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Stem Cells as Drug Delivery Methods: Application of Stem Cell Secretome for Regeneration

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Abstract

Mesenchymal stem cells (MSC) are a unique cell population defined by their ability to indefinitely self-renew, differentiate into multiple cell lineages, and form clonal cell populations. It was originally thought that this ability for broad plasticity defined the therapeutic potential of MSCs. However, an expanding body of recent literature has brought growing awareness to the remarkable array of bioactive molecules produced by stem cells. This protein milieu or “secretome” comprises a diverse host of cytokines, chemokines, angiogenic factors, and growth factors. The autocrine/paracrine role of these molecules is being increasingly recognized as key to the regulation of many physiological processes including directing endogenous and progenitor cells to sites of injury as well as mediating apoptosis, scarring, and tissue revascularization. In fact, the immunomodulatory and paracrine role of these molecules may predominantly account for the therapeutic effects of MSCs given that many *in vitro* and *in vivo* studies have demonstrated limited stem cell engraftment at the site of injury. While the study of such a vast protein array remains challenging, technological advances in the field of proteomics have greatly facilitated our ability to analyze and characterize the stem cell secretome. Thus, stem cells can be considered as tunable pharmacological storehouses useful for combinatorial drug manufacture and delivery. As a cell-free option for regenerative medicine therapies, stem cell secretome has shown great potential in a variety of clinical applications including the restoration of function in cardiovascular, neurodegenerative, oncologic, and genitourinary pathologies.

Keywords

Secretions; mesenchymal stem cells; tissue engineering; regenerative medicine; systemic therapy; mechanism of action

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1. Introduction

Adult mesenchymal stem cells (MSCs) comprise a unique cell population that was first described in the 1970s by Friedenstein *et al* [1]. This first description of bone marrow-derived adult MSCs in a series of animal studies and, later, of human embryonic stem cells in 1998 were seminal events in the field of stem cell research [1, 2]. MSCs are among the most well-studied and well-understood of stem cell types and much research has focused on their unique ability to indefinitely self-renew, differentiate into multiple cell lineages, and form clonal cell populations. These defining characteristics have generated much excitement for the use of this cell lineage for clinical therapeutic application. To date, most studies have explored methods to exploit the broad plasticity of stem cells and their ability to act as tissue-specific progenitors to repair tissue damage and restore function locally [3-7]. Similarly, these earlier works primarily attributed the therapeutic effects of stem cell therapy to this ability to locally engraft and differentiate into multiple tissue types. However, an expanding body of recent literature has also brought attention to the incredible array of bioactive molecules produced by stem cells [8-11]. This diverse protein assortment of cytokines, chemokines, angiogenic factors, and growth factors known as the “secretome” is being increasingly recognized for its role in the regulation of numerous physiological processes.

Investigation of the stem cell secretome often begins *in vitro* where recent advances in the field of proteomics have demonstrated its role in directing endogenous and progenitor cells to site of injury as well as in mediating apoptosis, angiogenesis, and tissue scarring [12-14]. Additionally, many studies have suggested that it is the secretome and its paracrine/autocrine roles rather than stem cell differentiation that may mediate many of the regenerative effects observed following therapeutic stem cell administration [12]. As such, there has been growing interest in the use of secretome in the clinical arena, particularly as it has several advantages over the traditional use of stem cells in regenerative medicine therapy, including increased ease of delivery, reduced concerns for oncogenic potential associated with stem cell use, lack of immunogenic reaction enabling allogeneic or off-the-shelf use, and wide potential for *in vitro* modulation of the protein milieu delivered [15]. Thus, stem cells can be thought of as combinatorial drug manufacture and delivery mechanism, the content of whose production can be adjusted for different clinical applications.

In this article, we begin with a brief overview of stem cells and potential mechanisms by which they aid in tissue repair, with a focus on the paracrine/autocrine function of stem cells. We then transition to a discussion of the stem cell secretome and the methods by which it has been studied *in vitro*. We then provide an overview of therapeutic applications for secretome with a focus on its potential use in the genitourinary tract. Finally, we highlight some of the challenges going forward in translating this promising research from the bench to the bedside.

