 3) immunosuppressing the local environment.

1- MSCs as hypoimmunogenic cells

Although there is a continuous controversy surrounding the exact composition of cell surface markers on MSCs, most studies describe MSCs as MHC class I positive (intermediate levels) and MHC class II negative. The expression of class I MHC on these MSCs is important because it protects these cells from certain NK cell mechanisms of deletion. As MHC class II proteins are potent alloantigens, their lack of expression on MSCs, under non-inflammatory

conditions, is another important factor by allowing them to escape recognition by alloreactive CD4+ T cells. In addition to this, MSCs do not appear to express the co-stimulatory molecules CD40, CD40L, CD80 or CD86 for effector T cell induction[163].

2- Effect of MSCs on the function of DC and other APC

Dendritic cells (DC) play important role in directing cellular and humoral immune responses against self and non-self antigens and contribute to the establishment of tolerance, especially in the periphery. Several studies have demonstrated that MSCs could prevent normal allogeneic responses by modulating DC function. Zhang et al[181] provides evidence that MSCs interfere with DC maturation by down-regulating CD1a, CD40, CD80, CD86 and HLA-DR expression. These findings were confirmed by Beyth et al.[182] who suggest that human MSCs converted APC into a suppressor cell via cell-to-cell contact, thus locking the DC into an immature state and thereby inducing peripheral tolerance. Similarly, Jiang et al.[183] reported that MSCs maintain DC in an immature state by inhibiting the upregulation of IL-12p70. All these results suggest that MSCs may be mediating allogeneic tolerance by directing APC toward a suppressor phenotype that results in an attenuated T cell response.

3- MSCs modulate the function T helper cells

Evidence has emerged that MSCs can also interact directly with T cells to suppress alloreactivity. The regular process of antigen specific $CD4^+$ T cell induction requires antigen capture and processing by APC, followed by a process of maturation and trafficking to local lymph nodes[180]. There is evidence showing that MSCs can direct $CD4^+$ T cells to a suppressive phenotype. Di Nicola et al.[171] as well as Tse et al.[163] showed that MSCs strongly suppressed

$CD4^+$ T cells in MLR, and attenuated the proliferation of T cell subsets. Studies of T cell differentiation have shown that in the presence of human MSCs, Th1 secretion of INF-γ dropped by 50% compared to controls. Conversely, effector T cells undergoing Th2 differentiation showed a significant increase in IL-4 secretion when co-cultured with human MSCs. These findings suggest an important counter-regulatory and anti-inflammatory role by directing cytokine-mediated immunity[180]. Furthermore, some studies have shown that MSCs influence control over cell division cycle pathways in cells of immunological relevance. Glennie et al.[185] have shown that T cells stimulated in co-cultures with MSCs exhibit an extensive inhibition of cyclin D2 and an upregulation of the cyclin dependent kinase inhibitor $p27^{kip\,t}$.

4- MSCs modulate $CD8^+$ T cells and NK cells

The role of MSCs on $CD8^+$ T cells and NK cells has also been addressed. There is evidence that MSCs inhibit the formation of $CD8^+$ T cells and appear to evade NK cell targeting mechanisms[185]. Furthermore, Rasmusson et al.[173] showed that NK cells in co-culture did not recognize MSCs although their lytic properties were still present.

5- Cytokines secreted by MSCs

The characterization of cytokines produced by MSCs is still provisional and is hindered by lack of standardization in isolation and culture conditions[180]. Some reports showed that MSCs do constitutively express mRNA for cytokines such as IL-6,-7,-8,-11,-12,-14,-27, leukemia inhibitory factor, macrophage colony stimulating factor and stem cell factor[186]. Although their role is still not fully understood, some of these cytokines provide critical cell-cell interactions

and promote HSC differentiation. As we saw previously, MSCs can also secrete other peptides such as HGF which is likely to contribute to creating a local immunosuppressive environment[171,174].

Although this remains controversial, IL-10 seems to be constitutively expressed by MSCs. This interleukin has a well-documented role in T cell regulation and in the promotion of the suppressor phenotype[180]. IL-10 can also antagonize IL-12 during induction of inflammatory immune responses.

Similarly, TGF-β1 seems to also be involved in T cell suppression by working with HGF in promoting the allo-escaping phenotype. Di Nicola et al.[171] showed that neutralizing antibodies to HGF and TGF-β1 restored the proliferative response in MLR suggesting that these factors are at least partially responsive,.

Furthermore, MSCs constitutively express PGE-2, which can influence numerous immune functions including suppression of B cell activation and induction of suppressor T cell formation[187]. Although there is evidence for PGE-2 secretion by MSCs, there is controversy surrounding the role of PGE-2 in inducing T cell suppression. Other prostaglandins and eicosanoids could definitely be involved in influencing alloresponses[180].

In contrast to immunosuppression through the secretion of soluble factors, suppression may also be mediated by withdrawal of factors in the microenvironment necessary for active immune responses. Indoleamine 2,3-dioxygenase (IDO) is known to catabolize L-tryptophan thereby depleting an essential amino acid from the local environment[169]. Recent evidence has shown that this mechanism is exploited by the mammalian fetal allograft to suppress T cell activity and prevent rejection[180].

To summarize, three broad mechanisms seem to contribute to this immunotolerance property. Firstly, MSCs are hypoimmunogenic, often lacking MHC class II and co-stimulatory molecule expression. Secondly, these stem cells prevent T cell responses indirectly through modulation of dendritic cells and directly by disrupting NK as well as $CD8^+$ and $CD4^+$ T cell function. Thirdly, MSCs induce a direct immunosuppressive effect on the local environment through the production of prostaglandins and IL-10 as well as by the expression of 2,3-dioxygenase which depletes the local milieu of tryptophan. This suppression mechanism appears to have no immunologic restriction because both autologous and third-party MSCs equally induce lymphocyte proliferation[167,188].

In order to understand better the mechanisms involved and the hierarchy of signals that control immunosuppression, research from other fields has been informative. In fact, in addition to the mechanisms involved in the maternal acceptance of the fetal allograft, the ways tumors evade our immune system may also reflect the survival mechanisms of MSCs.

6- MSCs immuno-modulatory properties and similarities with tumor evasion

Escape from immune surveillance is believed to be a primary feature of malignant disease in humans. Studies have shown that tumors develop multifactorial strategies to escape immune deletion[189]. These strategies may provide clues to how MSCs promote tolerogenic mechanisms during allogeneic engraftment. As seen with MSCs, modulation of tumor antigen expression, particularly MHC class I and II, accompanied by poor or non-expression of co-stimulatory molecules is a particularly common component of tumor immune evasion[180]. Furthermore, tumor cells have been shown to directly modulate DC and T cell function. Freshly isolated tumor-infiltrating T cells are usually inactive against autologous cancer cells but can be

reactivated *in-vitro* by the addition of IL-2[189]. Studies on MSCs by LeBlanc et al.[174] showed striking parallels to this form of suppression by showing an inhibition of the expression of IL-2 receptor thereby limiting T cell activation.

Tumors can also suppress $CD4^+$ T cell activity through secretion of immunosuppressive factors including TGF-β1, PGE-3 and IL-10. Studies have shown that tumor-derived prostaglandins increased the production of inhibitory cytokines such as IL-10, while suppressing Il-12 which is necessary for effective host cell-mediated antitumor immune response[189].

Despite similarities between MSCs and tumor cells, there are important differences between these 2 populations of cells. One fundamental difference resides in the control of cell division and apoptosis, which are tightly regulated in MSCs but dysregulated in tumor cells[180]. Furthermore, it is well documented that some tumor cells use FasL expression to escape immune recognition[180]. However, it seems that direct induction of apoptotic deletion is not a factor involved in MSCs interaction with T cells. It appears that MSCs retain certain aspects of the fetal allograft that promote tolerance, some of these mechanisms may be reactivated in neoplasia, the key difference being that MSCs perform these functions in an ordered and controlled way, whereas tumor cells do so in a manner that by definition has escaped normal cell controls[189].

For myocardial regeneration, MSCs may then be exploited as "universal donor cells" that can be isolated and expanded from donors irrespective of their MHC haplotype, tested for their functional capabilities well in advance, and be stored as an "off-the-shelf" reagent for immediate use when needed on any patient after an acute myocardial infarction.

Such cells can also be of great value in patients with genetic defects as well as in the sick elderly patients whose own MSCs may be dysfunctional. Furthermore, in older patients with

malignant diseases as well as hematological or bone disorders, sufficient MSCs may not be obtained for autologous use[174]. Moreover, simultaneous transplantation of MSCs might enhance hematopoietic engraftment after bone marrow transplantation[190] and might therefore be useful in controlling host-versus-graft diseases in clinical situations in which engraftment failure is high, such as HLA-mismatched sibling or umbilical cord blood transplantation.

Can differentiation still occur in a xenogeneic or allogeneic microenvironment?

Several studies indicate that pluripotent cells from one species can respond to signals from the microenvironment of another species and differentiate in situ into cells with mature phenotypes. This concept has been confirmed by Fukuhara et al.[191] who co-cultured mouse MSCs with rat cardiomyocytes and showed that mouse MSCs could differentiate into cardiomyocytes when direct cell-to-cell contact was allowed.

1- Why are the differentiated mature cells derived from xeno- or allogeneic stem cells not rejected?

If, as described previously, the hypoimmunogenicity of donor MSCs is the reason shy these cells are not rejected soon after implantation, why are stem cells that have fully differentiated into mature cardiomyocytes still tolerated even though they now have lost the characteristics of stem cells and fully express histocompatibility antigens like any other differentiated cells?

The classic concept of transplant immune response is that the recipient's T cell with unique MHC receptor will bind with a specific allo- or xenogeneic, which signals the T cell to be activated and to proliferate. According to this scenario, there is no reason why differentiated cells should not be rejected.

In an attempt to better understand this concept, the "stealth immune tolerance" hypothesis was proposed by our lab[192], which is an extension of the "danger model" theory proposed by Matzinger[168] who suggested that immune rejection of a transplanted organ is not due to mismatched MHC antigens from the donor organ alone, but also due to the presence of "danger signal" serving as the co-stimulant factor, as a result of an invasive surgical transplant procedure. Thus the "stealth immune tolerance" hypothesis is based on the fact that, in the particular scenario of stem cell transplantation, the expression of new foreign antigens on the maturing cells is temporally dissociated from the danger signals derived from the injury inflicted by an invasive implantation procedure. This is because it takes many weeks for the implanted cells to mature and fully express their MHC antigens. Thus, in cell transplantation, by the time the implanted cells differentiate, the effects to tissue injury would have subsided, so that the immune synapsis receives only the first "recognition" signal without the second "activation" signal[192]. According to the classic view of immunology and to the two-signal theory for immune synapsis, the T cells undergo apoptosis, and the implanted cells, now fully differentiated, are still tolerated.

It is important to note that this view although credible, remains so far a hypothetical one and that further *in vitro* study are currently underway to better understand this concept.

Conclusion

This introduction reviews the "state of the art" in stem cell research and highlights the finding that undifferentiated adult stem cells are not determined progenitor cells with limited differentiation potential. Rather, these cells seem to possess a much broader capacity for cellular differentiation that is dependent on the microenvironment of the engrafted site. As we

saw, a number of developmental regulatory pathways appear, perhaps not surprisingly, to be redundantly involved in regulating cell fates, engraftment, migration, lodgment, proliferation and differentiation. Although the basic mechanisms may be conserved among different stem cell lines, it is important to note that the cellular input of self-renewal or differentiation may be unique and confined to this particular stem cell and its microenvironmental niche. Furthermore, the multilineage potential of MSCs, their ability to elude detection by the host's immune system, and their relative ease of expansion in culture make MSCs transplantation a fascinating new approach for the management of heart disease. Ideally, MSCs can be harvested, expanded, and cryopreserved, ready for injection into patients following myocardial infarction.

REFERENCES

1- Orlic D, Hill J, Arai A. Stem cells for myocardial regeneration. Circ Res 2002; 91: 1092-1102

2- Nir S, David R, Zaruba M, Franz W, Eldor J. Human embryonic stem cells for cardiovascular repair. Cardiovasc Res 2003; 58: 313-23

3- Chiu RCJ, Zibaitis A, Kao RL. Cellular cardiomyoplasty: myocardial regeneration with satellite cell implantation. Ann. Thorac Surg 1995; 60:12-18

4- Taylor DA, Atkins BZ, Hungspreugs P et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med 1998 4:929-33

5- Leor J, Patterson M, Quinones MJ et al. Transplantation of fetal myocardial tissue into the infarcted myocardium of rat. A potential method for repair of infarcted myocardium? Circulation 1996; 94: II-332-6

6- Wang JS, Shum-Tim D, Galipeau J et al. Marrow stromal cells for cellular cardiomyoplasty: feasibility and clinical advantages. J Thorac Cardiovasc Surg 2000; 120:999-1006

7- Scorsin M., Hagege A., Vilquin JT., Fiszman M., Marotte F., Samuel JL., Rappaport L., Schwartz K., Menasche P. Comparison of the effects of fetal cardiomyocyte and skeletal myoblast transplantation on postinfarction left ventricular function. J. Thorac Cardiovasc Surg 2000; 119:1169-75

8- Fuchs S., Baffour R., Zhou YF., Shou M., Pierre A., Tio FO., Weissman NJ., Leon MB., Epstein SE., Kornowski R. Transendocardial delievery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J. Am Coll Cardiol. 2001; 37:1726-32

9- Edelman GM. Topobiology: an introduction to molecular embryology. London: HarperCollins Basic Books, 1988: 18,21.

