Chapter 13
Mesenchymal Stem Cell Therapy for Heart Disease

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Abstract Mesenchymal stem cells (MSC) are adult stem cells with capacity for self-renewal and multi-lineage differentiation. Initially described in bone marrow, MSC are also present in other organs and tissues. From a therapeutic perspective, facilitated by the ease of preparation and immunologic privilege, MSC are emerging as an extremely promising therapeutic agent for tissue regeneration and repair. Studies in animal models of myocardial infarction have demonstrated the ability of transplanted MSC to engraft and differentiate into cardiomyocytes and vasculature cells. Most importantly, engrafted MSC secrete a wide array of soluble factors that mediate beneficial paracrine effects and greatly contribute to cardiac repair. Together, these properties can be harnessed to both prevent and reverse remodeling in the ischemically injured ventricle. In proof-of-concept and phase I clinical trials, MSC therapy improved left ventricular function, induced reverse remodeling, and decreased scar size. This chapter reviews the current understanding of MSC biology and mechanism of action in cardiac repair of MSC therapy for cardiac disease.

Keywords Mesenchymal stem cells • Cardiomyocyte • Cardiac repair • Paracrine effects • Exosomes

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13.1 Introduction

The ability to mobilize and activate endogenous stem/progenitor cells in diseased organs or to introduce exogenous stem cells for tissue regeneration/repair may impact many diseases, including those affecting the brain, skeletal muscle, pancreas, and heart. The reports that embryonic and adult stem cells (ASC) can differentiate into cardiomyocytes (CMC), vascular smooth muscle cells (VSMC), and endothelial cells (EC) have stimulated studies investigating the use of stem cells as regenerative therapy for cardiovascular disease. Regenerative and reparative therapies would be particularly important for heart disease since, despite many recent advances in medical therapy and interventional techniques, ischemic heart disease and congestive heart failure (CHF) remain major causes of morbidity and mortality [1, 2]. The current therapeutic approaches to treat congestive heart failure merely delay the progression of the disease [3], thus generating a population of chronically ill patients. Heart transplantation is the only effective therapy for this otherwise deadly clinical condition. However, the limited number of organs donated is not enough to treat all patients who would require a transplant. Consequentially, the disability of a growing number of people with heart disease will continue to place a heavy burden on an already financially strained health-care system, and the socioeconomic costs are incalculable. Cellular therapy for treating these and other heart conditions is a growing field of basic and clinical research. Here, we examine the basic science that is the foundation of future clinical approaches to ASC therapy for heart diseases. In particular, we will focus our attention on mesenchymal stem cells (MSC), describing in detail the mechanisms through which MSC can repair damaged hearts.

13.2 Background

Acute myocardial infarction (AMI) is caused by the abrupt closure of a coronary artery primarily due to thrombus formation. The most effective therapy for AMI is represented by timely revascularization of the infarcted related artery (IRA), obtained with thrombolytic agents, percutaneous coronary intervention (PCI), or bypass surgery. With the advent of reperfusion therapies, the institution of intensive care units and the introduction of effective drugs like beta-blockers and ACE inhibitors, the occurrence of complications in patients with AMI has been reduced and life expectancy improved. Despite all these advances, AMI still produces significant morbidity and mortality especially in those patients who miss the window of opportunity for timely reperfusion. In patients with significant infarct size, ventricular remodeling ensues and often leads to CHF. Recently, stem cell administration has been under investigation as a possible regenerative/reparative therapy for AMI. This strategy is based on the hypothesis that certain multipotent stem cell types, once injected into the heart, would be able to repopulate the necrotic tissue and differentiate into new CMC, thus rescuing contractile function. Stem cell therapy has been tested also in models of chronic myocardial infarction (CMI) and chronic ischemic heart disease (CIHD).

13.2.1 Pathology of Acute Myocardial Infarct: The Traditional View

Following AMI, CMC begin to die starting from the endocardium. If blood supply is not restored within the first 6 h, all the cardiac tissue served by the IRA undergoes necrosis or apoptosis. The loss of myocardium initiates a complex multicellular process to repair the damaged tissue and maintain the structural integrity of the left ventricle. Different cell types are chemo-attracted to the infarcted area and participate in tissue repair. Inflammatory cells rapidly infiltrate the area of injury to remove necrotic and apoptotic CMC, biologically active mediators are activated and released, new blood vessels start sprouting from the native vessels surrounding the infarcted area [4]. This early inflammatory phase is normally followed by a fibrogenic phase. Fibroblast-like cells first appear at the border zone and slowly invade the infarcted region leading to scar formation. The surviving CMC become hypertrophic to compensate the loss of contracting tissue. In humans, the healing process takes from 6 to 8 weeks and leads to progressive changes in ventricular size, shape, and function. Until recently, the remodeling of the left ventricle was believed to be irreversible since the heart was considered a post-mitotic organ without any self-renewal capacity. However, recent evidence of cycling CMC in the postnatal heart [5–7] and the discovery of resident cardiac stem cells (CSC) [8–10] together with the demonstration of bone marrow (BM)-derived stem cells able to home in the heart and transdifferentiate into CMC [11, 12] have challenged the classic dogma that the adult heart is a post-mitotic organ and suggests the fascinating possibility that therapeutic myocardial regeneration might be achieved.

13.3 Adult Stem Cells for Cardiac Repair

The hypothesis that tissue regeneration/repair may be achieved by cells circulating in the bloodstream was proposed as early as the middle of the nineteenth century by Cohnheim [13]. Subsequent studies on wound repair focused on cells resident in the tissues such as pericytes, which are seen to proliferate during repair in most tissues. More recently, resident stem cells were discovered in a variety of tissues including muscle [14], fat [15] and liver [16], strengthening the theory of local repair as the sole mechanism for tissue regeneration. However, the recent observations on stem cell plasticity have largely revitalized Cohnheim's hypothesis and suggested that the stem cells found in most tissues may be replenished by stem cells for non-hematopoietic tissues mobilized from the BM. For these reasons, starting from the late 1990s, the cardiac regenerative capacity of a variety of multipotent ASC harvested from different sources has been experimentally tested both in vitro and in vivo.

Among ASC, CSC seem to possess all the properties required in order to achieve true cardiac regeneration, since they are autologous, can be expanded ex vivo, show proliferative restraint, and, most importantly, show the ability to differentiate into

EC, VSMC, and CMC that appear to become functionally integrated with the surrounding native myocardium [9, 17–19]. Skeletal myoblasts (SM) have been investigated both in experimental and clinical studies. However, their use in cardiac regenerative therapy has been questioned [20–22]. More recently, stem cells resident in other tissues such as fat, cord blood, and placenta have shown to rescue damaged hearts in animal models [23, 24]. However, much of the research in cardiovascular regenerative therapies, both in animals and in human beings, has been conducted using BM-derived stem cells. In particular, it has been demonstrated that administration of BM-MSC can rescue damaged hearts and improve cardiac function in MI animal models and improve vasculogenesis in chronic ischemia models [25]. In the following paragraphs we will analyze basic concepts that explain the therapeutic properties of MSC.

13.4 Mesenchymal Stem Cells

The BM stroma was originally thought to function mainly as a structural support for the hematopoietic stem and progenitor cells in the BM [26]. It is now clear that a heterogeneous population of cells including fibroblasts, adipocytes, EC, osteogenic cells, and adherent stromal cells compose the stroma. In the 1960s Ernest A. McCulloch and James E. Till first revealed the clonal nature of marrow stromal cells [27, 28]. In the 1970s Friedenstein and colleagues reported an in vitro assay for examining the clonogenic potentiality of stromal marrow cells [29–31]. In this assay, stromal cells were referred to as colony-forming unit fibroblasts (CFU-F). Subsequent experiments revealed the multipotentiality of marrow cells and how their fate was determined by environmental cues [32]. For instance, culturing marrow stromal cells in the presence of osteogenic stimuli such as ascorbic acid, inorganic phosphate, and dexamethasone promoted their differentiation into osteoblasts [33]; in contrast, the addition of transforming growth factor-beta (TGF-β) induced differentiation into chondrocytes [34]. Furthermore, it has been shown that these cells can differentiate into adipocytes, tendons, and muscle [35, 36].