2. Stem Cells in Regenerative Medicine

2.1 Definition of Stem Cells

Stem cells are a unique cell population defined by: 1) their ability to indefinitely self-renew, 2) the ability to form clonal cell populations derived from a single cell, and 3) their ability to differentiate into a number of different cell types. It is these special properties of broad plasticity and self-renewal that make these cells attractive for use in restoration of function to multiple tissue types.

Currently, the broad diversity of stem cells under clinical investigation can be divided into four categories: embryonic stem cells derived from the early stage embryo, stem cells derived from placenta or amniotic fluid with properties intermediate to those attributed to embryonic stem cells and adult stem cells, induced pluripotent stem cells or cells that have been "reprogrammed" via the use of specific transcription factors to achieve a pluripotent state, and, finally, adult stem cells [16, 17]. Mesenchymal stem cells, or multipotent adult progenitor cells, are a subset of adult stem cells that were first described in the literature by Friedenstein *et al.* in the 1970s [1]. They are the most well-studied and well-understood cell type in the field of stem cell therapy and, thus far, are the stem cell type whose secretome has been most extensively investigated for therapeutic applications. Since their discovery, MSCs have been identified throughout the body; classically, they were isolated from the bone marrow stroma although later work has also identified them in many other well-vascularized tissues [18]. MSCs may also be found in adipose, muscle, endometrium, and renal tissues, for instance, and, unlike tissue-specific progenitor cells, they can be induced to differentiate into multiple cell lineages including bone, neuronal, adipose, muscle, liver, lungs, spleen and gastrointestinal tissues [18]. Pericytes, or cells that reside in the endothelial lining and were traditionally thought to be important in maintaining the integrity of the vascular network, have also been recently classified as MSCs due to their ability to differentiate into multiple lineages [19]. While it has been suggested that all MSCs may be pericytes, there does seem to be difference between pericytes and MSCs derived from other sources. For instance, bone marrow-derived MSCs have shown efficacy in wound healing but pericytes have not been used to similar effect [20].

2.2 Stem Cell Use in Regenerative Medicine – Potential Mechanisms of Action

The therapeutic potential of stem cells can be attributed to 3 key mechanisms of action: 1) "homing" or the process whereby systemic stem cell delivery results in cell migration to specific areas of acute injury via chemical gradients, 2) local restoration of function by differentiating into multiple cell types to augment or replace damaged tissues, and 3) the secretion of bioactive factors with potential for affecting both local and systemic physiologic processes.

The process of innate systemic stem cell delivery to the site of injury is termed "homing" and, from a clinical/therapeutic perspective, can be taken advantage of in administering stem cells systemically rather than locally. Unlike tissue-specific progenitor cells, MSCs traverse the circulatory system with access to all tissues in the body but migrate or "home" to specific locations, such as areas of acute injury, following chemokine gradients where they

can engraft and facilitate healing and regeneration locally [21, 22]. It is hypothesized that MSCs migrate to target tissues via a process similar to that of leukocyte migration. Initial localization by means of chemoattraction is mediated by cell surface receptors such as the chemokine receptor C-X-C chemokine receptor type 4 (CXCR4) and its binding partner C-X-C motif ligand 12 (CXCL12) as well as the chemokine ligands – C-C chemokine receptor type 1 (CCR1), CCR4, CCR7, CCR10, CCR9, CXCR5, and CXCR6 [23, 24]. Adhesion to vascular endothelial cells at the target site is via integrins and selectins, although the exact role of these molecules in facilitating MSC-endothelial interactions is not well-established [25, 26]. Finally, transmigration of MSCs across the endothelium to the site of injury occurs via both leukocyte-like pathways involving vascular cell adhesion molecule 1 (VCAM-1) and G-protein-coupled receptor signaling, and via novel mechanisms such as paracellular and transcellular diapedesis [27]. Other factors involved in stem cell homing have been investigated in the *in vivo* setting with an aim to improve stem cell delivery to target tissues for clinical application. A study by Askari and colleagues identified stromal-cell-derived factor 1 (SDF-1) as a protein that could direct stem cells to injured myocardium. Since then, SDF-1 has emerged as a prominent stem cell homing factor that has been shown to be upregularted in many experimental models of cardiac injury and in patients with ischemic cardiac disease [28]. Stem cells secrete SDF-1 which serves to home innate circulating stem cells to that location where they presumably participate in repair and regeneration processes. Stem cell homing has also been found to be affected by a variety of other factors including age and passage number of the cells since MSC engraftment efficiency decreases with increased *in vitro* multiplication, culture conditions, and cell delivery method [22].