10- Tang G, Fedak P, Yau T, Weisel R, Kulik A, Mickle D, Li R. Cell transplantation to improve ventricular function in the failing heart. Eur J of cardiothorac Surg 2003; 23:907-916

11- Lovell M, Mathur A. The role of stem cells for treatment of cardiovascular disease. Cell prolif 2004; 37(1): 67-89

12- Campion DR. The muscle satellite cell: a review. Int Rev Cytol. 1984; 87:225-251

13- Menasche P., Hagege AA., Vilquin JT., Desnos M., Abergel E., Pouzet B., Bel A., Sarateanu S., Scorsin M., Schwartz K., Bruneval P., Benbunan M., Marolleau JP., Duboc D. Autologous skeletal myoblast transplantation for severe postinfarction left ventriculkar dysfunction. J Am Coll Cardiol. 2003; 41: 1078-83

14- Kao RL., Chiu RC. Cellular cardiomyoplasty: myocardial repair withg cell implantation. Austin, Tex: Landes Bioscience; 1997

15- Murry CE., Wiseman RW., Schwartz SM., Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. J Clin Invest 1996; 98:2512-2523

16- Kehat I., Kenyagin-Karsenti D., Snir M et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001; 108:407-14

17- Toma C., Pittenger MF., Cahill KS. Et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002; 105:93-98

18- Cohnheim J. Arch Path Anato Physiol Klin Med 1867; 40:1

19- Friedenstein AJ., Gorskoja V., Kulagina NN. Exp Hematol 1976; 4:276

20- Friedenstein AJ. Stromal mechanisms of bone marrow: cloning in vitro and retransplantation in vivo. Hamatol Bluttransfus 1980; 25:19-29

21- Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 1968; 6:230-247

22- Caplan AI. Mesenchymal stem cells. J Ortho Res 1991; 9:641-650

23- Peterson BE., Bowen WC., Patrene KD. Et al. Bone marrow as a potential source of hepatic oval cells. Science 1999l 284:1168-70

24- Pereira RF., O'Hara MD., Laptev AV et al. Marrow stromal cells as a source of progenitor cells for non hematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. Proc Natl Acad Sci U.S.A 1998; 95:1142-47

25- Ferrari G., Cusella De Angelis G., Coulta M et al. Muscle regeneration by bone marrow derived myogenic progenitors. Science 1998; 279: 1528-30

26- Gussoni E., Soneoka Y., Strickland CD. Et al. Dystrophin expression in the MDX mouse restored by stem cell transplantation. Nature 1999; 401:390-4

27- Brazelton TR., Rossi FM, Keshet GI et al. From marrow to brain: expression of neuronal phenotypes in adult mice. Science 2000; 290:1775-79

28- Krause DS, Theise ND, Collector MI et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001; 105:369-377

29- McKay R. Stem cell in the central nervous system. Science 1997; 274: 69-71

30- Pittenger MF, Mosca JD, McIntosh KR. Human mesenchymal stem cells: progenitor cells for cartilage, bone, fat and stroma. Curr Top Microbiol Immunol 2000; 251:3-11

31- Pittenger MF., MacKay AM, Beck SC. Et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284:143-7

32- Pittenger M, Martin B. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 2004; 95:9-20

33- Ringes-Lichtenberg SM., Jaeger MD, Fuchs M. Et al. Sustained improvement of left ventricular function after myocardial infarction by intravenous application of Sca-1+ Lin-/c-kit+ bone marrow derived stem cells. Circulation 2002; 106(Suppl.): II-14

34- Mangi AA., Kong D, He H. Genetically modified mesenchymal stem cells perform *in vivo* repair of damaged myocardium. Circulation 2002; 106(Suppl.): II-131

35- Deans R, Moseley A. Mesenchymal stem cells: biology and potential clinical uses. Exp Hem 2000; 28:875-884

36- Huang S, Terstappen LWMM. Formation of hematopoietic microenvironment and hematopoietic stem cells from single human bone marrow stem cell 1992; Nature 360:745

37- Waller EK, Olweus J, Lund-Johansen F et al. The "common stem cell" hypothesis reevaluated: human fetal bone marrow contains separate populations of hematopoietic and stromal progenitors. Blood 1995; 85:2422

38- Bittira B., Kuang JQ., Piquer S., Shum-Tim D., Al-Khaldi A., Chiu RCJ. The pathophysiological roles of bone marrow stromal cells in myocardial infarction. Circulation 2001; 104(Suppl.): II-523

39- Hoecht E., Kahnert H., Guan K et al. Cardiac regeneration by stem cells after myocardial infarction in transplanted human hearts. Circulation 2002; 106(Suppl.):II-132

40- Pereira RF., Halford KW., O'Hara MD et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc. Natl. Acad. Sci. U.S.A. 1995; 92:4857-61

41- Bittira B., Wang JS., Shum-Tim D., Chiu RCJ. Marrow stromal cells as the autologous adult stem source for cardiac myogenesis. Cardiac and Vascular Regeneration 2000; 1(3): 205-210

42- Rafii S, Avecilla S, Shmelkov S, Shido K, Tejada R, Moore M, Heissig B, Hattori K. Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment. Ann N.Y. Acad. Sci 2003; 996: 49-60

43- Chiu RCJ. Bone marrow stem cells as a source for cell therapy. Heart failure reviews 2003; 8:247-251

44- Lagasse E, Connors H, Al-Dhalimy M et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 2000; 6:1229-1234

45- Theise ND, Nimmakayalu M, Gardner R et al. Liver from bone marrow in humans. Hepatology 2000; 32:11-16

46- Verfaillie C. Stem Cell plasticity. Graft 2000; 3(6): 296-299

47- Orlic D., Kajstura J., Chimenti S. Et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc. Natl. Acd. Sci. U.S.A. 2001; 98:10344-49

48- Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines and stromal cells. Exp Hem 2002; 30:973-981

49- Lovell MJ, Mathur A. The role of stem cells for treatment of cardiovascular disease. Cell Prolif 2004; 37: 67-87

50- Richard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL. Isolation and characterization of osteoblast precursor cells from human bone marrow. J Bone Miner Res 1996; 11:312

51- Bianco P, Costantini M, Dearden LC and Bonucci E. Alkaline phsophatase positive precursors of adipocytes in human bone marrow. Br J Haematol. 68:401-403

52- Kuznetsov S, Robey P. A look at the history of bone marrow stromal cells. Graft 2000; 3(6): 278-283

53- Ashton BA, Allen TD, Howlett CR et al. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Ortho Rel Res. 1980; 151:294-307

54- Mezey E, Chandross KJ, Harta G et al. Turning blood into brain : cells bearing neuronal antigens generated in vivo from bone marrow. Science 2000; 290: 1779-82

55- Jackson KA, Majka SM, Wang H. Et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J. Clin. Invest. 2001; 107:1395-1402

56- Makino S, Fukuda K., Miyoshi S. et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999; 103:697-705

57- Mangi AA., Noiseux N., Kong D. Et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance in infarcted hearts. Nat. Med. 2003; 9:1195-1201

58- Bittira B., Kuang JQ., Al-Khaldi A. Et al. In-vitro preprogramming of marrow stromal cells for myocardial regeneration. Ann Thorac Surg 2002; 74:1154-1160

59- Wakitani S, Saito T, Caplan A. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 1995; 18:1417-1426

60- Xu W, Zhang X, Qian H, Zhu W, Sun X, Hu J, Zhou H, Chen Y. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocytes phenotype in vitro. Exp Biol and Med 2004; 229: 623-634

61- Hakuno D, Fukuda K, Makino S et al. Bone marrow-derived regenerated cardiomyocytes (CMG cells) express functional adrenergic and muscarinic receptors. Circulation 2002; 105: 93-8

62- Tomita S., Nakatani T., Fukuhara S. et al. Bone marrow stromal cells can differentiate into cardiac lineage and contract synchronously with cardiomyocytes by direct cell-to-cell interaction in vitro. Presented at the 82nd Annual Meeting of the American Association for Thoracic Surgery, Washington, DC, 2002.

63- Valiunas V, Doronin S, Valiuniene L, Potapova I, Zuckerman J, Walcott B, Robinson RB, Rosen MR, Brink PR, Cohen IS. Human mesenchymal stem cells make cardiac connexins and form functional gap junctions. J Physiol 2004; 555: 617-26

64- Bird SD, Doevandans PA, van Rooijen MA, Brutel de la Riviere A, Hassink RJ, Passier R, Mummery CL. The human adult cardiomyocyte phenotype. Cardiovasc Res 2003; 58: 423-34

65- Tomita S., Li RK., Weisel RD. et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999; 100:II-247-56

66- Chiu RCJ. Cellular cardiomyoplasty: the biology and clinical importance of milieu dependent differentiation. In Handbook of cardiac cell transplantation, 2004 Kipshidzen N., Serruys PW. (eds), Martin Dunitz Ltd, London UK:pp15-31

67- Wang JS, Shum-Tim D, Chedrawy E, Chiu RCJ. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. J Thorac Cardiovasc Surg 2001; 122:699-705

68- Shake JG, Gruber PJ, Baumgartner WA, Senechal G., Meyers J., Redmond JM, Pittenger MF, Martin BJ. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 2002; 73:1919-26

69- Davani S, Marandin A, Mersin N et al. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation 2003; 108(Suppl 1):II253-258

70- Dick AJ, Guttman MA, Raman VK, Peters DC, Pessanha BS, Hill JM, Smith S, Scott G, McVeigh ER, Lederman RJ. Magnetic resonance fluoroscopy allows targeted delivery of mesenchymal stem cells to infarct borders in swine. Circulation 2003; 108: 2899-2904

71- Kawada H. Fujita J., Kinjo K. et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 2004; 104:3581-7

72- Laflamme MA, Myerson D, Saffitz JE, Murry CE. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res 2002; 90: 634-640

73- Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Chimerism of the transplanted heart. New Engl J Med 2002; 346: 5-15

74- Taylor DA, Hruban R, Rodriguez R, et al. Cardiac chimerism as a mechanism for self repair; does it happen and if so to what degree? Circulation 2002; 106: 2-4

75- Botta R, Gao E., Stassi G. et al. Heart infarct in NOD-SCID mice: therapeutic vasculogenesis by transplantation of human CD34+ cells and low dose CD34+ KDR+ cells. FASEB J 2004; 18:1392-94

76- Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004; 94:678-85

77- Lee N, Thorne T, Losordo D, Yoon Y. Repair of ischemic heart disease with novel bone marrow-derived multipotent stem cells. Cell Cycle 2005; 4(7): 861-4

78- Terada N, Hamazaki T, Oka M et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002; 416:542-545

79- Wang X, Willenbring H, Akkari Y et al. Cell fusion is the principal source of bone-marrow derived hepatocytes. Nature 2003; 422:897-901

80- Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, et al. Fusion of bone marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 2003; 425:968-73

81- Anversa P., Nadal-Ginard B. Myocyte renewal and ventricular remodeling. Nature 2002; 415:240-3

82- Beltrami AP, Urbanek K, Kajstura J et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001; 344: 1750-7

83- Beltrami AP., Barlucchi L., Torella D. et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763-776

84- Messina E., De Angelis L., Frati G. et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004; 95:911-21

85- Martin C, Meeson A, Robertson S, Hawke T, Richardson J, Bates S, Goetsch S, Gallardo T, Garry D. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 2004; 265: 262-75

86- Chiu RCJ. Adult stem cell therapy for heart failure. Expert Opin. Biol. Ther. 2003; 3(2):215-25

87- Schofield R: the relationship between the spleen colony-forming cell and the hematopoietic stem cell. Blood Cells 1978; 4:7-25

88- Fuchs E, Tumbar T, Guasch G. Socializing with the Neighbors: stem cells and their niche. Cell 2004; 116: 769-778

89- Davey R, Zandstra P. Signal processing underlying extrinsic control of stem cell fate. Curr Opin Hematol 2004; 11: 95-101

90- Calvi LM, Adams GB, Weibrecht KW at al. Osteoblastic cells regulate the hematopoietic stem cell niche. Nature 2003; 425: 841-846

91- Marie-Rattis F, Voermans C, Reya T. Wnt signaling in the stem cell niche. Curr Opin Hematol 2004; 11: 88-94

92- Park I, Qian D, Kiel M, Becker M, Pihalja M, Weissman I, Morrison S, Clarke M. Bmi-1 is required for maintenance of adult self-renewing hematopoietic stem cells. Nature 2003; 423: 302-4

93- Duncan A, Rattis F, DiMascio L, Cougdon K, Pazianos G, Zhao C, Yoon K, Cook M, Willert K, Gaiano N, Reya T. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nature Imm 2005; 6(3): 314-321

94- Hirashima M, Ogawa M, Nishikawa S et al. A chemically defined culture of VEGFR2+ cells derived from embryonic stem cells reveals the role of VEGFR1 in tuning the threshold for VEGF in developing endothelial cells. Blood 2003; 101: 2261-7

95- Takamatsu Y, Simmons PJ, Morris HA, To LB, Levesque J-P. Osteoclast-mediated bone resorption is stimulated during short term administration of granulocyte colony-stimulating factor but is not responsible for hematopoietic progenitor stem cell mobilization. Blood 1998; 92:3465

96- Peled A, Petit I, Kollet O et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 1999; 283:845 CITED B/F

97- Peled A, Kellet O, Ponomaryov T et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4 and VLA-5 on immature human CD34+ cells: role in transendothelial/stromal migration and engraftment on NOD/SCID mice. Blood 2000; 95:3289

98- Sun M, Opavsky A, Stewart D, Rabinovitch M, Dawood F, Wen W, Liu P. Temporal response and localization of integrins β1 and β3 in the heart after myocardial infarction. Circulation 2003; 107: 1046-1052

99- Fujita J., Suzuki Y., Ando K. Et al. G-CSF improves postinfarction heart failure by mobilizing bone marrow stem cells but GM-CSF increases the mortality by deteriorating heart function in mice. Circulation 2002; 106(Suppl.): II-15

100- Miki T., Sakamoto J., Nakano A. et al. Mobilization of bone marrow cells by G-CSF/M-CSF improves ventricular function in heart undergoing postinfarct remodeling. Circulation 2002; 106(Suppl.) II-15

101- Fibbe WE, Pruijt JF, van Kooyk Y, Figdor CG, Opdenakker G, Willemze R. The role of metalloproteinases and adhesion molecules in IL-8 induced stem cell mobilization. Semin Hematol 2000; 37:19

102- Velders G, Fibbe W. Involvement of proteases in cytokine-induced hematopoietic stem cell mobilization. Ann. N.Y. Acad. Sci. 2005; 1044: 60-69

103- Sweeney EA, Priestley GV, Nakamoto B, Collins RG, Beaudet AL, Papayannopoulou T. Mobilization of stem,/progenitor cells by sulfated polysaccharides does not require selectin presence. Proc Natl Acad Sci U.S.A. 2000; 97:6544

104- Yamaguchi J, Kusano K, Masuo O, Kawamoto A, Silver M, Murasawa S, Marce M, Masuda H, Losordo D, Isner J, Asahara T. SDF-1 effects on ex vivo expanded EPC recruitment for ischemic neovascularization. Circulation 2003; 107: 1322-8

105- Papayannopoulou T, Nakamoto B. Peripheralization of hematopoietic progenitors in primates treated with anti-VLA4 integrin. Proc Natl Acd Sc U.S.A 1993; 90:9374

106- Levesque JP, Takamatsu Y, Nilsson SK, Haylock DN, Simmons PJ. Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoeitc progenitor cell mobilization by G-CSF. Blood 2001; 98:1289

107- Petit I, Szyper-Kravitz M, Nagler A et al. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and upregulating CXCR4. Nature Immunol 2002; 3:687

108- Heissig B, Hattori K, Dias S et al. In vivo inhibition of metalloproteinases blocks chemo-cytokine induced endothelial and hematopoietic stem cell mobilization. Blood 2000; 96:5-40a

109- Valenzuela-Fernandez A, Planchenault T, Baleux F et al. Leukocyte elastase negatively regulates stromal cell-derived factor-1 SDF/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4. J Biol Chem 2002; 277:15677

110- Heissig B, Hattori K, Dias S et al. Matrix recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002; 109:625

111- Flores I, Cayuela M, Blasco M. Effects of telomerase and telomere length on epidermal stem cell behavior. Science 2005; 309: 1253-6

112- Nilsson S, Simmons P. Transplantable stem cells: home to specific niches. Curr Opin Hematol 2004; 11:102-106

113- Frenette PS, Subbarao S, Mazo IB et al. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. Proc Natl Acad Sci U.S.A.1998; 95:14423-28

114- Katayama Y, Hidalgo A, Furie BC et al. PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha-4 integrin. Blood 2003; 102:2060-67

115- Papayannopoulou T, Craddock C, Nakamoto B. Et al. The VLA-4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgment of transplanted murine hematopoietic progenitors between bone marrow and spleen. Proc Natl Acad Sci U.S.A. 1995; 92:9647-51

116- Peled A, Petit I, Kollet O et al. Dependence on human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 1999; 283:845-848

117- Kollet O, Spiegel A, Peled A, Petit I, Byk T, Hershkoviz R, Guetta E, Barkai G, Nagler A, Lapidot T. Rapid and efficient homing of human CD34+CD38-CXCR4+ stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m mice. Blood 2001; 97: 3283-91

118- Solanilla A, Grosset C, Duchez P et al. Flt3-ligand induces adhesion of hematopoietic progenitor cells via a very late antigen VLA-4 and VLA-5 dependent mechanism. Br J Hematol 2003; 120:782-786

119- Driessen RL, Johnston HM, Nilsson SK: Membrane-bound SCF is a key regulator in the intial lodgment of stem cells within the endosteal marrow region. Exp Hematol 2003; 31:1284-1291

120- Mohle R, Boehmler AM, Denzlinger C et al. Nonpeptide mediators in the hematopoietic microenvironment. Ann NY Acad Sci 2003; 996:61-6