Since stromal cells showed self-renewal, differentiation, and characteristics typically associated with stem cells, many investigators referred to cultured stromal cells as MSC. These cells are rare and exist at an estimated frequency of about 1 in 100,000 BM cells [37]. However, the MSC can be isolated and expanded ex vivo, primarily taking advantage of their specific capacity to adhere to plastic surfaces. Briefly, the BM mononuclear cells are isolated using gradient techniques and plated in tissue culture-treated plastic dishes. By changing the culture medium, non-adherent cells are removed so that only the stromal cells remain in the dish. After few days, CFU-F start becoming visible. As for the endothelial progenitor cells (EPC), many investigators use the CFU assay as a method to quantify stromal progenitors. Interestingly, it appears that a strong correlation exists between age and proliferative potentiality, with decreasing progenitor proliferation associated with increasing age. The cells forming the CFU-F have already acquired the majority of the surface

markers thought to be typically expressed by the MSC. These cells can be expanded for many passages without altering their phenotype and biological properties.

MSC in cell culture have been characterized using a panel of specific antibodies; however, there is still a lack of consensus on the definition of MSC since the medium and serum used to culture the cells, the plating density as well as the oxygen tension may affect the phenotype. In general, it is well accepted that human MSC (hMSC) lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules while hMSC do express SH2 (CD105), SH3 and SH4 (CD73), CD90, CD29 and CD166 [32, 38] (Fig. 13.1). Aside from this consensus in terms of hMSC surface antigen expression, the precise phenotype of hMSC in human BM is still debated and the identification of hMSC prior to culture remains ambiguous. Several groups have developed protocols to prepare more homogeneous MSC populations, but none of them has gained widespread acceptance. Interestingly, Prockop and collaborators have reported the existence of a subpopulation of cells in cultures of hMSC that are small, proliferate rapidly, undergo cyclical renewal when the cells are replated, and are precursors of more mature cells in the same cultures. These cells were referred to as recycling stem cells (RS) [39].

Peculiar characteristics make MSC interesting for cell therapy and tissue engineering purposes. For example, MSC can be isolated, expanded ex vivo, and used in an autologous fashion, avoiding the problem of finding a compatible donor. Furthermore, several lines of evidence suggest that MSC may not be subject to allogenic rejection in human and animal models [37, 40, 41]. Three main mechanisms seem to contribute to such immunoprivileged profile. First of all, MSC are hypoimmunogenic since they lack HLA class II and co-stimulatory molecules expression. Secondly, it has been shown that MSC prevent a T-cell response indirectly through modulation of dendritic cells and directly by suppressing natural killer cells as well as $CD8^+$ and $CD4^+$ T-cell function. Thirdly, MSC induce a suppressive local microenvironment through the production of prostaglandins and interleukins. If it was confirmed that MSC truly avoid allogenic rejection, it would be reasonable to start thinking about the institution of an international cell bank of hMSC isolated from the BM of young and healthy subjects. However, other evidence has challenged such an optimistic view and urged for additional experimental studies [42]. Another advantageous characteristic of MSC is that they are easy to modify ex vivo using viral vectors [43]. By overexpressing genes of interest, the functionality of MSC can be increased. For instance, MSC overexpressing antiapoptotic genes have shown to be more resistant to hypoxic stimuli compared with non-modified MSC [44]. Furthermore, MSC might be used as platform to deliver specific soluble proteins to the site of injury. For example, it has been demonstrated that MSC overexpressing VEGF improve vascular regeneration compared with non-modified MSC [45].

Outside the BM, MSC have been recently isolated from many other tissues; among them, fat tissue, cord blood, and placenta are the most common [23, 24]. Circulating MSC have also been described but the results are debated and not always reproducible [46]. Verfaille's group has described a population of multipotent adult progenitor cells (MAPC), that share many of the same characteristics of MSC [47]. However, differently from MSC, MAPC are reported to expand indefinitely and

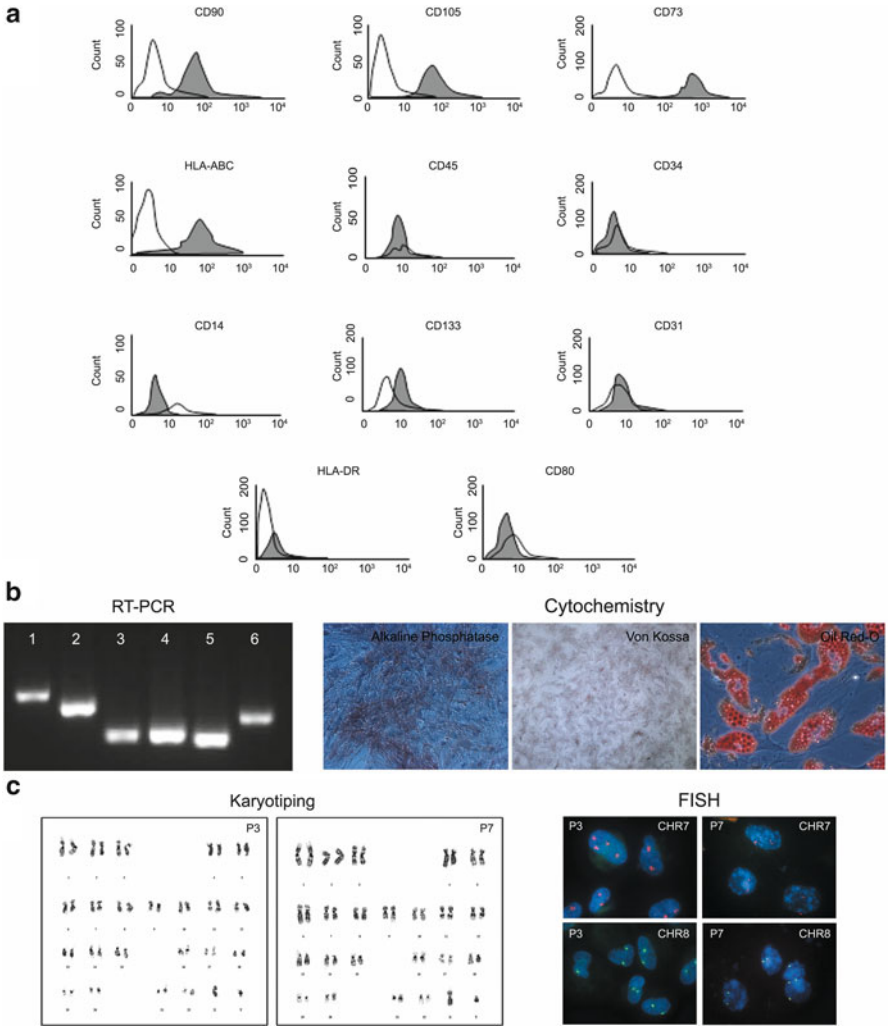

Fig. 13.1 Characterization of mesenchymal stem cells. (**a**) FACS analysis of MSC at P3 showing the typical antigen profile expressed by cells of mesenchymal origin. (**b**) MSC are multipotent. RT-PCR analysis (*left*) for osteocytes markers osteopontin (*1*), Cathepsin K (*2*) and Bone sialoprotein (*3*) or adipocyte markers ADFP (*5*) and PPARγ (*6*), show that using dedicated protocols it is possible to differentiate MSC into osteocytes and adipocytes. Glyceraldeyde 6-phophate dehydrogenase (*4*) was used as endogenous control. Cytochemical analysis (*right*) confirms the differentiation potential of MSC. The pictures show: alkaline phosphatase activity assay (phase contrast—10× magnification), Von Kossa staining (Bright field—2.5× magnification) and Oil Red O staining (phase contrast—40× magnification) after induction of MSC differentiation. (**c**) Karyotyping and FISH analysis of chromosome 7 (*red* signal) and 8 (*green* signal) with subtelomeric-specific probes in MSC at P3 and P7 does not show aberrations

appear to have an extended differentiation potential including ectodermal and endodermal lineages. Finally, a subpopulation of stem cells isolated at single-cell level and referred to as human BM-derived stem cells (hBMSC) has been identified [48]. The hBMSC self-renew without loss of multipotency for more than 140 population doublings and can differentiate into cells of all three germ layer.