121- Netelenbos T, van den Born J, Kessler FL et al. Proteoglycans on bone marrow endothelial cells bind and present SDF-1 toward hematopoietic progenitor cells. Leukemia 2003; 17:175-184

122- Nilsson SK, Haylock DN, Johnston HM et al. Hyaluronan is synthesized by primitive hematopoietic cells, participates in their lodgment at the endosteum following transplantation, and is involved in the regulation of their proliferation and differentiation in vitro. Blood 2003; 101:856-862

123- Gazitt Y. Homing and mobilization of HSC and hematopoietic cancer cells are mirror image processes, utilizing similar signaling pathways and occurring concurrently: circulating cancer cells constitute an ideal target for concurrent treatment with chemotherapy and anti-lineage specific antibodies. Leukemia 2004; 18: 1-10

124- Ahmed F, Ings SJ, Pizzey AR et al. Impaired bone marrow homing of cytokine-activated CD34+ cells in NOD/SCID model. Blood 2004; 103:2079-87

125- Liu B, Buckley SM, Lewis ID et al. Homing defect of cultured human hematopoietic cells in the NOD/SCID mouse is mediated by Fas/CD95. Exp Hematol 2003; 31:824-832

126- Zheng Y, Watanabe N, Nagamura-Inoue T et al. Ex-vivo manipulation of umbilical cord-blood derived hematopoietic stem/progenitor cells with recombinant human SCF can upregulate levels of homing-essential molecules to increase their transmigratory potential. Exp Hematol 2003; 31:1237-46

127- Gazzitt Y, Liu Q. Plasma levels of SDF-1 and expression of SDF-1 receptor on CD34+ cells in mobilized peripheral blood of non Hodgkin's lymphoma patients. Stem Cells 2001; 19:37

128- Pittenger MF, MacKay AM. Multipotential Human mesenchymal stem cells. Graft 2000; 3(6): 288-293

129- Reya T, Duncan AW, Ailles L et al. A role of Wnt signaling in self-renewal of hematopoietic stem cells. Nature 2003; 423: 409-414

130- Zhang J, Niu C, Ye L et al. Identification of the hematopoietic stem cell niche and control of the niche size. Nature 2003; 425: 836-841

131- Abbott J, Huang Y, Liu D, Hickey R, Krause D, Giordano F. SDF-1 plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 2004; 110: 3300-5

132- Saito T., Kuang JQ., Bittira B., Al-Khaldi A., Chiu RCJ. Xenotransplant cardiac chimera: immune tolerance of adult stem cells. Ann. Thorac. Surg. 2002; 74:19-24

133- Wang L, Li Y, Chen J, Gautam SC, Zhang Z, Lu M, Chopp M. Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. Exp Hematol 2002; 30:831-6

134- Devine SM., Bartholomew AM., Mahmud N et al., Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol 2001; 29:244-255

135- Sorger JM, Despres D, Hill J, Schimel D, Martin BJ, McVeigh ER. MRI tracking of intravenous magnetically labeled mesenchymal stem cells following myocardial infarctions in rats. Circulation 2002; 106:2-16

136- Rombouts WJ, Ploemacher RE. Primary murine MSCs show highly efficient homing to the bone marrow but lose homing ability following culture. Leukemia 2003; 17:160-170

137- Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, Dicorleto PE, Topol EJ, Penn MS. Effect of SDF1 on stem cell homing and tissue regeneration in ischemic cardiomyopathy. Lancet 2003; 362: 697

138- Takahashi T, Kalka C, Masuda H et al. Ischemia and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999; 5:434-8

139- Harada M, Qin Y, Takano H, Minamino T, ZouY, Toko H, Ohtsuka M, Matsuura K, Sano M, Nishi J, Iwanaga K, Akazawa H, Kunieda T, Zhu W, Hasehawa H, Kunisada K, Nagai T, Nakaya H, Yamauchi K, Komuro I. G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nature Med 2005; 11(3): 305-311

140- Bhatia R, Hare J. Mesenchymal stem cells: future source for reparative medicine. CHF. 2005; 11:87-91

141- Liesveld JL, Rosell K, Panoskaltsis N, Belanger T, Harbol A, Abboud CN. Response of human CD34+ cells to CXC, CC and CX3C chemokines: implications for cell migration and activation. J Hematother Stem Cell Res 2001; 10: 643-5

142- Eghbali-Webb M. The role of cardiac fibroblast in cardiac regeneration. In Handbook of cardiac cell transplantation, 2004 Kipshidzen N., Serruys PW. (eds), Martin Dunitz Ltd, London UK:pp73-90

143- Heissig B, Hattori K, Dias S et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002; 109:625-637

144- Heissig B, Werb Z, Rafii S, Hattori K. Role of ckit/kit ligand signaling in regulating vasculogenesis. Thromb Haemost 2003; 90: 570-6

145- MacDonald D, Burstein B, Shum-Tim D et al. Feasibility of intravenous adult bone marrow stromal cell administration in cellular cardiomyoplasty. Paper presented at the International Society of Stem Cell Research, June 10-13, 2004: 2nd Annual Meeting; Boston, MA.

146- Geng Y. Molecular mechanisms for cardiovascular stem cell apoptosis and growth in the hearts with atherosclerotic coronary disease and ischemic heart failure. Ann. N.Y. Acad. Sci 2003; 1010: 687-97

147- Griese DP., Grumbeck B., Kunz-Schughardt L. et al., Genetic engineering of postnatal hematopoietic stem cells for gene and cell-based therapy of ischemic heart disease in rats. Circulation 2002; 106(Suppl.)II-15

148- Yau TM, Fung K., Fujii T. Et al. Enhanced myocardial angiogenesis by gene transfer using transplanted cells. Circulation 2001; 104:1218-1222

149- Chuah MK, Brems H, Vanslembrouck V, Collen D, Vandendriessche T. Bone marrow stromal cells as targets for gene therapy of Hemophila A. Hum Gene Ther 1998; 9:353

150- Alessandri G, Emanueli C, Madeddu P. Genetically engineered stem cell therapy for tissue regeneration. Ann N.Y. Acad Sci 2004; 1015: 271-284

151- Schwartz EJ, Alexander GM, Prockop DJ et al. Multipotential marrow stromal cells transduced to produce L-DOPA: engraftment in a rat model of Parkinson's disease. Hum Gene Ther 1999; 10:2539-49

152- Assmus B, Schachinger V, Teupe C et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) Circulation 2002; 106: 3009-17

153- Strauer BE, Brehm M, Zeus T et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Cicrulation 2002; 106: 1913-18

154- Perin EC, Dohmann HF, Borojevic R et al. Transendocardial autologous bone marrow cell transplantation for severe chronic ischemic heart failure. Circulation 2003; 107:2294-2302

155- Wollert KC, Meyer GP, Lotz J et al. Intracoronary autologous bone marrow cell transfer after myocardial infarction: the BOOST randomized controlled trial. Lancet 2004; 364: 141-8

156- Kuethe F, Richartz BM, Sayer HG, Kasper C, Werner GS, Hoffken K, Figulla HR. Lack of regeneration of myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans with large anterior myocardial infarctions. Int J Cardiol 2004; 97: 123-127

157- Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, Zhang JJ, Chunhua RZ, Liao LM, Lin S, Sun JP. Effect on left ventricular function of intracoromary transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction. Am J Cardiol 2004; 94: 92-95

158- Lazarus H, Koc O. Culture-expanded human marrow-derived MSCs in clinical hematopoietic stem cell transplantation. Graft 2000; 3(6): 329-333

159- Peschle C, Condorelli G. Stem cells for cardiomyocyte regeneration: state of the art. Ann. N.Y. Acad. Sci 2005; 1047:376-385

160- Reffelmann T, Kloner R. Cellular cardiomyoplasty- cardiomyocytes, skeletal myoblasts, or stem cells for regenerating myocardium and treatment of heart failure. Cardiovasc Res 2003; 58: 358-68

161- Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol 2003; 31: 890-6

162- Bartholomew A, Sturgeon C, Satskas M et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002; 30: 42-48

163- Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003; 75:389-397

164- Liechty KW., Mackenzie TC., Shaaban AF. Et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after *in utero* transplantation in sheep. Nat. Med. 2000; 11:1282-6

165- MacDonald D, Saito T, Shum-Tim D, Bernier PL and Chiu RCJ. Persistence of marrow stromal cells implanted into acutely infarcted myocardium: Observations in a xenotransplant model. J Thorac Cardiovasc Surg 2005; *In press*

166- Klyushnenkova E, Mosca JD, McIntosh KR. Human mesenchymal stem cells suppress allogeneic T cell responses in vitro: implications for allogeneic transplantation. Blood 1998; 92: 642a

167- Chou S, Kuo T, Liu M, Lee O. In utero transplantation of human bone-marrow derived multipotent mesenchymal stem cells in mice. J Orthop Res 2006; 24(3): 301-12

168- Matzinger P. The Danger Model: a renewed sense of self. Science 2002; 296(5566): 301-305

169- Meisel R, Zibert A, Laryea M et al. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-deoxygenase-mediated tryptophan degradation. Blood 2004; 103: 4465-72

170- Aggarwal S, Pittenger M. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005; 105: 1815-22

171- Di Nicola M, Carlo-Stella C, Magni M et al. Human bone marrow stromal cells suppress T lymphocyte proliferation induced by cellular or non-specific mitogenic stimuli. Blood 2002; 99: 3838-3843

172- Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naïve and memory antigen-specific T cells to their cognate peptide. Blood 2003; 101: 3722-3729

173- Rasmusson I, Ringden O, Sundberg B, LeBlanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation 2003; 76: 1208-1213

174- LeBlanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57:11-20

175- Koc ON, Day J, Nieder M et al. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy and Hurler syndrome. Bone Marrow Transplant. 2002; 30: 215-222

176- Horwitz EM, Gordon PL, Koo WK et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. Proc Natl Acad Sci USA 2002; 99: 8932-8937

177- LeBlanc K, Rasmusson J, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004; 363:1439-1441

178- Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats: similarities to astrocyte grafts. Proc Natl Acad Sci U.S.A. 1998; 95: 3908-13

179- Dai W, Hale S, Martin B et al. Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short and long-term effects. Circulation 2005; 112: 214-223

180- Ryan J, Barry F, Murphy J and Mahon B. Mesenchymal stem cells avoid allogeneic rejection. J of Inflammation 2005; 2(8):1-11

181- Zhang W, Ge W, Li C et al. Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. Stem Cell Dev 2004; 13:263-271

182- Beyth S, Borovsky Z, Mevorach D et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T cell unresponsiveness. Blood 2005; 105:2214-2219

183- Jiang XX, Zhang Y, Liu B et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood 2005; *in press*

184- Glennie S, Soeiro I, Dyson PJ, Lam EW and Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 2005; 105:2821-2827

185- Djouad F, Plence P, Bony C et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003; 102: 3837-3844

186- Silva WAJ, Covas DT, Panepucci RA et al. The profile of gene expression of human marrow mesenchymal stem cells. Stem Cells 2003; 21: 661-669

187- Arikawa T, Omura K, Morita I. Regulation of bone morphogenic protein-2 expression by endogenous prostaglandin E2 in human mesenchymal stem cells . J Cell Physiol 2004; 200: 400-406

188- Grinnemo K, Mansson A, Dellgren D et al. Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. J Thorac and Cardiovasc Surg 2004; 127(5): 1293-1300

189- Mitra R, Singh S, Khar A. Antitumor immune responses. Expert Rev Mol Med 2003; 2003: 1-22

190- Koc ON, Gerson SL, Cooper BW et al. Rapid hematopoeitic recovery after coinfusion of autologous blood stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol. 2000; 18:307-20

191- Fukuhara S, Tomita S, Yamashiro S et al. Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro. J Thorac Cardiov Surg 2003; 125: 1470-80

192- Chiu R. "Stealth immune tolerance" in stem cell transplantation: potential for "universal donors" in myocardial regenerative therapy. J Heart and lung transplantation 2005; 24(5): 511-516

Chapter 2

MATERIAL & METHODS

Study Rationale:

Background information

Coronary artery disease accounts for 50% of all cardiovascular deaths and remains a major cause of morbidity and mortality[1]. Cellular transplantation is a promising strategy that can improve heart function through several mechanisms including myogenesis[2], angiogenesis[3] and through paracrine effects attenuating left ventricular remodeling[4,5]. In recent years, we and others have reported that autologous marrow stem cells (MSCs), when transplanted into infarcted myocardium, can differentiate into cells of various phenotypes and improve ventricular function[4,6-9]. The observed beneficial effects of cell transplantation have then led to many human clinical trials[10-13].

Despite the promising early results, such clinical application remains limited by the logistic, economic and timing issues when harvesting autologous cells from elderly sick patients. Furthermore, a number of recent studies have documented a significantly reduced capacity for neovascularization, proliferation and differentiation as well as increased levels of apoptosis *in vitro* and *in vivo* in MSCs obtained from elderly donors and from patients with diabetes or ischemic heart disease[14-18]. These impairments clearly limit the therapeutic potential of autologous MSCs and highlight therefore the clinical advantages of "universal donor cells" for cellular transplantation.

Recently rodent and porcine MSCs have been reported to be uniquely immune tolerant, both in the *in vitro* mixed lymphocyte co-culture studies[19,20] and in the *in vivo* allo- and xeno-transplant models[4,21-24]. Although there is a substantive body of literature that supports the notion that human MSCs are immunosuppressive *in vitro*, it is not yet clearly proven whether their

immunoprivileged properties are retained universally *in vivo*, and whether these cells still possess their ability to improve ventricular function within a xenogeneic environment. Thus we transplanted human MSCs into infarcted rat myocardium without the use of any immunosuppression and evaluated whether these cells could survive, differentiate and contribute to the improvement in heart function.

Objectives of this research project are:

To implant human MSCs into infarcted rat myocardium without immunosuppression and to see whether these cells 1) survive, 2) differentiate and 3) contribute to the improvement in the overall cardiac function

Animals

Immunocompetent female syngeneic Lewis rats (200-250 g, Charles River, Quebec, Canada) were used in this study. All procedures were approved by the Institutional Animal Care Committee at the McGill University Health Center and performed by one author (R.A.) in compliance with the *Guide for the Care and Use of Laboratory animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care.

Experimental design

A total of 90 female rats underwent open-chest coronary artery ligations and were randomly assigned to 3 groups (**figure 1**). In group I (n=40), lac-Z labeled human male MSCs (3×10^6) were injected directly into the peri-ischemic region of rat myocardium 5 minutes after ligation of the proximal left coronary artery. In group II (n=10), lac-Z labeled isogenic rat MSCs (3×10^6) were similarly injected around the peri-infarcted myocardial region. In group III (n=10), a similar volume of cell-free culture medium was injected into control animals after coronary ligation. No immunosuppression was given at any time. All animals underwent blinded assessment with transthoracic echocardiography at 4-5 days after implantation and 8 weeks after injection. Heart specimens from group I were serially harvested at 1, 3, 6 and 8 weeks and processed for immunohistochemical and polymerase chain reaction (PCR) analyses. Rats in group II and III were sacrificed 8 weeks after cell implantation.

Additionally, fully differentiated human male fibroblasts (3×10^6) were directly injected into infarcted rat myocardium and served as immunologic controls (group IV, n=12). Hearts were serially harvested and examined for the presence of cellular infiltration and surviving labeled fibroblasts by immunohistochemistry and PCR analyses.

Cell Isolation, culture and labeling

Rat MSCs

Isolation and culture of rat MSCs were performed according to Caplan's method[25]. Briefly, after an overdose of pentobarbital (100mg/kg) given intraperitoneally, bone marrow stromal cells were harvested by flushing the femurs and tibias with Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100U/mL penicillin G, 100 μg/mg streptomycin, 0.25 μg amphotericin B; All obtained from Gibro Laboratories, Boston, MA) in a humidified atmosphere of 5% CO_2.