13.5 Structural and Functional Effects of Mesenchymal Stem Cells on Infarcted Hearts

BM-MSC isolated from mouse, rat, swine, and humans have been administrated in experimental models of permanent coronary ligation, ischemia/reperfusion (I/R), and cryoinjury. The timing of administration varied from few minutes after injury to 4 weeks, when the acute inflammatory response to ischemia has subsided. Different routes of administration have been tested: direct intramyocardial injection, local coronary delivery, systemic intravenous infusion. A great variety of read-outs have been employed to quantitate the effect of stem cell transplantation into injured hearts. Traditional morphometric analyses documented an overall reduction in infarct size, less severe ventricular remodeling, and improved vascularization [49, 50]. Furthermore, BM-MSC administration ameliorated ventricular function in most cases. Cardiac performance in rodents has been measured both ex vivo, using Langendorff-perfused heart preparations, and in vivo, using intraventricular pressure transducers. Echocardiographic analyses have been performed to monitor function in both rodents and larger animals following cell transplantation. Additionally, ultrasonic crystals have been used to determine regional wall motion across the infarcted region of the heart of larger animals following cell transplantation.

These anatomical and functional assays demonstrated beneficial effects of BM-MSC but were unable to identify the underlying mechanism of stem cell action, that is distinguishing between direct function of the donor cells versus a beneficial effect imparted upon the surviving host myocardium. Understanding the mechanistic basis for limitation of ventricular remodeling and improved cardiac function is of critical importance when attempting to effect modifications aimed at enhancing the intervention. To determine the mechanism of action it is crucial to track the cells after transplantation and follow-up their fate in the heart.

13.6 Mechanisms of Action of Mesenchymal Stem Cells in Cardiac Repair

Transdifferentiation of transplanted stem cells into CMC and into vascular lineage cells has been originally proposed as the principal mechanism underlying the therapeutic action of MSC [37, 51, 52]. More recently, other investigators have failed to

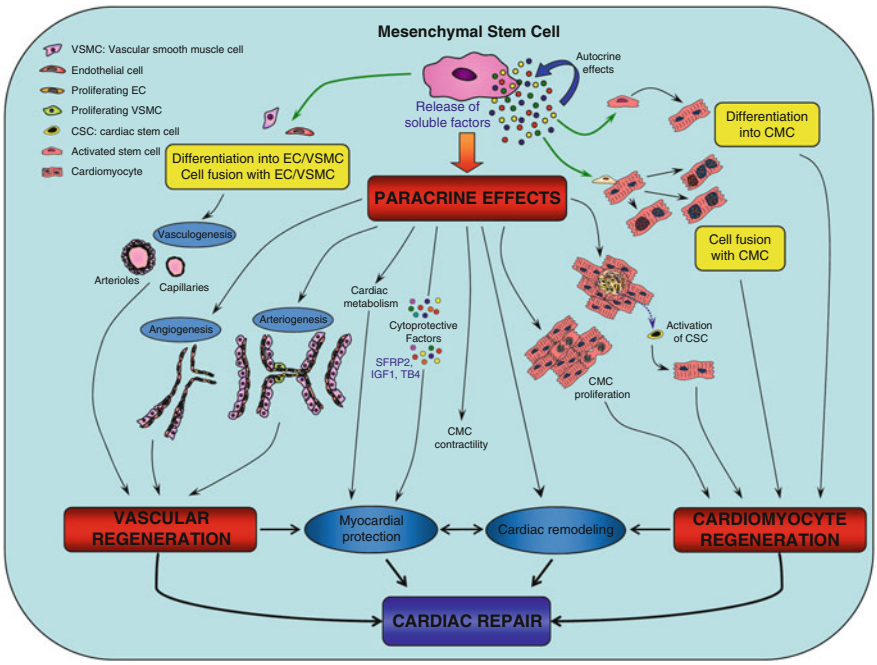

Fig. 13.2 Mechanisms of mesenchymal stem cell action in cardiac regeneration and repair. Cardiomyocyte and vascular regenerations represent the two mechanisms of action originally proposed to explain the reparative effects observed after MSC therapy in ischemic heart disease models. More recently, it has been demonstrated that soluble factors produced and released by MSC determine a series of beneficial paracrine effects, resulting in myocardial repair (see text for details)

detect permanent engraftment of transplanted BM-MSC [53, 54]. Furthermore, cell fusion of BM-derived donor cells with recipient CMC has been reported [55, 56]. Finally, so far it has not been possible to reproducibly induce a functional cardiac phenotype in BM-MSC in vitro using physiological growth factors or nontoxic chemical compounds. These negative results have questioned the plasticity of both endogenous and transplanted BM-MSC.

Regardless of whether stem cells transdifferentiate via a fusion-dependent or -independent mechanism, it has been shown that in many cases the number of newly generated CMC is too low to justify functional improvements. Therefore, it has been proposed that the functional benefits observed after MSC transfer in animal models of cardiac injury might be related to secretion of soluble factors that act in a paracrine fashion, protect the heart, cause attenuation of pathological ventricular remodeling, and promote neovascularization [57–59]. Accordingly, three mechanisms of action have been proposed for adult BM-MSC in heart repair: (1) CMC regeneration, (2) vasculogenesis, and (3) paracrine effects (Fig. 13.2). Regardless of the mechanism of action, there is a general agreement that BM-MSC transplantation is safe and has beneficial effects on infarcted hearts.

13.6.1 Cardiomyocyte Regeneration

Before examining the results supporting the cardiomyogenic potentiality of MSC, it is helpful to do a mathematical exercise in order to better understand what cell number is needed for the regeneration of myocardial infarct in humans. An adult heart contains approximately 20 million CMC per gram of tissue [60]. The average human left ventricle weighs ~200 g and therefore contains approximately four billion CMC. It has been estimated that the loss of 25 % of the left ventricle, corresponding to the loss of one billion CMC, leads to CHF [61]. Therefore, true cardiac regeneration would require restoring approximately one billion CMC synchronously contracting with the host myocardium. We anticipate that with the currently available technology this task is not achievable. However, substantial steps forward have been made and many investigators believe that this goal can be eventually accomplished. Several independent groups have reported cardiomyogenic differentiation of BM-MSC, both in vitro and in vivo [62–66].