Whole marrow was plated in tissue culture dishes, medium was replaced every 3 days and the nonadherent cells were discarded. Each primary culture was passaged twice to 3 new plates, and the cell density of the colonies was grown to approximately 90% confluence. To prevent the MSCs from differentiating or slowing their rate of division, each primary culture was replated to 3 new plates when the cell density within colonies became 80% to 90% confluent, approximately 2 weeks after seeding.

Once these cells were nearly confluent, MSCs were transfected as described previously [26] with pMFG-lac Z plasmid containing β-galactosidase gene for identification of the transplanted cells in the myocardial scar tissue. The resulting MSCs expressing lacZ were expanded for 4 weeks before transplantation. The cells were allowed to proliferate until at least a 70% confluence was achieved before using them for transduction in order to achieve a high retrovirus titer. Twenty-four hours before transduction, the MSCs were trypsinized with 0.05% Trypsin + 0.53 mM EDTA (Gibco Labs) and replated. The next day, these cells were transduced with lac Z retroviral particles twice per day for 3 consecutive days with lipofectamine (3μL of

lipofectamine 2mg/mL solution for each 1mL of virus medium). At each transduction, the MSCs medium was replaced with the supernatant from the lacZ-GP + AM12 cells.

Human MSCs

Human MSCs were isolated, cultured and prepared by Cambrex Inc.[27]. Briefly, bone marrow aspirates were passed through a density and hematopoietic cells, fibroblasts, and other nonadherent cells were washed away during medium changes. The remaining purified MSCs population was further expanded in culture to form a clonal homogeneous population of cells, fully characterized by specific cell surface markers using flow cytometry, being uniformly positive for CD 166, CD 105, CD44, CD29 and negative for hematopoeitic markers such as CD14, CD34 and CD45. Furthermore, their capacity to differentiate along adipogenic, chondrogenic, and osteogenic lineages was assessed as described elsewhere[26]. They were then shipped to our laboratory at 4°C for cell transplantation. Upon arrival, the cells were resuspended in DMEM supplemented with 10% FBS. After transfecting them with lac-Z encoding gene, they were maintained in a humidified atmosphere containing 5% CO_2.

Human fibroblasts

Human fibroblasts were harvested by outgrowth from a piece of chest skin taken from male donors. Attached cells in culture flasks were then transfected with lac Z reporter gene as described above.

X-gal staining for detection of β-galactosidase activity

Seventy-two hours after the last transduction, MSCs were trypsinized and parts of the cells were plated in a 35-mm dish for histochemical staining for β-galactosidase activity, in order to determine the percentage of cells expressing β-galactosidase. The medium was aspirated from the plates and the cells rinsed with PBS. The cells were fixed at 4^0C in fix solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) for 15 minutes and rerinsed with PBS. Staining for β-gal was performed with a solution containing 1 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactoside (Xgal), 1 mmol/L ethyleneglycol-bis(β-aminoethyl-ether)-N,N'-tetraacetic acid, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6O$. $3H_2O$, 2mmol/L magnesium chloride, and 0.01% sodium deoxycholate was added. The cells were then incubated at 37^0C and protected from light for 16 hours. The presence of blue-labeled cells was then confirmed under phase microscopy.

Preparation of cells for injection

Cells isolated from the rat bone marrow, as well as human MSCs were cultured in complete medium in tissue culture dishes. After labeling, the medium was aspirated and the cells were washed with 6mL of Hank's balanced salt solution (HBSS). The HBSS was aspirated and 2mL of trypsin-EDTA was added to detach the cells from the bottom of the dish. The detached cell suspension was then placed in a flask with 2mL of complete medium and placed in a hemocytometer for counting. A volume consisting of $3x10^6$ cells was then collected and centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 0.5 cm^3 of complete medium.

Creation of the infarction and transplantation of MSCs

Female rats were anesthetized with 5% isoflurane (MTC Pharmaceuticals, Cambridge, Ontario, Canada). Animals were intubated with an 18-gauge intravenous catheter and connected to a Harvard rodent ventilator (Harvard Apparatus Co. Inc, Boston, MA) at 85 breaths/minute. Anesthesia was maintained with 3% isoflurane and oxygen at 5-6L/min.

A 1.5 cm left anterolateral thoracotomy was performed in the fifth intercostals space under sterile conditions to expose the heart, and the left coronary artery was ligated approximately 1 to 2 mm from its origin with a 7-0 polypropylene suture (Ethicon, Inc, Somerville, NJ) **(figure 2)**. Successful performance of coronary occlusion was verified by observation of the development of a pale color in the left ventricle after ligation. Under direct vision, human male MSCs and rat MSCs (3×10^6 cells suspended in 150 μL Dulbecco Modified Eagle Medium) were injected directly at 3 different sites into the peri-infarcted area of the left ventricle using a 28-gauge insulin syringe 5 minutes after coronary artery ligation. Small blebs under the injected area were confirmed in every case. An equal volume of cell-free culture medium and fibroblasts (3×10^6) were injected into the control animals (group III and IV) respectively. After achieving hemostasis, the muscle layers and skin were closed separately with 4-0 monofilament sutures. Once spontaneous respiration resumed, the animals were extubated and placed in a temperature-controlled chamber until they resumed full alertness and mobility. Furthermore, Buprenorphine hydrochloride (0.01 to 0.05mg/kg SC) was given postoperatively for pain.

Functional Assessment

Transthoracic echocardiography was performed on all surviving animals in group I (n=23), II (n=10) and III (n=10) at 4-5 days (baseline) and at 8 weeks after coronary ligation. Echocardiograms were obtained with a commercially available system (Sonosite, Titan-Washington) equipped with a 15-MHz transducer. We decided *a priori* to exclude any rat that had an ejection fraction above 45% after the first echocardiogram. After sedating the animals with 2% isoflurane, echocardiography was performed as described elsewhere[28]. Briefly, parasternal long- and short-axis views were obtained with both M-mode and 2-dimensional images. End-diastolic (LVEDD) and end-systolic (LVESD) diameters of the LV were measured with M-mode tracings between the anterior and posterior walls from the short-axis view just below the level of the papillary muscles of the mitral valve. This was done according to the American Society of Echocardiology leading-edge method from at least 3 consecutive cardiac cycles[28]. Fractional shortening (FS) was determined as [(LVEDD-LVESD)/LVEDD] x 100 (%). LV end-diastolic volume (LVEDV) was calculated as 7.0 x $LVEDD^3$ / (2.4+LVEDD), and LV end-systolic volume (LVESV) as 7.0 x $LVESD^3$ / (2.4+LVESD. The ejection fraction (EF) was estimated as (LVEDV-LVESV) / LVESV x 100 (%).

All measurements were performed by one experienced observer who was blinded to the treatment groups.

Tissue processing and staining for β-galactosidase activity

Hearts from group I were serially harvested at different time intervals for up to 8 weeks. Five rats were sacrificed at 1 week, 12 rats at 3 weeks, 13 rats at 6 weeks and 10 rats at 8 weeks. All the hearts from group II and III were processed 8 weeks after cell implantation (n=10 each).

Specimens were serially harvested at 1, 3, 5, 8, 10, 12 and 14 days in group IV and processed for histological analyses.

The hearts were collected and rinsed with PBS, fixed in 2% paraformaldehyde and stained for β-galactosidase activity as described previously[26]. After X-gal staining, the hearts were cut longitudinally and embedded in paraffin.

Histological and Immunohistochemical Analyses

Serial coronal sections of 5 μm in thickness were then mounted on a set of gelatin-coated glass slides such that serial sections could be used for different stains. A series of section from each heart specimen were stained with hematoxylin and eosin to depict nuclei, cytoplasm and connective tissue and assess cell survival as well as the extent of cellular infiltration. In order to examine the extent of differentiation, immunohistochemical staining on other serial sections from each heart was done as previously described[26] using cardiomyocyte-specific markers for troponin IC (Santa-Cruz Biotechnology Inc, Santa Cruz, Calif) and connexin-43 (Zymed Laboratories, Inc, San Francisco, CA). Cellular infiltration was detected by hematoxylin and eosin staining and confirmed by staining with CD68 monoclonal antibodies against macrophages (Abcam Inc, Cambridge, MA).

1- Hematoxylin and Eosin

A series of sections from each heart specimen were stained with hematoxylin and eosin in the following method:

Staining procedure

1- Xylene	2 minutes

2- Xylene	2 minutes
3-100% alcohol	2 minutes
4- 100% alcohol	2 minutes
5- 95% alcohol	2 minutes
6- 80% alcohol	2 minutes
7- 70% alcohol	2 minutes
8- Tap water wash	2 minutes
9- Distilled water	6 dips
10- Harris' Hematoxylin	Up to 5 minutes if necessary*
11- Tap water	2 minutes or until clear
12- Acid alcohol *70% alcohol 1000cc + hydrochloric acid, concentrated 10cc)*	3 dips
13- Tap water	2 minutes
14- Ammonia water *2 mL NH_4OH + 1L of distilled water*	7 dips
15- Tap water	2 minutes
16- Eosin	1 dip
17- 100% alcohol	2 dips
18- 100% alcohol	2 dips
19- 95% alcohol	2 dips
20- Xylene	2 minutes
21- Xylene	2 minutes

22- Slides were then dried and coverslips applied with Perimount mounting media (Fisher Scientific)

* Slides may be dipped in Hematoxylin for 1-2 minutes at a time, then rinsed under tap water and viewed under a light microscope for adequate staining

Immunostaining

Immunostaining with anti-connexin 43, troponin I-c and CD-68 was performed in the following manner:

Dewaxing paraffin slides

1- Incubate slides in xylene	5 minutes
2- Incubate slides in xylene	5 minutes
3- 100 % ethanol twice	10 minutes each
4- 95% ethanol twice	10 minutes each
5- Wash in deionized water	5 minutes

Heat-induced epitope retrieval (for Connexin 43 only)

1- After slides were deparaffinized as described above, they were washed with distilled water 3 times for 2 minutes each
2- Slides were put in a slide rack and placed in a 1 L glass beaker containing 500 mL of 0.01 M citrate buffer
3- The beaker was placed on a hot plate and the solution heated it boiled for 10 minutes
4- After heating, the beaker was removed from removed from the hot plate and allowed to cool down for at least 10-20 minutes at room temperature
5- Slides were rinsed with PBS and the immunostaining protocol started

Immunoperoxidase staining procedure

All steps were carried out at room temperature in a humidified chamber

Blocking endogenous peroxidase activity

1- Slides were incubated for 10 minutes in 0.3% hydrogen peroxide diluted in methanol at room temperature to quench endogenous peroxidase activity

2- Slides were washed with PBS twice for 5 minutes each

3- A tissue adhesion pen was used to encircle all specimens on the slide

Blocking non-specific antigenicity

Sections were incubated for 1 hour with 1.5% blocking serum in PBS. Excess blocking serum was blotted from all slides

Primary antibody application

Sections were incubated with primary antibody overnight in a humidified chamber at 4^0C. Optimal antibody concentrations were determined by titration, diluted in 1.5% blocking serum in PBS. Optimal antibody concentrations for troponin I-C and connexin-43 used were 1μg/mL.

Secondary antibody application

Slides were washed with PBS 3 times, for 5 minutes each time after the application of primary antibody. Sections were incubated for one hour with 10μL of biotinylated secondary antibody at a concentration of 1μg/mL (alpha-rabbit IgG made in goat, Vector laboratories).

ABC Horseradish peroxidase application

1- ABC solution was mixed and allowed to stand for 15 minutes prior to use. ABC solution consisted of 1 μL avidin (solution A) in 998 μL of 10% lamb serum/PBS with 1μL botinylated horseradish peroxidase solution (solution B).

2- Slides were washed 3 times with PBS

3- Sections were incubated with 100μL of 1:1000 ABC solution at room temperature for 60 minutes

4- Slides were washed with 3 changes of PBS for 5 minutes each

Chromagen visualization

1- Diaminobenzidine tetrahydrochloride (DAB) solution consisted of 100 mL 0.1 M tris, pH 7.6; 11.1 mM D-glucose; 7.5 mM NH_4Cl; 60 units glucose oxidase; and 1.16 mM DAB

2- Slides were incubated in solution at room temperature for 5-10 minutes

3- The AEC substrate kit for peroxidase was prepared as follows:

   a. 2 drops of Buffer Stock solution was added to 5 mL of distilled water and mixed well

   b. 3 drops of AEC stock solution were added and mixed well

   c. 2 drops of hydrogen peroxide solution was added and mixed well

   d. Tissue sections were incubated with the substrate at room temperature until suitable staining developed. Development times varied based on the tissue thickness and were determined by incubating sections 2 minutes at a time, stopping the reaction in distilled water and then viewing of the colored reaction product under light microscopy

Counterstaining after immunostaining

Counterstaining after immunostaining was performed with Hematoxylin alone in order to show the morphology of the labeled cells in relation to surrounding myocardium. The procedure is as follows:

1- Distilled water rinse	6 dips
2- Harris' Hematoxylin	Up to 5 minutes
3- Tap water	2 minutes or until clear
4- Acid alcohol	Dips
5- Tap water	2 minutes
6- Ammonia water	7 dips
7- Tap water	2 minutes

8- Slides were then dried and coverslipped with Permount mounting medium.

Cells derived from the implanted MSCs or fibroblasts (lac-Z labeled) were identified by their blue nuclei under Olympus light microscopy (BX41-TF, Olympus, Tokyo, Japan). Digital images were then transferred to a computer equipped with Image Pro Software (Media Cybernetics, Silver Spring, MD).

Furthermore, the fluorescent in situ hybridization technique (FISH) was used to confirm our results by allowing the detection of nucleic acid sequences specific to the human Y chromosome. The FISH procedure has been previously described[29].

In brief, the paraffin slides were baked at 60^0C for 2 hours, and after removing the paraffin, the rehydration process was performed using 95%, 70% and 50% ethanol respectively. The slides

were microwave-treated in citrate buffer (10 mmol/L, pH 6) for 1.5 minutes at 750W followed by 4.5 minutes at 50W. A mixture of 0.5 mL pepsin and 50 mL of HCl (150 μg/mL) was applied to each slide and incubated at 37^0C for 20 minutes and in 2 x sodium citrate-sodium chloride buffer (SSC) with 0.1% igepal for 10 minutes. The slides were then incubated in 1% formaldehyde/50mmol/L $MgCl_2$/PBS for 10 minutes at 20^0C and in PBS for another 5 minutes, following by dehydration. Then 3μL FISH probe cocktail (Vysis Inc, Downers Grove) was applied to each section. After this step, simultaneous denaturation of probe and target DNA was carried out at 74^0C for 10 minutes. The slides were then incubated overnight in a humidified chamber at 39^0C to allow hybridization of probe and target DNA. After several washes, the remaining probe molecules were stained with antifade containing 4,6-diamino-2-phenylindole (DAPI). Slides were then examined by an independent observer using an Olympus fluorescent microscope (Tokyo, Japan).

PCR analysis

Random samples from group I and IV were also processed for PCR analysis to confirm the survival of the implanted male cells into female hearts at different time intervals. This method was previously shown to be highly sensitive and specific for the detection of viable transplanted cells[30].

Genomic DNA was purified using Dneasy (Quiagen, Valencia, CA) according to the manufacturer's instructions and the presence of living human male cells in female hearts was confirmed by targeting a specific microsatellite sequence within the human Y chromosome (*DYS390*). The primer pairs used were TATATTTTACACATTTTTGGGCC and TGACAGTAAAATGAACACATTGC. A pair of primers for rat aconitase gene was used as a

control (forward: 5'-TTTCAAACCCTGTCAACAAATG-3'; reverse: 5'-CTTCCAAGTGAGCGAAGACC-3') in parallel PCR reactions and genomic DNA from both male human MSCs and infarcted female rat heart tissues with no MSCs implantation were processed as a positive and negative control respectively.

The PCR reaction mixture contained 150nM of each primer and 100 ng of sample DNA template with the following thermal protocol: 5 minutes activation step at 95°C for the HotStarTaq *Plus* DNA polymerase (Quiagen, Valencia, CA) followed by 35 cycles of denaturation (94°C for 30sec), annealing (58°C for 30 sec) and extension (72°C for 1 min) as well as a final extension step (72°C for 10 min).

Statistical Analysis

All data are expressed as mean ± SEM. Repeated echocardiographic variables at 4-5 days and after 8 weeks were compared by means of 1-way repeated-measures analysis of variance (ANOVA). If a significant F ratio was obtained, a Bonferroni post hoc test was used to assess pair-wise differences. A value of $P<0.05$ was considered statistically significant.

Chapter 3

RESULTS

MSCs culture

MSCs (rat and human) proliferated in the culture medium, adhered to the bottom of the culture dish, and developed spindle-shaped morphology (**figure 3**). They were observed under phase microscopy and assessed for proliferation and morphology at each medium change. To trace the fate of MSCs after implantation, the cells were transfected with replication-defective retrovirus carrying the *lacZ* reporter gene. B-galactosidase staining *in vitro* demonstrated that transfection efficiency was nearly 100% and 80% in rat and human MSCs respectively (**figure 4**).

Mortality and sample size

The overall mortality was 20% (18/90) occurring mainly during the first 48 hours after coronary ligation. There was no significant difference in the mortality rate among the different groups. No late postoperative death was observed. Furthermore, there were neither transplant-related mortalities nor morbidities associated with immunorejection.

Echocardiographic measurements were done in 43 rats, histological and immunohistochemical analyses in 72 rats and PCR in 20 rats. All rats had an EF < 45% after ligation and were all therefore included in the analyses.

Histologic and immunohistochemical assessment of engrafted cells

Gross examination of the infarcted hearts revealed a fibrous scar in the left ventricle that was clearly delineated from the normal myocardium. After x-gal staining, all hearts in group I and II revealed sparse areas of blue discoloration suggesting the presence of labeled cells. This was in contrast with the hearts in the control group **(figure 5).**

As could be expected, isogenic rat MSCs were shown to engraft within the injured rat myocardium **(figure 6A).**

Histologic examination of serial sections of group I confirmed the successful engraftment of human MSCs within the xenogeneic environment at 1, 3, 6 and 8 weeks after cell implantation **(figures 6 and 7).** This finding was primarily assessed by histochemistry and confirmed by FISH analyses **(figure 8).**

It is of interest to note that at 1 and 3 weeks, the MSCs were scattered throughout the ventricle having different shapes and sizes **(figures 6B and 7A).** However, at 6 and especially at 8 weeks, some transplanted cells started to acquire a more elongated and mature phenotype and to align with other host cardiomyocytes **(figures 6C and D).** Furthermore, positive connexin-43 staining was found between grafted cells and neighboring host cardiomyocytes **(figure 7D).**

Although a mild inflammatory reaction was seen as expected in all groups at an early stage, no significant inflammatory response suggestive of immune rejection remained in any cross-sections of group I after 1 week, even after the expression of cardiac-specific markers. This was in contrast with Group IV where extensive mononuclear cellular and macrophage infiltration was noted early after xenogeneic fibroblast implantation **(figures 9A-C)**. This was accompanied by a rapid loss of labeled fibroblasts with time, with none remaining 8 days after ligation.

PCR results

In addition to Xgal staining, we confirmed the survival of human MSCs within the xenotransplant environment with PCR, targeting a specific region within the human Y chromosome. Genomic DNA purified from the recipients' left ventricles was amplified and the

DNA product was detected at all time points in all samples that were randomly harvested from rats in group I **(figure 10).** This was in contrast with hearts from group IV where human fibroblasts were implanted. In this group, no gene product could be amplified beyond 8 days. This finding correlated with the histological results. Furthermore, as expected, genomic DNA from female rats (group III) produced no detectable PCR product.

Assessment of cardiac function

The left ventricular ejection fraction (EF), fractional shortening (FS) and left ventricular end-systolic (LVESD) and end-diastolic diameters (LVEDD) measurements were not significantly different among the 3 groups preoperatively. However, it should be noted that this model resulted in severe cardiac injury with reductions in contractile function and dilatation of the left ventricle noted in all groups to a similar degree 3-4 days after infarction **(figure 11).**

Six to eight weeks after cell implantation, a significant improvement on the EF and FS was observed in groups 1 and 2 compared with the control group ($p<0.001$) indicating a beneficial effect of transplantation **(figure 12).** Furthermore, a significant increase in the ejection fraction from 35.2%±5.5% to 43.8%±6.1% ($P<0.001$), and in fractional shortening (15.1%±2.8% to 17.4%±3.6%; $P=0.04$) were noted in group I over time. Similar changes were seen in group II. In contrast, there was a reduction in the ejection fraction (36.7%±4.1% to 25.6%±5.7%; $P=0.002$) and fractional shortening (14.7%±2.1% to 9.5%±1.1%; $P=0.002$) in group III.

Although the LV dimensions were similar in all groups 3-4 days after ligation, they continued to worsen over time in group III whereas these parameters were steady in the transplanted groups indicating a favorable effect on LV remodeling **(figure 12).** This was also apparent on

gross morphology **(figure 13).** Although a trend could easily be observed in both diameters in the transplanted groups, statistical significance was reached with only LVESD.

Chapter 4

DISCUSSION

The major findings of this study were that 1) human MSCs implanted into infarcted rat myocardium survived for at least 8 weeks, as demonstrated by histochemistry, and confirmed by *in situ* hybridization and PCR analyses; 2) no significant inflammatory reaction was seen in the MSC-transplanted rats, despite the lack of any immunosuppressive therapy; and 3) the implanted MSCs significantly contributed to the improvement in ventricular function and attenuated LV remodeling.

Survival of Implanted Cells

Perhaps the most significant observation in this study was the successful engraftment and survival of human MSCs in a xenogeneic immunocompetent environment for at least 8 weeks after implantation. It is important to emphasize that clinically, we would still favor the use of allogeneic MSCs for cellular transplantation, thus to avoid risks such as trans-species viral infections. However, we decided to confirm their immunotolerance property in an extreme xenogeneic mismatch model, since this would be immunologically more challenging.

We previously reported the formation of stable cardiac chimera in a mice-to-rat[22,31] and pig-to-rat[23] models. However, in this current study, we used a clonally homogeneous population of human MSCs, fully characterized by specific cell surface markers and by their confirmed potential to differentiate *in vitro* into multiple cell lineages[28].

In accordance with our previous results using mice[22,31] or pig MSCs[23], no significant inflammatory reaction was observed in the MSC-transplanted groups. This also confirms the *in vitro* findings reported by Grinnemo et al. where human MSCs were tolerated when co-cultured with rat lymphocytes[32]. However, this was in contrast with our immunological control group where human male fibroblasts were implanted. In this case, and as expected, a massive

macrophage and monocellular infiltration was seen early after implantation and the human fibroblasts were rapidly rejected and eliminated.

Extent of differentiation

In this current study, we again confirmed the importance of the microenvironment to supply the proper conditions for cardiomyocytic differentiation of the MSCs. These results are consistent with previous observations reported by our group[6,8,26] and by other laboratories[2,4,7,9,28,33,34]. It is of interest to note that at 3 weeks, none of the β-gal$^+$ cells expressed cardiac-specific markers. At 6 weeks, some transplanted cells were shown to acquire a more mature elongated phenotype and to better integrate within the cardiomyocytic network. This was more apparent at 8 weeks after transplantation when some cells started to express cardiac-specific markers and to develop connexin 43-positive gap junctions with other host cardiomyocytes. Although the extent of complete differentiation was not observed in all the slides, some transplanted cells seemed to acquire with time a more mature phenotype, appearing more rod-shaped with a centrally located nuclei and aligning themselves within the muscle fibers.

Orlic et al.[2] reported that intramyocardial injection of bone marrow-derived cells led to the regeneration of >60% of contracting myocardium. Similarly, after transplanting MSCs into rat myocardium, Tomita et al.[7] reported the expression of cardiac-specific proteins 8 weeks later. This was also confirmed by Toma et al.[33] and Min et al.[34] after implanting human MSCs in immunosuppressed rat and pig model, respectively. In contrast, Balsam et al.[35] and Murry et al.[36] reported fusion of implanted cells with the host cells, without evidence of donor cell differentiation. Thus, whether MSCs could fully differentiate into cardiomyocytes remains

controversial. It may be noted, however, the cells used by Balsam et al and Murry et al appear to be the CD 34-positive hematopoietic stem cells (HSCs), while our cells are CD 34-negative MSCs obtained from the marrow stroma, which characteristically adhere to culture dish, in contrast to the HSCs.

Our results also confirmed *in vivo* the concept that the "milieu-dependent differentiation" may not be species specific. Fukuhara et al.[37] had shown that co-culture of mouse MSCs with rat cardiomyocytes could successfully induce the former to undergo cardiomyocytic differentiation.

Our results reported here were in contrast with those of Grinnemo et al.[32] who similarly transplanted human MSCs into infarcted rat myocardium. In their study, human MSCs could not be detected 1 week after implantation and a massive infiltration was observed in the immunocompetent rats. However there seems to be some subtle differences in our experimental designs. For example, in their study, MSCs were harvested from the sternum of patients undergoing cardiac surgery[32]. Such patients tend to be older and sick. A number of recent studies have shown that MSCs harvested from elderly patients and from patients with coronary artery disease exhibit a lower capacity for differentiation, survival and proliferation[14-17]. In our study, human MSCs were collected from young healthy donors with no history of cardiac or other systemic diseases. In fact, the same group had previously demonstrated the immunotolerant properties of human MSCs in several *in vitro* studies[19,20,38]. It is of interest to note that in all these studies, as well as in the *in vitro* studies by others [39], human MSCs used

were harvested from young healthy donors. Still, further studies to clarify reasons for such contradictory findings will be highly desirable.

The immunotolerance property of MSCs

Our findings of MSCs immune privilege are consistent with many recent observations made in the *in vitro* mixed lymphocyte co-culture studies[19-21]. In addition to being hypoimmunogenic and expressing low levels of MHC antigens and co-stimulatory molecules[40], both differentiated and undifferentiated MSCs have been shown to suppress allo- and xeno-reactive lymphoproliferative responses and to modulate T and NK cell activity by altering the cytokine secretion profile of antigen-presenting cells[19,30,41]. The secretion of anti-inflammatory cytokines may also augment the immunosuppressive effects of regulatory T cells. In addition to this *in vitro* evidence, there is mounting observations that MSCs are immuno-privileged cells *in vivo* as well. The injection of allogeneic MSCs in baboons was tolerated without immunosuppression[42], was shown to prolong skin graft survival[21], to alleviate autoimmune disorders[43], to engraft in the brains of albino rats[44] and to reduce severe graft-versus host disease during bone marrow allotransplantations[45]. Furthermore, Liechty et al. reported the survival of human MSCs in fetal sheep and their differentiation along multiple lineages, even after the development of immune competence[46]. Additionally, our group had reported previously similar results in mouse-to-rat[22] and pig-to-rat[23] xenotransplant models.

Effects of MSCs on Cardiac Function

In the present study, MSCs transplantation was accompanied by a significant improvement in ventricular function which was greater in the MSC-transplanted groups at all time points.

These results extend the previously reported findings of MSCs transplantation in several other studies in animal models[6,9,34] and in humans[10-13]. Although the exact mechanism for improved heart function was not determined in this study, it has been suggested that heart function might be improved by the contractile properties of the neocardiomyocytes[2,6], by preventing ventricular remodeling and dilatation[4], enhancing neoangiogenesis[3,4,7], and through other paracrine mechanisms altering the extracellular matrix and reducing scar formation and expansion[5,48]. Based on the small number of cells retained after implantation[49] and given the magnitude of the effects on ventricular function, it is likely that the improvement in regional function seen in our study probably resulted from a combination of these factors, although further mechanistic studies are definitively warranted.

Furthermore, our observations suggest that, in addition to the improved contractile function, MSCs contributed as well in attenuating LV remodeling and preventing LV dilatation as demonstrated by the relative stabilization of the LV dimensions in the transplanted groups over time. This is consistent with our previous findings[31] and may be related to the paracrine action of engrafted MSCs after myocardial infarction involving a number of angiogenic and growth factors, and the downregulation of proapoptotic proteins[5,47,48].

Study Limitations

This study has several limitations. Although we did not quantitatively assess cell survival after injection, we and others have previously demonstrated that a relatively small proportion of cells is retained after injection, mainly because of mechanical losses attributed to the injection of

cells into a beating heart[49]. This important issue is under intensive study by us to improve the efficiency of cell delivery.

Although we did not assess the question of chronic rejection, we confirmed the survival of these cells for up to 6 months after transplantation in a previous study implanting pig MSCs into rat myocardium[23]. Recently, a similar finding in an allogeneic model was reported also by Dai et al.[4]. Further studies will however be required to determine whether the survival of human MSCs and their beneficial effects are preserved with longer follow-up.

Conclusion

In summary, our present study is the first, to our knowledge, to confirm the *in vivo* immunotolerance property of human MSCs and their contribution in improving heart function in an extreme model of xenogeneic mismatch. By attenuating contractile dysfunction and pathologic remodeling, these cells significantly contributed to a remarkable recovery in ventricular performance after myocardial infarction.

The potential importance of these findings for the treatment of ischemic heart disease is apparent. In addition to their powerful replicative capacity, MSCs can easily be harvested from bone marrows, expanded ex vivo, and differentiated into many cell type lineages, if desired. Another recent and important attribute that was confirmed in this study is their unique immunotolerance property and their ability to be transplanted in an allo- or xenogeneic setting. We suggest that this unique attribute would allow these cells to be used as "universal donor cells" with fascinating therapeutic implications. From a clinical perspective, these cells could be harvested and mass-produced well in advance, tested for their functional capabilities, and

stored as a standardized cell population for immediate "off the shelf" use on any patient without delay following an acute myocardial infarction. Such logistic advantages are not available with the use of autologous MSCs which is currently the cell source of choice. Perhaps more importantly, since such allogeneic MSCs can be obtained from young healthy donors, they could be of great value in patients with genetic cardiomyopathies and in the elderly patients with ischemic heart disease or diabetes whose own MSCs could to be dysfunctional.

FIGURES

Figure 1

Experimental design

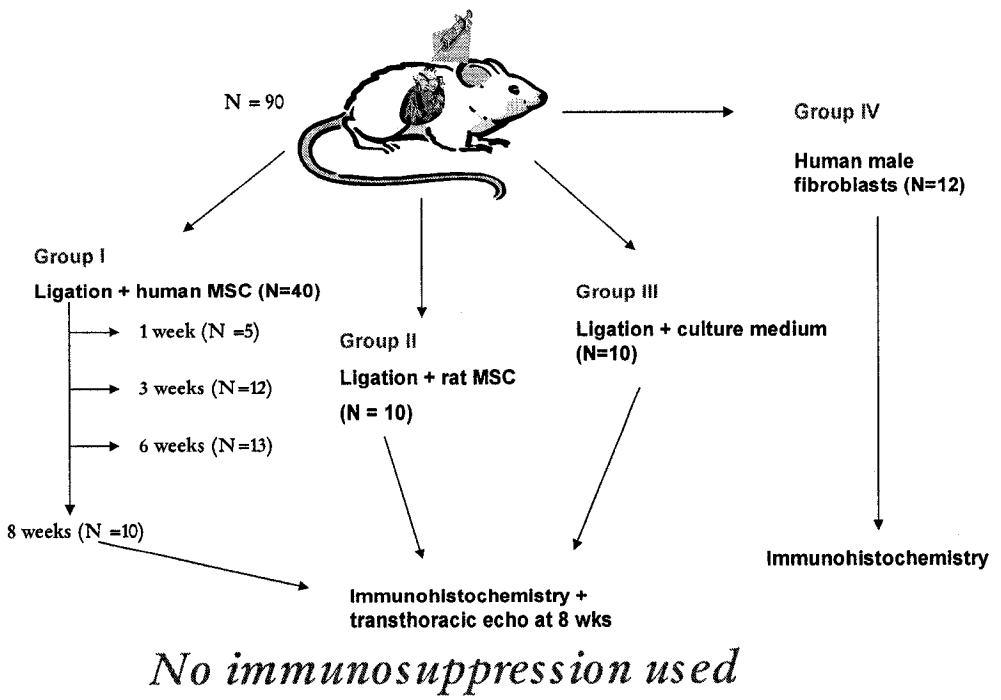

Figure 2

After exposing the heart via a left thoracotomy incision, the left coronary artery was ligated proximally with a 7.0 polypropylene suture (in blue). A pale infarcted area could be seen in the LV (white arrow).