13.6.1.1 Cardiac Differentiation In Vitro

Cardiomyogenic differentiation of mouse BM-MSC in vitro has been reported using culture medium supplemented with the demethylating agent 5-aza-cytidine (5-AZA) at a concentration of 3 μmol/l for 24 h [64]. Under these culture conditions, Makino and collaborators reported that the morphology of almost 30 % of the cells changed from fibroblast-like shape to a ball-like form and, with time, to the characteristic rod-shape myofibers. These differentiating cells tended to fuse in a syncytium resembling a myotube and started expressing fetal CMC markers [64]. In particular, the β-isoform of myosin heavy chain (β-MHC) was much more expressed than the α-isoform and specific transcription factors of the cardiac and myocyte lineage, including GATA-4, Nkx2.5, and HAND1/2 [62]. Furthermore, alternative splicing forms of the myocyte enhancer factor 2 (MEF2) gene were expressed by 5-AZA stimulated MSC. Indeed, MEF2A and MEF2B detected in early passage cells were replaced by MEF2C and MEF2D in late passage MSC, suggesting that the developmental program of gene expression would recapitulate the one observed during prenatal life. Electron microscopy revealed a CMC-like structure, such as the presence of sarcomeres, centrally positioned nuclei, and atrial granules. Importantly, it has been shown that MSC-derived CMC-like cells express functionally competent α- and β-adrenergic and muscarinic receptors on the membrane [63]. The differentiated cells are reported to beat spontaneously and synchronously in vitro and the rate of contraction increases after exposure to isoproterenol, whereas the addition of a selective β1 blocker inhibits contractile activity [63]. Other groups proposed that CMC-mediated contact is essential to induce MSC differentiation towards cardiac lineage [65, 66]. In one study hMSC were cocultured with human CMC in a ratio of 1:1 or cultured alone in the presence of medium conditioned by CMC. After 48 h of coculture, immunocytochemistry revealed that differentiating MSC expressed

sarcomeric myosin, β-MHC, cardiac troponin-T (cTnT), and cardiac troponin-I (cTnI) that were not expressed by the hMSC exposed to the conditioned medium [65]. The importance of cell-to-cell contact has been confirmed by Ashraf and collaborators, who cocultured MSC from green florescent protein (GFP)-transgenic mice with rat neonatal CMC [66]. After 7 days of coculture, ~14–32 % of MSC acquired the cardiac phenotype and started contracting synchronously with surrounding CMC. The presence of gap junctions between MSC-derived cardiac cells and neonatal CMC was documented by positive connexin-43 staining. Differentiation was confirmed by transmission electron microscopy analysis, showing a CMC-like ultrastructure, including sarcomeres, abundant glycogen granules, and a number of mitochondria. In a concomitant experiment, MSC separated from CMC by a semipermeable membrane did not differentiate into cardiac cells, confirming the pivotal importance of cell-to-cell contact.

13.6.1.2 Cardiac Differentiation In Vivo

Strong evidence in favor of MSC multipotency is derived from a study testing the fate of hMSC after systemic administration into fetal sheep early in gestation [67]. In this xenogenic system, hMSC engrafted and persisted in multiple tissues for as long as 13 months after transplantation. The cells underwent site-specific differentiation into chondrocytes, adipocytes, BM stromal cells, myocytes, and CMC. The presence of human cells was detected with an antibody specific for β-2 microglobulin or with in situ hybridization for human ALU sequences, and the differentiation into cardiac cells was established both by morphology and by staining with an antibody against SERCA-2. This study not only demonstrated that systemically administered hMSC can migrate across endothelial barriers, stably integrate into the heart, and differentiate into cardiac cells, but also that hMSC have unique immunologic characteristics that allow stable engraftment and the capacity to differentiate in a xenogenic environment. The cardiomyogenic potentiality of hMSC was further tested by Pittenger and collaborators, who injected LacZ labeled MSC into the left ventricular cavity of immunodeficient CB17 SCID/beige adult mice [51]. It was estimated that ~0.44 % of the injected cells survived in the myocardium 4 days after injection but much fewer hMSC were still present at later time points. The engrafted hMSC became with time morphologically similar to the surrounding CMC and aligned with them. Cardiac differentiation was confirmed by double staining with an anti-β-galactosidase antibody and specific cardiac markers. None of the hMSC expressed cardiac proteins 4 days after injection, but started to stain positive for desmin and cTnT at day 14. After 60 days the β-gal$^+$ cells also expressed α-actinin, β-MHC, and phospholamban. High-magnification view showed sarcomeric organization of the α-SA and cTnT positive cells, further demonstrating the ability of adult hMSC to undergo striated muscle differentiation in the heart.

Additional in vivo evidence of the cardiomyogenic potentiality of MSC came from Prockop's laboratory where the integration and differentiation of rat BM-MSC

were examined after transplantation into organogenesis-stage embryos [68]. GFP-tagged MSC were infused into 1.5- to 2-day-old chick embryos and surviving grafted cells were identified as GFP^+ cells 4 days after injection by quantitative PCR and immunohistochemical analysis. MSC expanded 1.3- to 33-fold in one-third of surviving embryos and integrated into multiple host tissues. In particular, the most common site of detection was the heart, even though the site of injection might have played a role in this particular model. Some of the GFP^+ cells found at the heart level expressed α-MHC and some cardiotin, a protein found in the longitudinal sarcoplasmatic reticulum of mature CMC. To exclude cell fusion as a mechanism of differentiation, the GFP^+ cells were retrieved by cell sorting from the dispersed embryos and karyotyping was performed. All the GFP^+ cells contained the normal complement of 42 rat chromosomes, and therefore they were distinctly different from chick cells that contain 78 chromosomes.

Taken together, these results support the concept that MSC can home to the intact myocardium and differentiate into cardiac-like cells. However, can MSC do the same in the presence of myocardial injury and regenerate the lost tissue? One of the most convincing answers to this question came from Fukuda's laboratory [69]. In a first set of experiments, a single hematopoietic $Lin^-CD34^-c\text{-}kit^+Sca1^+$ cell or BM-derived cells harvested from mice ubiquitously expressing GFP were transplanted into lethally irradiated syngeneic mice. AMI was then induced by coronary ligation and BM-derived cells were mobilized by granulocytes colony-stimulating factor (G-CSF) administration. Eight weeks after AMI only three $GFP^+/actin^+$ cells were found in the group transplanted with the single hematopoietic cell, while more than 5,000 $GFP^+/actin^+$ cells were detected in animals receiving BMC. These data suggested that most of the $GFP^+/actin^+$ cells were derived from non-hematopoietic BM-derived cells, most likely from MSC. To confirm this hypothesis, clonally purified MSC were tested using the same protocol. The MSC were transfected with a plasmid encoding GFP under the control of a cardiac-specific myosin light chain promoter. PCR analysis proved the engraftment of MSC in the BM of all recipients. Eight weeks after AMI a total of 1,034 GFP^+ cells were detected in the heart, indicating that MSC mobilized from the BM homed to the infarcted tissue and differentiated into cardiac cells. These data provide strong evidence that MSC can home to the heart after MI, engraft and differentiate into CMC. Furthermore, the results strongly suggest that the majority of BM-derived CMC homing to the heart after myocardial damage is MSC. However, the number of MSC-derived CMC is too low to achieve cardiac regeneration. It is then important to understand if an exogenous administration of MSC can do the job. As already reported, several studies have tested MSC transplantation in experimental myocardial injury models. Overall, the results show that the milieu surrounding the infarcted tissue seems to attract the MSC and promote their engraftment. Indeed, MSC preferentially home to the infarcted area and to the border zone when injected systemically, while their number in the remote areas is limited [70]. Importantly, it has been shown that after a few weeks, MSC engrafted at the border zone and differentiate into cells expressing a variety of cardiac-specific markers [70–73]. These cardiac-like cells seem also to be functionally connected with the native CMC since they express both connexin-43

and N-caderin [73], proteins responsible for cell-to-cell connection and electrical coupling. Unfortunately, even after direct intramyocardial injection, the efficiency of cell engraftment is low and no data are available regarding the replicative potentiality of MSC retained in the heart. Furthermore, the MSC engrafted in the infarct scar appear primarily fibroblast-like and lack the features typical of complete myogenic differentiation such as mature sarcomeric organization and intercalated discs [72]; this may speak to the importance of the local extracellular milieu in driving MSC differentiation. On the other hand, the fact that MSC present at the border zone do turn into cardiac-like cells may support the hypothesis that cell contact with intact CMC is crucial for proper MSC differentiation. Like for other BM-derived stem cell types, cellular fusion may represent a confounding factor when testing the regenerative capacity of MSC. For example, using a *Cre-lox* recombination system, our group has shown that mouse BM-MSC injected into infracted hearts can fuse with resident CMC [55]; however, the frequency of cell fusion was low.

Beside all the mechanistic hypotheses, it is reasonable to conclude that MSC can differentiate into cardiac-like cells in vivo. However, the low efficiency of cardiac regeneration from donor MSC is not sufficient to explain the important beneficial effects observed by the majority of the researchers in terms of both ventricular remodeling and cardiac function after MSC administration. More studies are needed to better understand the signals addressing MSC differentiation towards cardiac lineage in order to be able one day to achieve cardiac regeneration using MSC.

13.6.2 Vasculogenesis

Generating a functional and stable microvasculature network remains one of the major challenges in tissue regeneration and repair. The development of mature vessels relies not only on endothelial proliferation and migration, since cooperation between endothelial cells and pericytes is fundamental for vascular development and maturation. Blood vessels derive from mesodermal precursors called angioblasts early during embryogenesis [74]. In this process, termed vasculogenesis, precursor angioblasts differentiate into EC forming a vascular network. This primordial plexus is refined into a functional network by a process where vessels undergo extensive elongation and maturation [75].

In contrast to the embryonic heart vasculature, the adult heart vessels are quiescent. Only when under stress or pathologic conditions, like MI, the coronary vascular bed expands [74]. Postnatal neovascularization encompasses three different mechanisms: the first is referred to as angiogenesis and consists in the sprouting of new vessels from preexisting vessels. The second mechanism is collateral enlargement and muscolarization, namely arteriogenesis. Recently, a third mechanism has been demonstrated, postnatal vasculogenesis, that consists of the assembly of new blood vessels by differentiation of endothelial precursors originating from the BM [76]. Based on this rationale, administration of BM-MSC has been proposed as a novel strategy to induce therapeutic vasculogenesis. Almost all the experimental

studies testing the potential of MSC to induce vascular regeneration have shown an increase in capillary density and improvement in tissue perfusion. However, it is still debated if differentiation of MSC into EC and VSMC rather than generation of new pericytes and/or release of paracrine mediators represents the main mechanism of action [37, 49, 77]. In support of the first hypothesis, it has been reported that after 15 days in culture, MSC start expressing αSM actin and β-actin filaments, which are, respectively, specific to smooth muscle and non-muscle cells, but they do not express CD31 [78]. Immunofluorescence studies revealed that, once injected into infarcted hearts, some engrafted MSC expressed the smooth muscle phenotype (αSM actin$^+$) while some acquired an endothelial phenotype (CD31$^+$); furthermore, vessel density was augmented in the MSC group in comparison with the control group. In another experimental model of myocardial infarction in rats, MSC differentiation into endothelial phenotype enhanced microvascular density and improved heart function [79]. Also, when tested in chronically ischemic dog hearts, MSC treatment resulted in a trend toward reduced fibrosis and greater vascular density with immunohistological evidence of colocalization of engrafted MSC with EC and smooth muscle cells markers [80]. More recently, it has been confirmed in a pig model that MSC can differentiate into EC as early as 72 h and persist in chimeric vessels at least up to two weeks even though the number of differentiated cells was low [81].

Despite this evidence, some investigators believe that MSC, rather than differentiate into EC and VSMC, participate in vasculogenesis by turning into pericytes that stabilize and favor the maturation of the new vessels [82]. For example, it has been shown that co-implantation of EPC with human MSC into immunodeficient mice results in formation of extensive vascular networks after one week [83]. The presence of human EPC-lined lumens containing erythrocytes throughout the implants indicated not only a process of vasculogenesis from the two cell types, but also the formation of functional anastomoses with the host circulatory system. Importantly, MSC were shown to reside in perivascular locations around the engineered lumens, confirming their active participation in blood vessel assembly. The results are similar and support another report, where human MSC combined with human umbilical vein EC were shown to facilitate blood vessel assembly and adopt a perivascular location and phenotype [84]. Similar to normal pericytes, human MSC-derived perivascular cells contracted in response to endothelin-1 in vivo. Importantly, the authors remarked that they could not detect differentiation of human MSC into endothelial cells in vitro, and that MSC alone could not form conduit for blood flow in vivo [84]. Importantly, there is evidence showing that MSC may have a perivascular origin in multiple human organs; thus, blood vessel wall harbors a reserve of progenitor cells that may be integral to the origin of MSC [85].

Beyond all the controversies, in the majority of the animal studies only a limited number of engrafted MSC stained positive for EC and VSMC markers, suggesting that the direct role of MSC in neo-vasculogenesis is limited. In contrast, as discussed in more detail below, there is solid evidence supporting the key role of MSC as regulators of vascular regeneration via paracrine mechanisms.

13.6.3 Paracrine Effects

There is a growing body of evidence supporting the hypothesis that paracrine mechanisms mediated by factors released by the MSC play an essential role in the reparative process observed after stem cell injection into infarcted hearts. Paracrine secretion has been recognized for more than 15 years, since Haynesworth et al. [86] reported that MSC synthesize and secrete a broad spectrum of growth factors and cytokines such as VEGF, FGF, HGF, insulin growth factor-1 (IGF-1), SDF-1, and thrombopoietin. The mechanisms mediating the effects of these paracrine factors are numerous. Cytokines and growth factors may favor neovascularization, cytoprotection, and endogenous cardiac regeneration. Furthermore, the post-infarction inflammatory and fibrogenic processes, cardiac contractility, and cardiac metabolism may also be influenced in a paracrine fashion (Fig. 13.2).

Despite evidence that BM-MSC incorporate into vascular structures, as discussed earlier, several studies suggest that only a small number of vessels contain donor cells. Nevertheless, BM-MSC lead to a significant increase in capillary density and collateral development when transplanted into ischemic tissues. The molecular processes leading to angiogenesis and arteriogenesis include the pivotal role of nitric oxide, VEGF, bFGF, HGF, angiopoietin, and others. These molecules lead to EC and VSMC migration, proliferation, vessel enlargement and maturation, and synthesis of extracellular matrix. Interestingly, it has been shown that BM-MSC express several pro-angiogenic and pro-arteriogenic factors. Accordingly, it has been proposed that the release of these factors by transplanted stem cells may play an important role in determining the increase in capillary density and collateral development observed in ischemic tissues of animals treated with MSC.

Epstein and collaborators have suggested that local delivery of MSC augments collateral perfusion through paracrine mechanisms [59]. These authors injected 1×10^6 MSC in the adductor muscle of mice 24 h after femoral artery legation. Compared with controls injected with medium or mature EC, distal limb perfusion improved and conductance vessels increased in number and total cross-sectional area. Surprisingly, labeled MSC were tracked dispersed between muscle fibers, but were not seen incorporated into mature collaterals. On the other hand, protein levels of VEGF and bFGF were significantly increased in the muscle of MSC-treated animals compared with controls. Furthermore, colocalization of VEGF and transplanted MSC within adductor tissue was documented. Consequently, the authors concluded that MSC contributed to collateral remodeling through paracrine mechanisms. Gene expression profiling of MSC grown under normal conditions or under hypoxia allowed to document that these cells express a wide range of arteriogenic cytokines at baseline and that several of them are up-regulated by hypoxia [87]. The gene array data were confirmed using ELISA assays and immunoblotting of the MSC conditioned media (CM). Furthermore, it was shown that MSC-CM promoted proliferation and migration of EC and VSMC in a dose-dependent manner in vitro and enhanced collateral flow recovery and remodeling in a model of hind limb ischemia in vivo. Other studies, testing MSC transplantation in experimental

infarcted hearts, reported an increase in capillary density in treated animals compared with controls, despite the presence of few EC of donor origin [70, 88, 89]. In these cases, even though not directly proven, a pro-angiogenic paracrine action seems to be the most reasonable explanation to the effects observed.