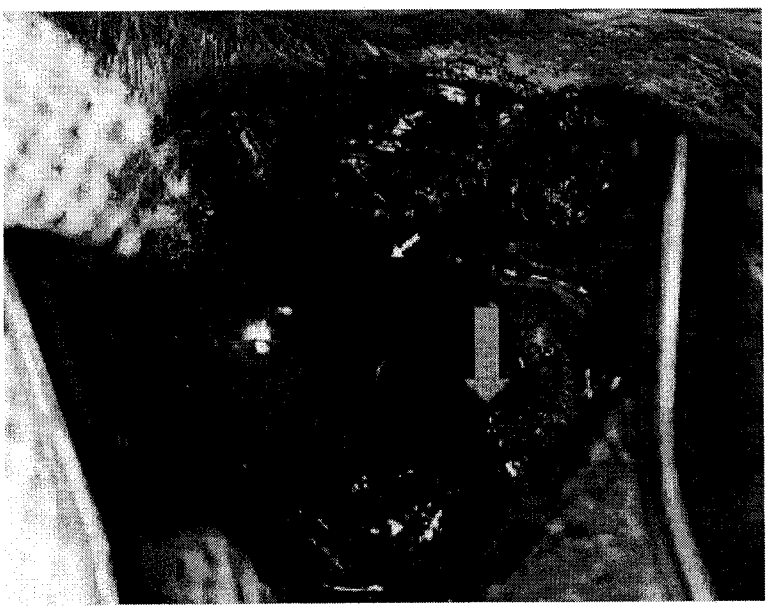

Figure 3

Spindle-shape appearance of MSCs in a culture dish

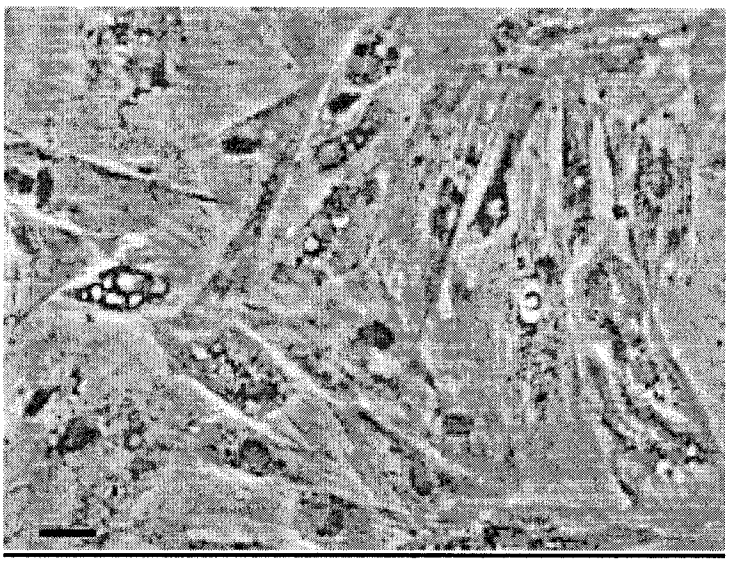

Figure 4:

X-gal staining of labeled human MSCs in culture flasks

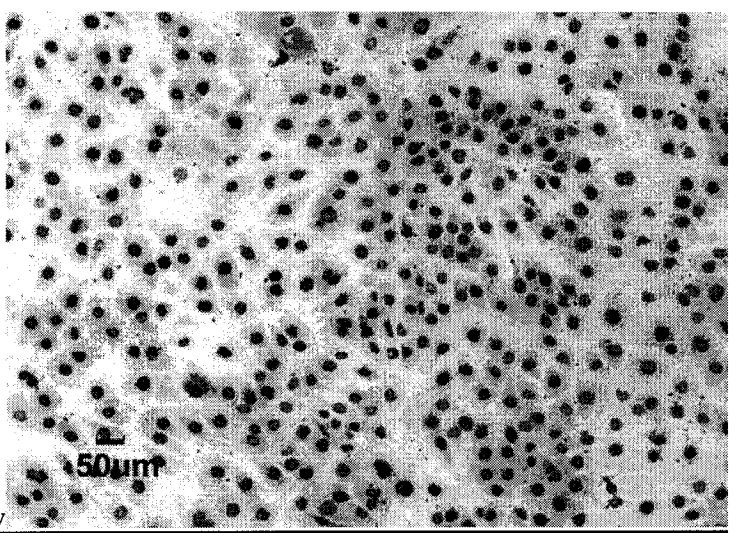

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Figure 5

Gross heart specimen after human MSCs implantation and staining for β-galactosidase activity. Note the blue discoloration seen around the infarcted area (**A**).

(**B**) represents a transmural scar lesion 1 week after coronary ligation (Original magnification x100).

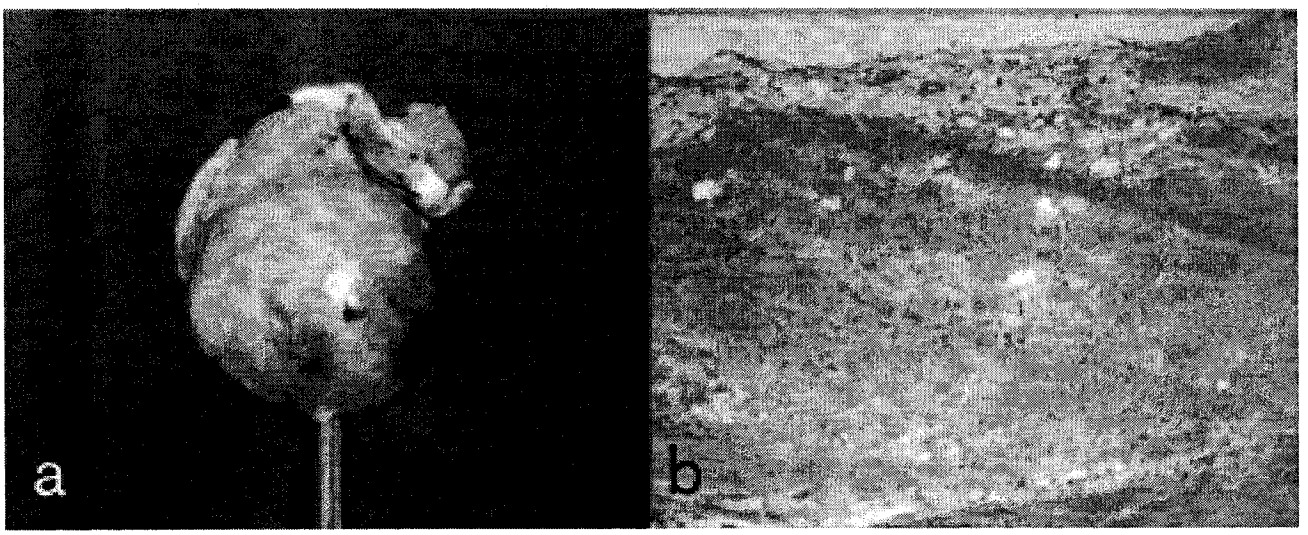


Figure 6

Representative sections (stained with H&E and X-gal) of rat myocardium with evidence of engraftment of human MSCs (in blue) harvested at 1(**B**); 6 **(C)** and 8 weeks (**D**) after coronary ligation. **A** represents a section with rat MSCs implantation.

At 6 and 8 weeks, β-gal positive cells were more elongated and aligned within the muscle fibers compared to the cells harvested at an earlier stage. Note the absence of any significant inflammatory reaction despite the lack of immunosuppression.

Original magnification: x 100 (A and B); x400 (C and D).

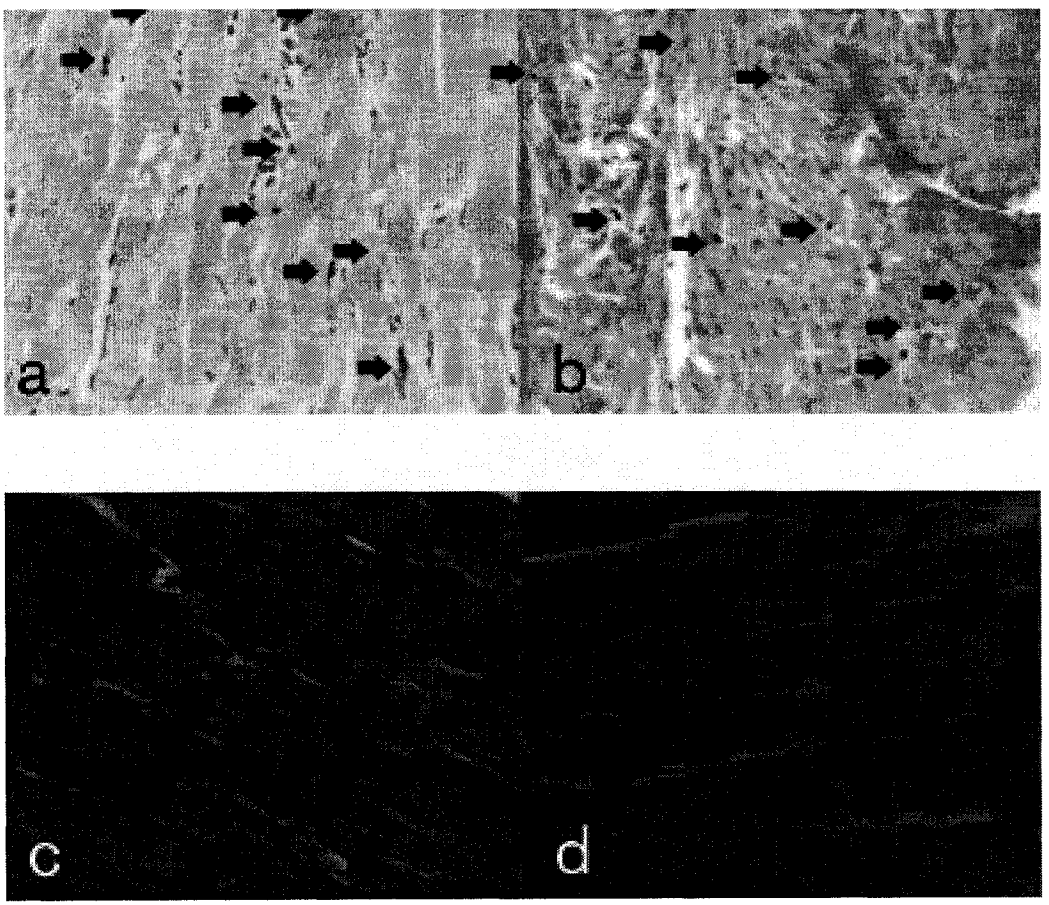


Figure 7

Sections of infarcted myocardium immunostained with antibodies against troponin IC (**A**) connexin-43 (**B-D**). Note the connexin-43 positive gap junctions (white arrows) between an engrafted cell and host cardiomyocytes (blue arrow) (**D**).

Original magnification: x 100 (A and B); x400 (C and D).

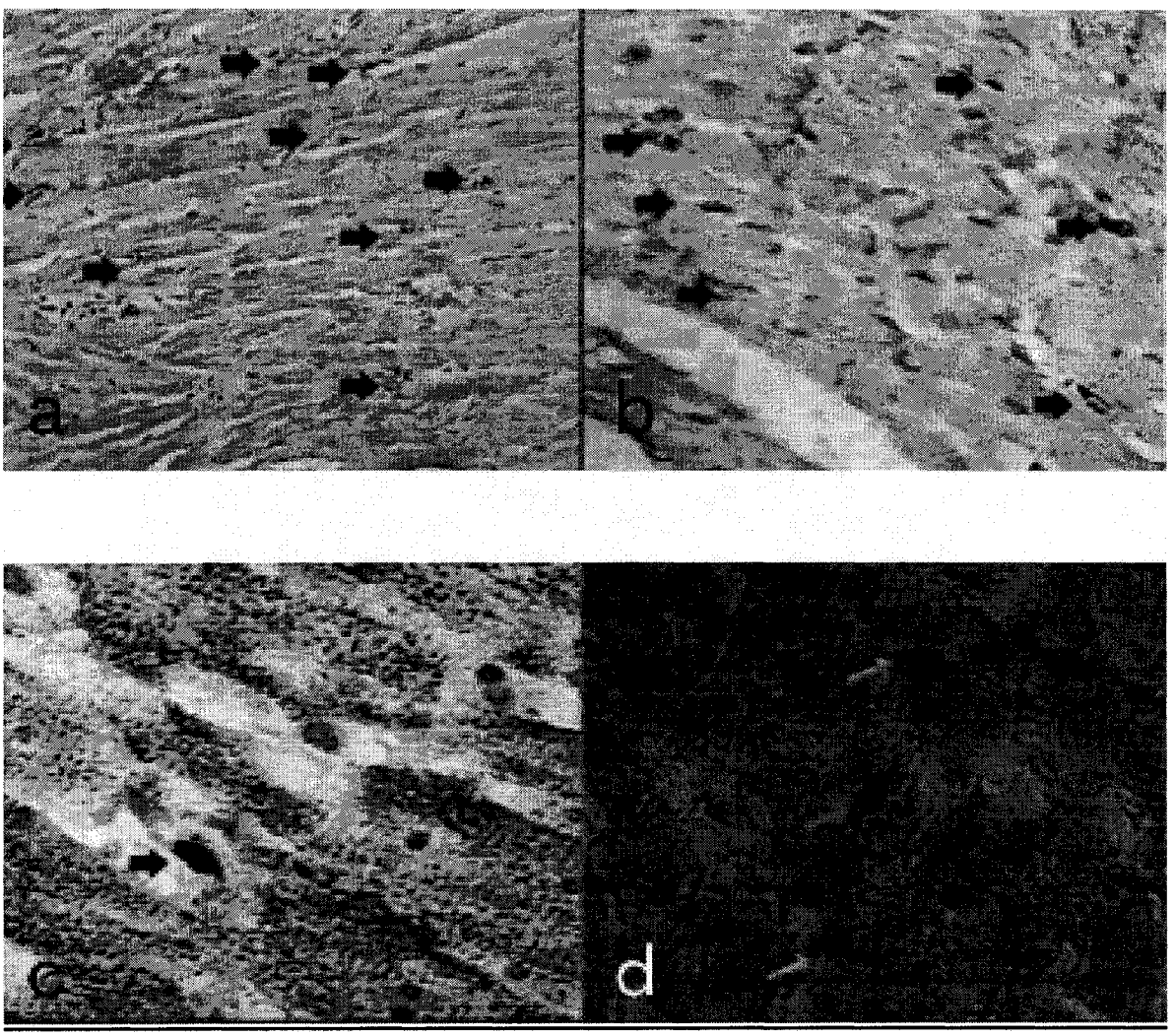


Figure 8

Sections taken 8 weeks after human MSCs transplantation. In situ hybridization of the Y chromosome in human MSCs stains red (x100).