Our group expanded the spectrum of stem cell paracrine actions by demonstrating that BM-MSC exert direct cytoprotective action on ischemic CMC. In particular, we clearly showed that cell culture medium conditioned by hypoxic MSC can reduce apoptosis and necrosis of isolated rat CMC exposed to low oxygen tension [57]. The cytoprotective effect was greatly enhanced in MSC overexpressing the gene Akt-1 (Akt-MSC) in vitro. To further validate the protective properties of the Akt-MSC, we studied the effect of the CM in vivo, using a rat experimental model of permanent coronary occlusion. Concentrated CM (C-CM) obtained by ultrafiltration was injected into the heart at the infarct border zone 30 min after left coronary occlusion. After 72 h, the infarct size and the CMC apoptotic index were significantly lower in animals treated with C-CM from Akt-MSC compared to controls. Of note, C-CM from non-modified MSC reduced infarct size compared with saline but the results were not statistically significant, confirming that Akt overexpression enhanced the production of cytoprotective factors. In a follow-up study we confirmed our earlier results and documented how the limitation of the infarct size was matched by preservation of cardiac function. Importantly, the data obtained with C-CM injection essentially replicated the results observed with MSC transplantation in terms of both infarct size and cardiac function, confirming that cytoprotection was the main mechanism of stem cell action in our experimental model. To verify whether Akt overexpression truly up-regulates the expression of secreted factors, we tested by quantitative RT-PCR some candidate genes encoding molecules known to be released by the MSC. Our data showed that VEGF, bFGF, HGF, insulin growth factor 1 (IGF-1), and thymosin ß4 (TB4) were significantly up-regulated in the Akt-MSC at baseline normoxia and increased further after exposure to low oxygen tension. Hypoxic non-modified MSC also up-regulated VEGF, bFGF, HGF, and TB4 even though significantly less than the Akt-MSC. Interestingly, it has been reported that direct injection of TB4 limits myocardial infarct size and induces functional recovery in the same experimental model used in our studies [90]. Our original findings in rodents have been successfully replicated by others also in a large animal model. Indeed, Akt-MSC injected into pig infarcted hearts led to limitation of infarct size and preservation of heart function [91]. Furthermore, other groups have confirmed the paracrine cytoprotective effects exerted by BM-derived stem cells on ischemic CMC [92–94].

Beside cytoprotection, paracrine factors released by transplanted stem cells may alter the extracellular matrix, resulting in more favorable post-infarction remodeling and strengthening of the infarct scar. For example, it has been shown that direct hMSC injection into ischemic rat hearts decreases fibrosis, apoptosis, and left ventricular dilatation while increases myocardial thickness. This resulted in the preservation of systolic and diastolic cardiac function without evidence of myocardial regeneration [95]. It is likely that MSC achieve this preservation of cardiac function, in addition to myocardial salvage, by acutely increasing cellularity and decreasing

production of extracellular matrix protein collagen type I, collagen type II, and tissue inhibitor of metalloproteinase-1 that results in positive remodeling [96]. Furthermore, stem cells may also produce and release local signaling molecules that limit local inflammation when injected into injured tissues. This hypothesis seems to be supported by the fact that expression profiling of adult progenitor cells reveals characteristic expression of genes associated with enhanced DNA repair, up-regulated antioxidant enzymes, and increased detoxification systems.

It has also been suggested that ASC may positively influence cardiac metabolism and contractility. Feygin and collaborators demonstrated that the border zone of infarcted pig hearts is affected by profound bioenergetic abnormalities which are partially attenuated after MSC transplantation [97]. Because of the low cell engraftment, the authors postulated that MSC did not provide a structural contribution to the damaged heart and concluded that the observed beneficial effects likely resulted from paracrine repair mechanisms. We have recently reported that Akt-MSC prevent metabolic remodeling in infarcted rat hearts [98]. Treatment with Akt-MSC spared phosphocreatine stores and significantly limited the increase in 2-DG uptake in the residual intact myocardium compared with the saline- or the MSC-treated animals. Furthermore, Akt-MSC-treated hearts had normal pH, whereas low pH was measured in the saline and MSC groups. We have also observed that cell shortening, maximal rate of relengthening ($+dL/dt$), and maximal rate of shortening ($-dL/dt$) of isolated adult rat CMC are significantly improved in the presence of CM from hypoxic MSC, particularly Akt-MSC, compared with standard conditions. Results from other groups seem to confirm that ASC can release inotropic factors [99].

Other evidence suggests a further intriguing hypothesis: exogenous stem cell transplantation may activate resident CSC and/or stimulate CMC replication via paracrine action, thus improving endogenous cardiac regeneration. For example, it has been shown that intramyocardial administration of HGF and IGF-1 at the infarct border zone induces CSC migration, proliferation, and differentiation [9]. Since MSC release both HGF and IGF-1, particularly under hypoxic stimulation [58], it is reasonable to hypothesize that MSC injected into ischemic hearts may attract and activate resident CSC. Indirect evidence supporting this hypothesis has been documented in a study in which MSC were injected into infarcted pig hearts [71]. Immunohistochemical analysis performed after 10 days revealed the presence, only in MSC-treated animals, of newly formed CMC, some of which stained positive for c-kit and others for Ki67. Unfortunately, the co-staining for c-kit and Ki67 was not performed, so that the true origin of those replicating CMC could not be determined. However, the authors concluded that endogenous cardiac regeneration was present. Also, the administration of hBM-MSC seems to determine proliferation of host CMC [48]. The RNA levels of HGF, IGF-1, VEGF, and bFGF were significantly up-regulated in hBM-MSC-treated hearts compared with controls. Interestingly, in the same study the paracrine cytoprotective and pro-angiogenic actions exerted by BM-derived stem cells were further confirmed. Recently, more direct evidence that BM-MSC stimulate proliferation and differentiation of endogenous CSC has been produced [81]. Transendocardial injection of GFP-MSC was performed in a pig model of ischemia/reperfusion injury: a detailed tissue analysis clearly showed that

MSC stimulate endogenous CMC turnover in two likely related ways: by stimulating endogenous c-kit^{+} CSC and by enhancing CMC cell cycling.

13.7 Discovery of Mesenchymal Stem Cells-Derived Therapeutic Molecules

The demonstration that BM-MSC, particularly those which are genetically modified, may secrete therapeutic molecules provides a potential breakthrough in that, rather than administering cells, one may be able to administer specific proteins or other soluble factors produced by these cells for cardiac therapy [43]. In this contest, Akt-MSC revealed themselves as a new model to identify possible novel cytoprotective molecules. In general, testing the properties of CM is particularly straightforward, both in vitro and in vivo. However, identifying the nature of specific factors involved in cardiac repair is a much more demanding task, although extremely relevant and worth pursuing (Fig. 13.3). Hypothesizing that the paracrine mediators are proteins or peptides, there are basically two approaches to identify them: transcriptomics and

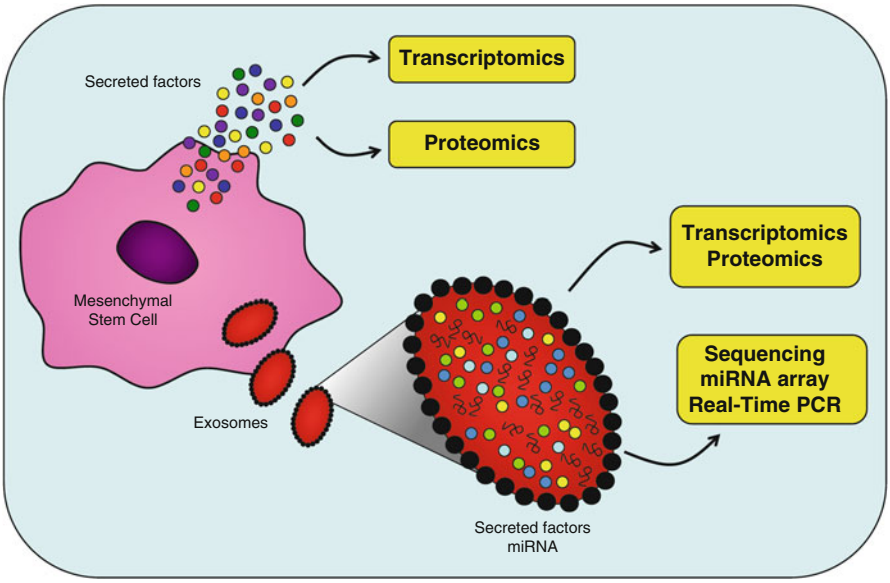

Fig. 13.3 Different approaches to identify putative paracrine mediators. To identify secreted paracrine factors, multiple experimental approaches can be used. The most common are transcriptomics and proteomics. The discovery that exosomes are involved in paracrine-mediated effects, has opened new scenarios. It is thought that exosomes act either by activating specific cell receptors or by releasing proteins and miRNA inside the target cells. Accordingly, to profile the content of exosomes it is possible to use transcriptomics, proteomics, or miRNA array profiling

proteomics. Each of these methods has pros and cons that will be discussed together with specific examples of paracrine factor discovery.

Among the different approaches possible, our group chose to perform a wide microarray expression analysis of Akt-MSC vs native MSC [100]. Although the genomic approach, as compared with a proteomics, can overlook important post-transcriptional events such as alternative splicing, it is currently more comprehensive, technically less demanding, and enables easier identification of novel genes with previously unknown cell survival effects. Approximately 650 transcripts were differentially regulated between Akt-MSC and control MSC. A sub-analysis of the data revealed 62 transcripts encoding for 51 unique genes potentially contributing to the paracrine effects of Akt-MSC. Among these up-regulated genes, the secreted frizzled related protein 2 (Sfrp2) was the most dramatically up-regulated. Microarray data were confirmed by quantitative RT-PCR and by Western blot analysis. We have demonstrated that Sfrp2 exerts cytoprotection on ischemic CMC and that the pro-survival effect of Akt-MSC was markedly attenuated upon knockdown of Sfrp2 with siRNA. Furthermore, we have shown that the mechanism of action of Sfrp2 is through modulation of Wnt signaling pathway. The cardioprotective and additional beneficial properties of Sfrp2 on damaged hearts were recently confirmed by other investigators [101].

Transcriptomic analysis was used to identify factors responsible for tissue repair observed after intravenous (iv) injection of hMSC in a mouse model of MI [102]. Administration of hMSC results in a high number of cells trapped predominantly in the lung. Data from microarray gene expression analysis indicated that after embolization in lung, 451 human transcripts of hMSC were up-regulated. These transcripts were examined to identify putative genes of interests and TNFAIP6 (TSG-6), previously shown to be a powerful anti-inflammatory factor, emerged as one of the most solid candidates, with a 47-fold increase. Knock-down experiments showed that iv injection of hMSC, but not hMSC transduced with TSG-6 siRNA, limits inflammatory responses and infarct size [102]. Moreover, iv administration of recombinant TSG-6 also reduced inflammatory response and infarct size, confirming the importance of this soluble factor in tissue repair.

Proteomic analysis is an alternative approach to identify putative paracrine factors, although sample preparation still represents a major hurdle. One of the problems with this approach is that proteins are usually secreted at low concentration in the culture media, making it difficult to recover these factors. In addition, culture media are rich in salts and other compounds that interfere with most proteomic techniques, making selective precipitation of proteins almost mandatory for a correct analysis. In addition, the presence of serum proteins, even in trace amounts, dramatically influences the dynamic range of the sample and consequently the identification of secreted proteins. Thus, the development of reliable methods for profiling secretory proteins is highly desirable. Efficient methodologies should be developed for the enrichment and analysis of the secretome of different cell lines, free of essential contaminants. These new methods should encompass the optimization of cell incubation conditions in serum-free medium, the sub-fractionation of the CM with appropriate chromatographic techniques, the establishment of

biochemical assays to monitor the paracrine effects of the isolated protein, and the use of the CM in heterologous cell systems for biological assays.

Despite these limitations, several interesting results have already been obtained with proteomics. For instance, two-dimensional liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used to globally profile the proteome of murine MSC (mMSC) [103]. Using this technique, it was possible to identify 258 proteins specifically expressed by mMSC, 54 of which were classified as secreted proteins. In another study, a chemically defined serum-free medium was conditioned by MSC derived from hESC using a clinically compliant protocol [104]. The CM was analyzed by multidimensional protein identification technology and cytokine antibody array analysis and revealed the presence of 201 unique proteins. Computational analysis predicted that these factors are involved with three major groups of biological processes: metabolism, defense response, and tissue differentiation, including vascularization, hematopoiesis, and skeletal development. Furthermore, several of these proteins are known to be activators of important signaling pathways in cardiovascular biology, bone development, and hematopoiesis such as Jak-STAT, MAPK, Toll-like receptor, TGF-β, and mTOR.

The administration of soluble factors instead of stem cells may be more easily translated into the clinical arena since it has several obvious advantages. Indeed, this strategy would bypass most of the issues associated with cell-based therapy, i.e., immune compatibility, tumorigenicity, xenozootic infections and waiting time for ex vivo expansion of autologous cell preparations. Such an approach would have a greater potential for the development of "off-the-shelf" stem cell-derived products. However, there are technical problems related to protein/peptide administration. The most obvious limitation of protein therapy is represented by the necessity to maintain therapeutic concentrations in order to induce the desired effect for the necessary length of time. Establishing the threshold concentration and the necessary time remains to be determined and represents a difficult task. Different actions may require different concentrations and timing. Substantial differences between animal models and humans further complicate the scenario. For example, it has been shown that a single dose of specific growth factors is effective in enhancing neovascularization in animals but not in humans [105]. Other possible hurdles are represented by protein stability and pharmacokinetic. To overcome these problems, a variety of strategies have emerged for manipulating protein properties, stability, specificity, immunogenicity, and pharmacokinetic [106]. Mechanisms for altering these properties include manipulation of primary structure, incorporation of chemical and post-translational modifications, and utilization of fusion partners. The protein and peptide therapeutics have already become an important class of drugs due to advancements in molecular biology and recombinant technologies. Currently, most therapeutic proteins are administered by the parenteral route which has many drawbacks. Various delivery strategies have evolved over the past few years to improve delivery of proteins and peptides, including the use of biopolymers and nanomaterials for controlled release of proteins [107], and delivery via noninvasive routes such as subcutaneous release or dermal patches. Noninvasive approaches remain challenging due to poor absorption and enzymatic instability, pharmacokinetics and

pharmacodynamics of protein therapeutics. Development of an oral dosage form for protein therapeutics is still most desirable, although it presents a greater challenge. Even though the road to reach optimal protein therapy has numerous hurdles, we anticipate that the constant development and application of rational protein design technology will enable significant improvements in the efficacy and safety of existing protein therapeutics, as well as allow the generation of entirely novel classes of proteins and modes of action. In this case, curing AMI with a single protein or, most likely, with a cocktail of proteins may become reality.

13.8 Exosomes

Recently, it has been proposed that the beneficial paracrine effects observed after MSC therapy are mediated by exosomes [108] (Fig. 13.3). Lai and colleagues demonstrated, through size fractionation studies, that the active component in CM was a large complex 50–200 nm in size. They purified exosomes from CM of MSC derived from human embrionic stem cells (hESC) by size exclusion using high-performance liquid chromatography and demonstrated that exosomes reduce infarct size while the CM deprived of exosomes do not [108]. The secretion of cardioprotective exosomes is not unique to hESC-MSC and was also found in MSC derived from different sources [108]. Altogether, these observations suggest that the secretion of protective exosomes is a characteristic of MSC and may be a reflection of the stromal support role of MSC in maintaining a microenvironmental niche for other cells such as hematopoietic stem cells. Besides cytoprotection, it has also been suggested that exosomes can act directly through the interaction ligand/receptor or indirectly on angiogenesis by modulating soluble factor production involved in endothelial and progenitor cell differentiation, proliferation, migration, and adhesion [109]. For instance, exosomes generated from platelets play an interesting beneficial pro-angiogenic role in a model of myocardial ischemia by delivering a cocktail of pro-angiogenic proteins, such as VEGF, basic fibroblast growth factor, and PDGF, has been demonstrated [110].