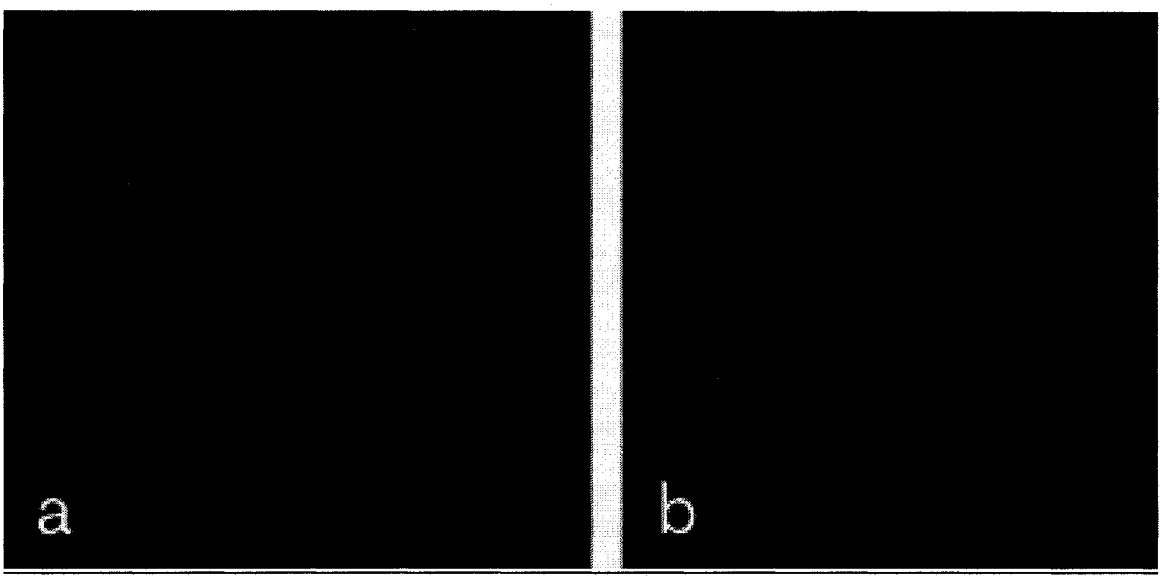


Figure 9

Extensive cellular infiltration noted at 3 (**A**) and 8 (**C**) days after transplantation of human fibroblasts. Only few surviving labeled fibroblasts were found at 8 days. Immunostaining with CD68 showing massive infiltration of macrophages (brown spots) at 5 days after injection of fibroblasts (**B**). This is in contrast to the minimal infiltration seen at 1 week after human MSCs transplantation (**D** (x100).

Arrows point to the cellular infiltration around the implanted cells.

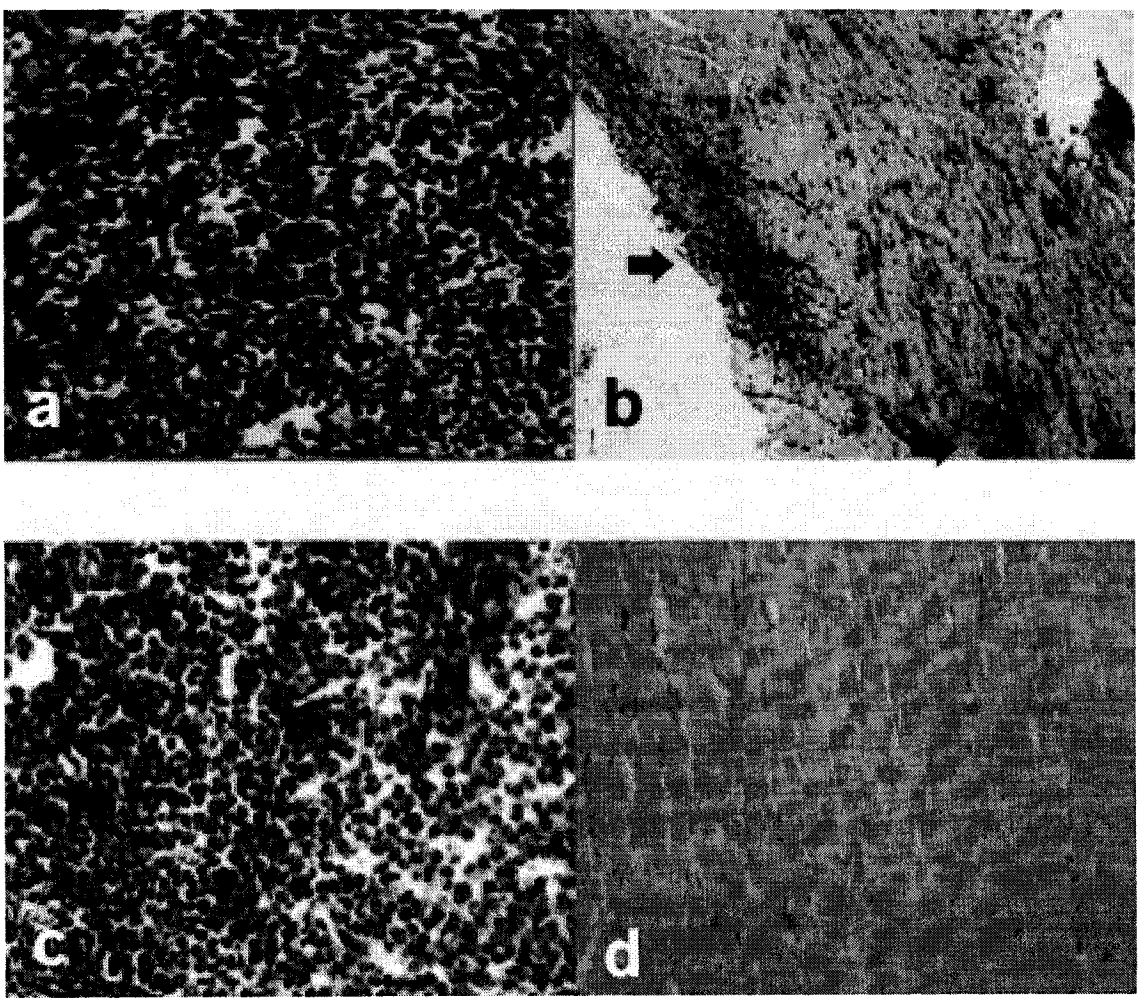


Figure 10

PCR product specific for the human Y chromosome (*DYS390* sequence). On the left, a positive band is seen in all the female rat hearts that were transplanted with human male MSCs at 6 (**T1,T2**) and 8 weeks (**T3-5**) after coronary ligation. Human male MSCs and myocardium from untreated female rats were used as positive (**P**) and negative (**U**) controls respectively.

On the right, a positive band is seen in rat hearts after human fibroblasts implantation at 3 (**F1**) and 5 days (**F2**) after ligation. A very light signal is seen at 8 days after ligation (**F3**). No signal is seen in the samples taken at 10 (**F4**) and 12 days (**F5**), suggesting complete rejection of the human fibroblasts. Human male skin fibroblasts and myocardium from untreated female rats were used as positive (**P**) and negative controls (**U**) respectively.

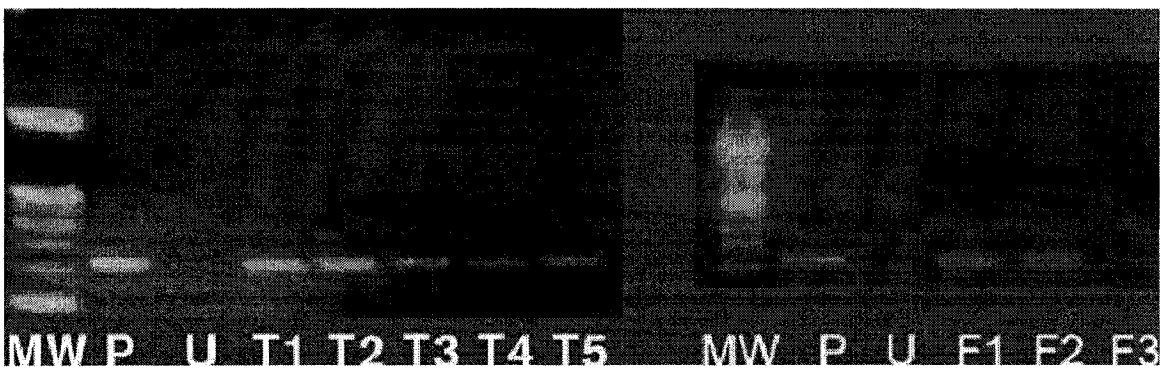
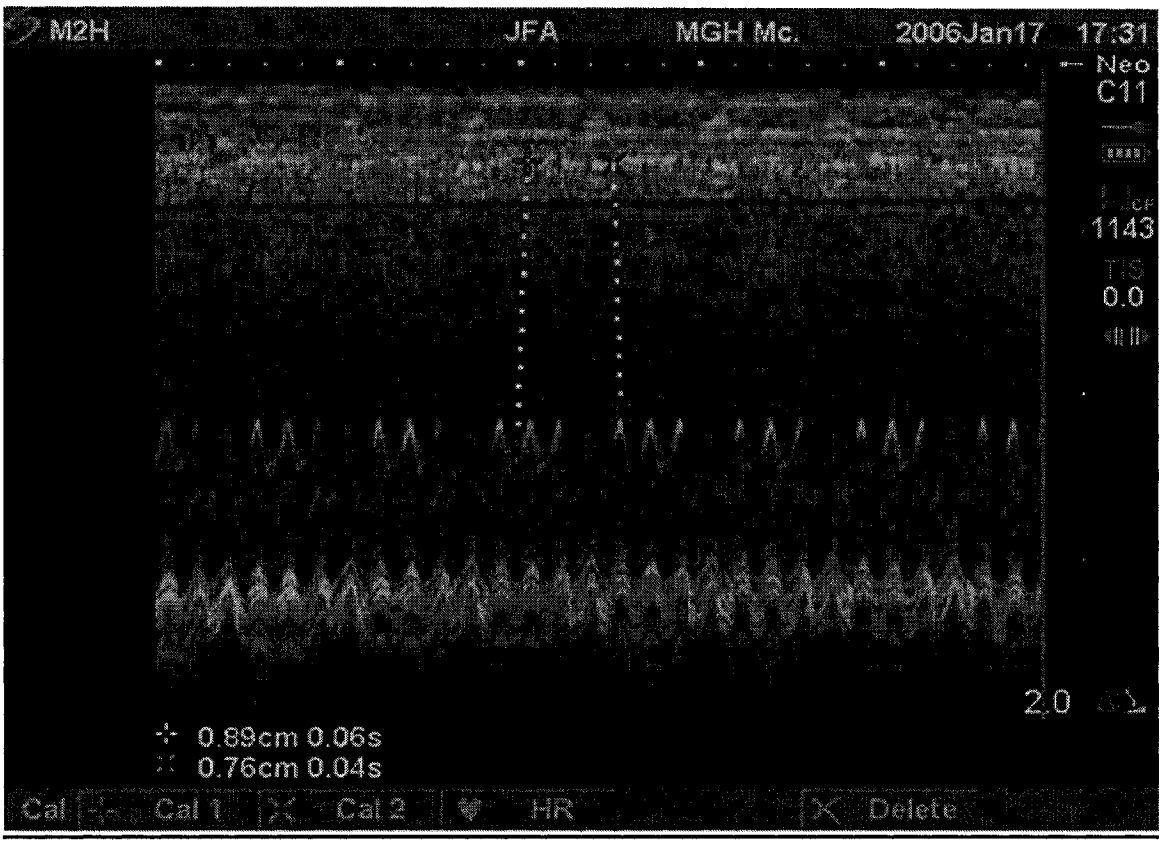


Figure 11

Representative echocardiograms of infarcted rat hearts with culture medium injection (top) and human MSCs implantation (bottom). Note the difference in the movement of the anterior wall.



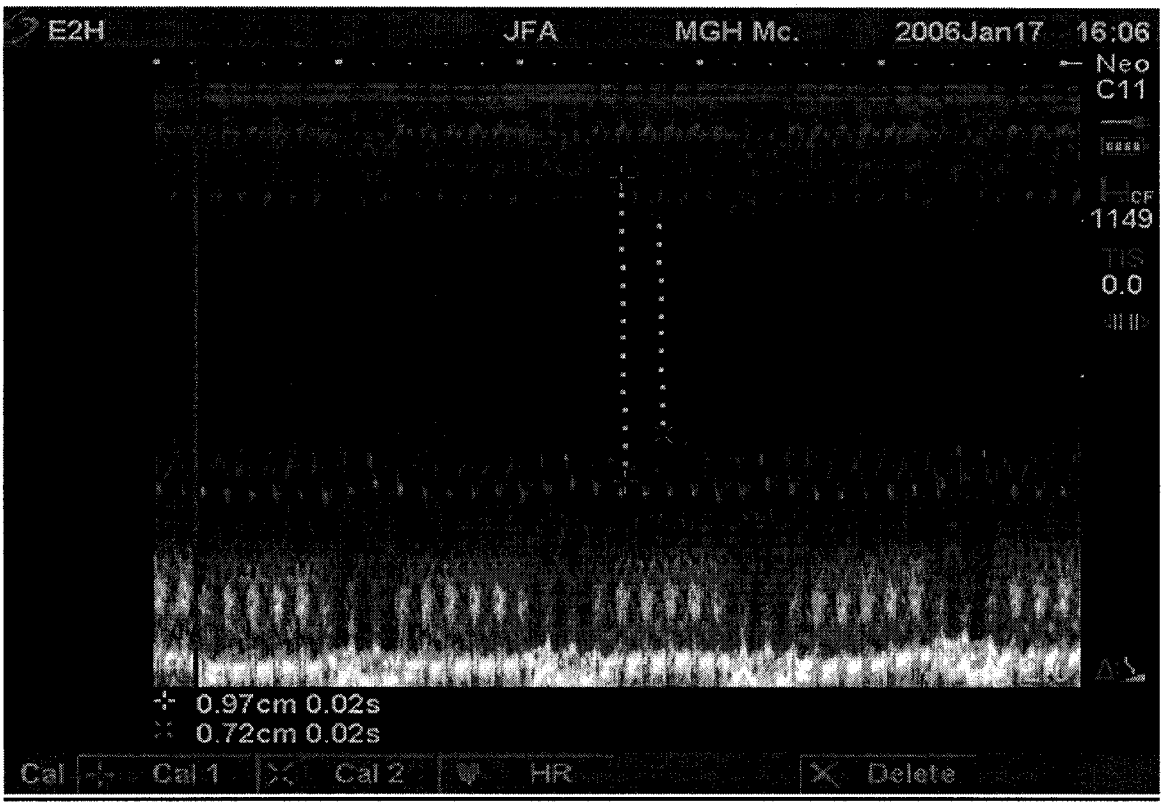

E2H
JFA
MGH Mc.
2006Jan17
16:06
Neo
C11
CF
1149
TIS
0.0
0.97cm 0.02s
0.72cm 0.02s
Cal
Cal 1
Cal 2
HR
Delete

Figure 12

Contribution of MSCs to ventricular function and LV remodeling. At 8 weeks after MSCs implantation, EF and FS were significantly higher in the MSC-transplanted groups (I and II) and continued to decline in the control group (III).

No significant changes in the LVESD and LVEDD in the MSCs transplanted groups over time, in contrast with the increase in both dimensions over time in the control group.

$p < 0.05$ when compared to group III at 8 weeks after ligation; † $p < 0.05$ when compared to results at 3-4 days post ligation. Data are represented as mean ± SEM.

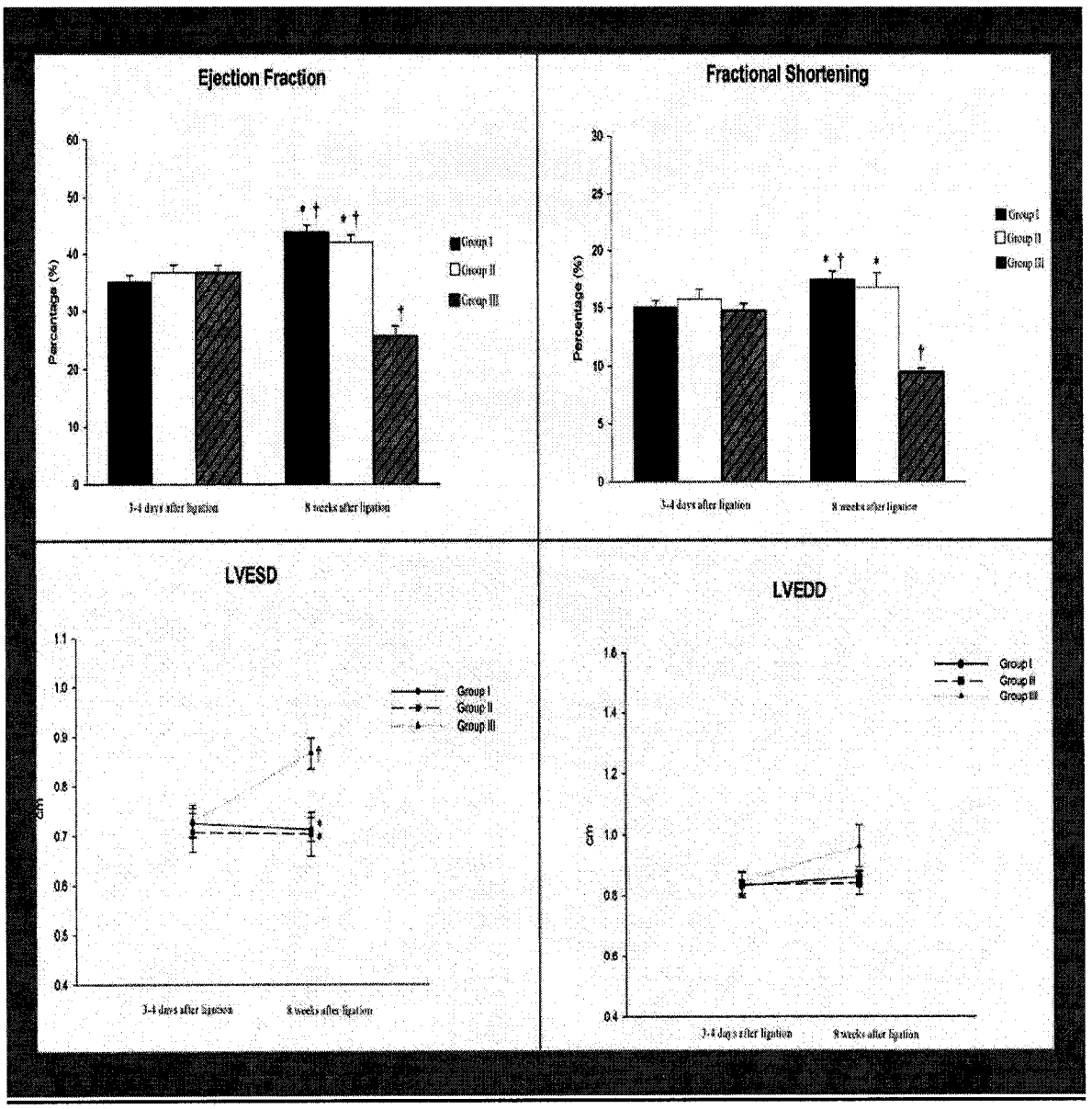
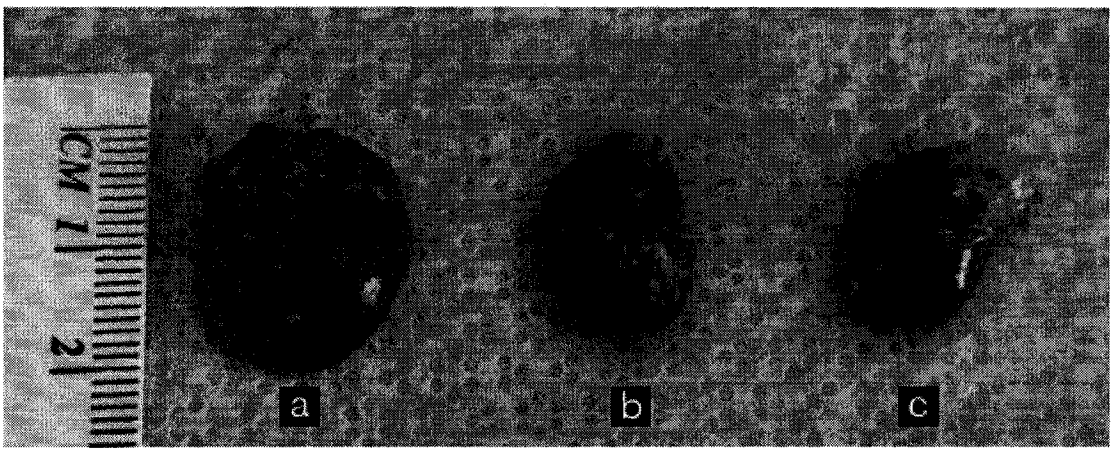

Ejection Fraction
Percentage (%)
Group I
Group II
Group III
3-4 days after ligation
8 weeks after ligation
Fractional Shortening
Percentage (%)
Group I
Group II
Group III
3-4 days after ligation
8 weeks after ligation
LVESD
cm
Group I
Group II
Group III
3-4 days after ligation
8 weeks after ligation
LVEDD
cm
Group I
Group II
Group III
3-4 days after ligation
8 weeks after ligation

Figure 13

Representative photomicrograph of infarcted heart specimens taken 8 weeks after culture-medium injection (**A**) and human MSCs transplantation (**B**). (**C**) represents a normal rat heart.



Chapter 5

FUTURE DIRECTIONS

Cellular therapy will definitely play a major role in the future clinical practice. The attractive characteristics of cells as tissue building units and the accumulating experience with cell culture, handling, and genetic engineering will promote cell therapy in various fields of medicine.

Recent clinical trials have shown the feasibility of adult autologous cell therapy in patients following a myocardial infarction. However, interventions aimed at enhancing donor cell retention, survival, homing and proliferation are still definitely required to achieve a better level of cardiomyocyte engraftment.

The multilineage potential of MSCs, their ability to elude detection by the host's immune system, and their relative ease of expansion in culture make MSCs transplantation a fascinating new approach for the management of heart disease. Ideally, MSCs can be harvested, expanded, and cryopreserved, ready for injection into any patient following an acute myocardial infarction.

Several unresolved questions are still however open for future research. In addition to defining which stem cell is best suitable, we must also define which patient groups are suitable for this therapy, and what is the optimum timing, the optimal angiogenic milieu, the dosage and the method of delivery. Furthermore, long-term side effects and arrhythmogenic potential are still unknown as most of the clinical studies are fairly recent. Further fundamental questions relating to the biology of MSCs are still unresolved. What are the specific signals and mechanisms involved in their homing, engraftment and differentiation? What are the exact mechanisms behind the improvement in ventricular function? What are the potential benefits of such therapy in nonischemic heart failure? In addition, rigorous criteria are needed to better

assess the efficacy of cellular cardiomyoplasty, as well as the long-term stability and function of the transplanted cells.

It is therefore clear that, in spite of the great promise of stem-cell cardiomyoplasty, many challenges remain to be solved. Investigations at both experimental and clinical levels are being pursued, and it is hoped that this fascinating therapeutic approach could prove to be a potent therapeutic tool aimed to deal with the failing human heart.

REFERENCES

1- Braunwald E, Bristow MR. Congestive heart failure: fifty years of progress. Circulation 2000; 102: 14IV-23

2- Orlic D, Kajstura T, Chimenti S, Bodine BM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 401: 701-705

3- Davani S, Marandin A, Mersin N, Royer B, Kantelip B, Herve P, Etievent JP, Kantelip JP. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation 2003; 108II-253-258

4- Dai W, Hale S, Martin B, Kuang JQ, Dow JS, Wold LE, Kloner RA. Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short and long-term effects. Circulation 2005; 112: 214-223

5- Tang Y, Zhao Q, Qin X, Shen L, Cheng L, Ge J, Phillips MI. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005; 80: 229-37

6- Saito T, Kuang J, Lin C, Chiu R. Transcoronary implantation of bone marrow stromal cells ameliorates cardiac function after myocardial infarction. J Thorac Cardiovasc Surg 2003; 126: 114-123

7- Tomita S., Li RK., Weisel RD., Mickle DA, Kim EJ, Sakai T, Jia ZQ. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999; 100:II-247-56

8- Bittira B, Shum-Tim D, Al-Khaldi A, Chiu RC. Mobilization and homing of bone marrow stromal cells in myocardial infarction. Eur J Cardiothorac Surg 2003; 24(3): 393-8

9- Kajstura J, Rota M, Whang B, Cascapera S, Hosoda T, Bearzi C, Nurzynska D, Kasahara H, Zias E, Bonafe M, Nadal-Ginard B, Torella D, Nascimbene A, Quaini F, Urbanek K, Leri A, Anversa P. Bone marrow cells differentiate in cardiac cell lineages after infarction independently of cell fusion. Circ Res 2005; 96: 127-137

10- Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) Circulation 2002; 106: 3009-17

11- Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H . Intracoronary autologous bone marrow cell transfer after myocardial infarction: the BOOST randomized controlled trial. Lancet 2004; 364: 141-8

12- Kang HJ, Kim HS, Zhang SY, Park KW, Cho HJ, Koo BK, Kim YJ, Soo D, Sohn DW, Han KS, Oh BH, Lee MM, Park YB. Effects of intracoronary infusion of peripheral blood stem-cells mobilized with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction; the MAGIC cell randomized clinical trial. Lancet 2004; 363: 751-6

13- Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, Yu J, Corti R, Mathey D, Hamm C, Suselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher A. Intracoronary bone-marrow derived progenitor cells in acute myocardial infarction. N. Engl J Med 2006; 355:1210-21

14- Heeschen C, Lehmann R, Honold J, Assmus B, Aicher A, Walter DH, Martin H, Zeiher AM, Dimmeler S. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. Circulation 2004; 109: 1615-22

15- Stolzing A, Scutt A. Age-related impairment of mesenchymal progenitor cell function. Aging cell 2006 1: 1-12

16- Scheubel R, Zorn H, Silber R, Kuss O, Morawietz H, Holtz J, Simm A. Age-dependent depression in circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting. J Am Coll Cardiol 2003; 42: 2073-80

17- Zhang H, Fazel S, Tian H, Mickle DA, Weisel RD, Fujii T, Li RK. Increasing donor age adversely impacts beneficial effects of bone marrow but not smooth muscle myocardial cell therapy. Am J Physiol Heart Circ 2005; 289: 2089-2096

18- Mieno S, Boodhwani M, Sodha N, Clements R, Ramlawi B, Feng J, Bianchi C, Selke FW. Circulation 2006; 114 (suppl): II-413

19- LeBlanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp. Hematol 2003; 31: 890-6

20- Rasmusson I, Ringden O, Sundberg B, LeBlanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation 2003; 76: 1208-1213

21- Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002; 30: 42-48

22- Saito T, Kuang JQ, Bittira B, Al-Khaldi A, Chiu RCJ. Xenotransplant cardiac chimera: immune tolerance of adult stem cells. Ann Thorac Surg 2002; 74: 19-24

23- Luo J, Shum-Tim D, Chiu RCJ. Marrow stromal cells as universal donor cells for cardiac regenerative therapy: Fact or fancy? In *Stem Cell Therapy in Ischemic Heart Disease*. Eds. Dimarakis I. et al. Imperial College Press, London, UK. *In press.*

24- Amado L, Saliaris D, Schuleri K, St John M, Xie J, Cattaneo S, Durand D, Fitton T, Huang J, Stewart G, Lehrke S, Baumgartner W, Martin B, Heldman A, Hare J. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci U.S.A. 2005; 102(32): 11474-9

25- Caplan AI. Mesenchymal stem cells. J Orthop Res 1991; 9: 641-50

26- Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RCJ. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. J Thorac Cardiovasc Surg 2000; 120: 999-1006

27- Pittenger MF, MacKay AM, Beck SC, Jaiswal R, Douglas R, Mosca J, Moorman M, Simonetti D, Craig S, Marshak D. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284:143

28- Davani S, Marandin A, Mersin N, Royer B, Kantelip B, Herve P, Etievent JP, Kantelip JP. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation 2003; 108II-253-258

29- Muller P, Pfeiffer P, Koglin J, Schafers H, Seeland U, Janzen I, Urbuschat S, Bohm M. Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. Circulation 2002; 106: 31-35

30- Muller-Ehmsen J, Peterson K, Kedes L, Wittaker P, Dow J, Long T, Laird P, Kloner R. Long-term survival of transplanted neonatal rat cardiomyocytes after myocardial infarction and effect on cardiac function. Circulation 2002; 105: 1720-6

31- MacDonald D, Saito T, Shum-Tim D, Bernier PL and Chiu RCJ. Persistence of marrow stromal cells implanted into acutely infarcted myocardium: Observations in a xenotransplant model. J Thorac Cardiovasc Surg 2005; 130: 1114-21

32- Grinnemo K, Mansson A, Dellgren D et al. Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. J Thorac and Cardiovasc Surg 2004; 127(5): 1293-1300

33- Toma C., Pittenger MF., Cahill KS, Byme B, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002; 105:93-98

34- Min JY, Sullivan M, Yange Y et al. Significant improvement of heart function by cotransplantation of humam mesenchymal stem cells and fetal cardiomyocytes in postinfarcted pigs. Ann Thorac Surg 2002; 74: 1568-75

35- Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weismann I, Robbins R. Hematopoietic stem cells adopt mature hematopoeitic fates in ischemic myocardium. Nature 2004; 428: 668-673

36- Murry CE, Soonpaa MH, Reinecke H et al. Hematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004; 428: 664-668

37- Fukuhara S, Tomita S, Yamashiro S et al. Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro. J Thorac Cardiov Surg 2003; 125: 1470-80

38- Rasmusson I, Ringden O, Sundberg B, LeBlanc K. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. Exp Cell Res 2005; 205:33-41

39- Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005; 105: 1815-1822

40- LeBlanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57:11-20

41- Beyth S, Borovsky Z, Mevorach D et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T cell unresponsiveness. Blood 2005; 105:2214-2219

42- Devine SM, Bartholomew AM, Mahmud N et al. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol 2001; 29:244-55

43- Kushida T, Inaba M, Takeuchi K, Sugiura K, Ogawa R, Ikehara S. Treatment of intractable autoimmune diseases in MRL/ipr mice using new strategy for allogeneic bone marrow transplantation. Blood 2000; 95: 1862-8

44- Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats: similarities to astrocyte grafts. Proc Natl Acad Sci U.S.A. 1998; 95: 3908-13

45- LeBlanc K, Rasmusson I, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004; 363:1439-1441

46- Liechty K, MacKenzie T, Shaaban A et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nature 2000; 6(11): 1282-6

47- Fazel S, Chen L, Weisel R et al. Cell transplantation preserves cardiac function after infarction by infarct stabilization: augmentation by stem cell factor. J Thorac Cardiovasc Surg 2005; 130: 1310-8

48- Gnecchi M, He H, Liang OD et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005; 11: 367-368

49- Teng C, Luo J, Chiu RC, Shum-Tim D. Massive mechanical loss of microspheres with direct intramyocardial injection in the beating heart: implications for cellular cardiomyoplasty. J Thorac Cardiovasc Surg 2006; 132(3): 628-32