Compared with other secreted vesicles, exosomes have much better defined biophysical and biochemical properties. They are small membrane vesicles (between 30 and 100 nm in diameter) of endocytotic origin that are secreted by most cells in culture. They seem to form by invagination and budding from the limiting membrane of late endosomes, resulting in vesicles limited by a lipid bilayer containing cytosol from the producing cells and exposes the extracellular domain of various transmembrane proteins at their surface. As a bi-lipid membrane vesicle, exosomes not only have the capacity to carry a large cargo load, but also protect the contents from degradative enzymes or chemicals: proteins, RNA and microRNA (miRNA) contained in MSC exosomes are protected from degradation by trypsin and RNase as long as the lipid membrane is not compromised [108, 111]. Most exosomes have an evolutionary conserved set of proteins, but they have a unique tissue/cell type specific proteins that reflect their cellular source. They have been shown to be

secreted by many cell types [112–119]. They are also found in physiological fluids such as normal urine [120], plasma [121], and bronchial lavage fluid [122].

Exosomes are known to bear numerous membrane proteins that have binding affinity to other ligand on cell membranes or the extracellular matrix, such as the transferrin receptor, tumor necrosis factor receptors, integrins, and tetraspanin proteins (e.g., CD9, CD63, and CD81). These membrane bound molecules provide a potential mechanism for the homing of exosomes to a specific tissue or microenvironment. For example, integrins on exosomes could home exosomes to CMC that express ICAM1, a ligand of integrins after myocardial I/R injury [123], or to VCAM-1 on EC [124]. Tetraspanin proteins, which function primarily to mediate cellular penetration, invasion and fusion events, could facilitate cellular uptake of exosomes by specific cell types.

Several studies have analyzed the biological activities of exosomes, but little is known about their possible functions in vivo. They are believed to be important for intercellular communication. Exosomes may also facilitate the uptake of therapeutic proteins, RNA or miRNA into injured cells. It was observed that the efficiency of exosome uptake correlated directly with intracellular and microenvironmental acidity [125]. This may be a mechanism by which MSC exosomes exert their cardioprotective effects on ischemic CMC that have a low intracellular pH [126]. During myocardial I/R injury, the restoration of blood and oxygen to ischemic myocardium paradoxically exacerbates the ischemia-induced cellular insults. It has been hypothesized that with their complex cargo, exosomes would have adequate potential to participate in a wide spectrum of biochemical and cellular activities, simultaneously target and correct the various ischemia-induced cascades, and prevent occurrence of the paradoxical reactions induced by reperfusion. In addition, many of the proteins in the exosomes are enzymes. Since enzyme activities are catalytic rather than stoichiometric and are dictated by their microenvironment (e.g., substrate concentration or pH), the enzyme-based therapeutic activities of exosomes could be activated or attenuated according to the release of injury-associated substrates, which in turn, is proportional to the severity of disease-precipitating microenvironment.

Despite this encouraging evidence, the translation of cardioprotective MSC-derived exosomes into a therapeutic agent presents several unique challenges. The major challenge would be to produce good manufacturing practices (GMP) grade exosomes from non-autologous cell sources. Finding a robust scalable and highly renewable cell source will be central to the development of a commercially viable manufacturing process for the production of MSC exosomes in sufficient quantity and quality to support clinical testing or applications. To address this issue, it has been proposed to immortalize ESC-derived MSC by *Myc* [127]; as this modification is thought to not compromise the quality or yield of exosomes. The translation of MSC-derived exosomes into clinical applications is also complicated by the relative novelty of exosomes with few precedents in the regulatory and safety space of biopharmaceuticals. This will require the formulation of new standards for manufacture, safety, and quality control.

13.9 Future Perspectives

Although MSC therapy holds promise in the future treatment of heart disease such as AMI, CIHD, and CHF, its current use is significantly hampered by biological and technological challenges. One of the major problems is represented by the extensive loss of cells after transplantation. Many studies have shown that the majority of cells successfully delivered to the heart die within the first weeks [77]. The causes of cell death in AMI setting are multifactorial and are influenced by the ischemic environment, which is devoid of nutrients and oxygen, coupled with the loss of survival signals from matrix attachments and cell–cell interactions. We and others have conceptualized the idea of improving cell survival by overexpressing protective genes [128]. To optimize this approach, one may consider the use of viral vectors encoding multiple cytoprotective genes, acting on different cell death and apoptosis pathways. Recently, it has been proposed that preconditioning of stem cells with different cytokines may result in improved cell engraftment [129]. The combination of genetic modification and preconditioning may further enhance cell survival and engraftment. An alternative method would be to seed cell ex vivo on a biodegradable polymeric scaffold, followed by in vivo engraftment instead of injecting the cells directly into the site of injury [130]. Improvement of MSC survival will ultimately allow us to address cell scalability and to make cell-based therapies more easily applicable to humans.

Safety concerns regarding the proarrhythmic effects following transplantation of MSC for cardiac repair must be taken into consideration. So far, BM-derived stem cells have not shown proarrhythmic effects in early clinical trials. However, it is not known whether increasing the number of cells that survive or the number of the cells injected will provoke an arrhythmogenic reaction. Tailored preclinical studies should be carried out in small and large animal models with the specific aim to rule out completely that MSC can induce arrhythmias.

Other unresolved issues are the efficacy and safety concerns surrounding the changes in gene expression and functional properties of MSC with advancing age and disease [131]. The properties of self-renewal and lineage potentiality of cells harvested from high-risk patients may be affected. We know that both the age and the presence of disease status adversely influence several aspects of the intrinsic characteristics of ASC. For instance, EPC from patients with cardiovascular disease display varying degrees of functional impairment and an inverse correlation has been reported between the number of circulating EPC and the prevalence of risk factors for CAD [132]. If these deficiencies were also demonstrated for MSC, they may limit the therapeutic application of individualized treatment using a patient's own isolated MSC.

Importantly, the morphological and histological complexity of the myocardium should not be overlooked when designing cell-based protocols for cardiac grafting. The expectation that injecting a stem cell may result in the regeneration of new functionally competent cardiac tissue may be excessively simplistic. Even though the anatomy and the physiology of the heart are not as complicated as other organs

such as the pancreas or the kidney, rebuilding new cardiac tissue may not be an easy task. The myocardium consists of a variety of cell types including CMC, fibroblast, EC, and other vascular cells embedded in a complex extracellular matrix that provides the scaffolding for the three-dimensional alignment of the various components which is required for proper mechanical and structural function. This level of complexity raises caution against designing overly simplistic grafting protocols based on a single cell type. It may well be that the optimal grafting procedure for cardiac repair requires more than one cell type and/or biomaterials to produce a graft that is able to recapitulate normal cardiac function. In this regard, we strongly believe that tissue engineering will likely play a pivotal role and will advance the field of regenerative medicine [133].

Finally, the demonstration of paracrine/autocrine mechanisms improves our understanding of MSC biology and action in tissue repair and regeneration. It is evident that the improvement in cardiac function following MSC therapy can be attributed mainly to the release of key paracrine factors by stem cells in the injured myocardial microenvironment. A growing body of evidence strongly suggests that these secreted molecules mediate a number of protective mechanisms including cell survival, neovascularization, remodeling, and proliferation. The regulatory machinery governing paracrine factor release appears to be complex and dependent on spatiotemporal parameters. Advances in profiling technologies continue to identify significant secreted factors that mediate cardiac repair mechanisms. The potential for magnifying stem cell-mediated paracrine effects using "engineered," "conditioned," or other ex vivo manipulated stem cells will significantly propel this type of therapy forward and provide invaluable information regarding stem cell biology.

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