Classically, the main therapeutic benefit of stem cells was thought to be derived from their ability to locally restore function by differentiating into multiple cell types to augment and/or replace damaged tissues. Differentiation of stem cells to local tissue types has been demonstrated in application to a variety of pathologies, including stress urinary incontinence, in which it is thought that MSCs restore function by differentiating into the multiple cell lineages of the urethra, replacing and augmenting damaged urethral muscle, nerves, and connective tissue [6, 29-31].

3. The Stem Cell Secretome: A Brief Overview

More recent work suggests a complex role of stem cells in functional recovery since many studies of stem cells in animal models suggest that MSCs are relatively short-lived after delivery and do not engraft and differentiate to form new permanent tissues [31-34]. Additionally, stem cells delivered into animal models have been found to exert therapeutic benefits despite engraftment distant from the site of actual injury. Contemporary studies have demonstrated that, in addition to differentiating into target tissue types, stem cells likely exert a therapeutic effect via the secretion of bioactive factors that have antiapoptotic, antiscarring, and neovascularization effects, as well as immunomodulatory properties [21, 35]. The investigation of this protein milieu or “secretome” is a subject of growing interest with the increasing recognition of the paracrine/autocrine role of cell secretions in the regulation of many physiological processes and their potential for therapeutic application.

3.1 What is the Stem Cell Secretome?

Stem cell secretome is defined as the complex set of secreted molecules from stem cells that are crucial to many biological functions including cell growth, replication, differentiation, signaling, apoptosis, adhesion and angiogenesis. The secretome is thought to be encoded by approximately 10% of the human genome and includes a diverse array of serum proteins, growth factors, angiogenic factors, hormones, cytokines, extracellular matrix proteins, extracellular matrix proteases, hormones, and even, in low abundance, lipid mediators and genetic material [36, 37]. These secreted molecules are released by stem cells through classical and non-classical secretion mechanisms, including protein translocation, exocytosis, and vesicle or exosome encapsulation [38, 39]. The soluble factors and vesicles secreted by stem cells may act directly by mediating intracellular pathways in injured cells, or indirectly, by inducing the secretion of functionally active products from adjacent tissues.

Proteins released via the classical or conventional pathway translocate from the lumen of the endoplasmic reticulum to the Golgi complex where they are subsequently excreted from the cell via exocytosis. This process is typically mediated by a signal sequence associated with the secreted protein that directs ribosomes to the endoplasmic reticulum during polypeptide synthesis [38]. Further post-translational processing including signal peptide cleavage, folding, and linking of carbohydrate chains occurs in the lumen of the endoplasmic reticulum. Folded proteins then undergo further processing in the Golgi complex prior to exiting the cell via exocytosis.

However, some proteins including several key regulators of the immune response, angiogenesis, cell growth, and cell differentiation are secreted via a non-classical mechanism that does not involve the endoplasmic reticulum-Golgi complex [39]. The mechanism of secretion of these molecules, which include fibroblast growth factor and members of the interleukin family, may involve direct membrane translocation of proteins, or the export of proteins in membrane- or protein-coated vesicles [37]. Long regarded as repositories for cellular “garbage”, such extracellular vesicles (EVs) or exosomes derived from MSCs are now known to contain a variety of proteins, lipids, and functional RNAs (mRNAs and microRNAs) and may play an important role in intercellular communication [40, 41]. The role of these secreted vesicles is a subject of growing interest within the secretome research community given their potential to transport a host of signaling molecules to distant cell targets. To date, stem cell EVs are not well characterized due to their complex composition, and most studies have focused on the genetic component of stem cell-secreted vesicles. Interestingly, while secreted EVs share some common features across all cell types, EVs also contain material that reflect the function of the originating cell [42]. The first observation of this EV functionality was reported by Raposo *et al* in 1996 who observed that B lymphocytes secreted antigen-presenting EVs [43]. Subsequent studies have implicated EVs in various other immune functions and in cancer development. While some studies report that tumor cells release EVs that result in anti-tumor effect, tumor cells have also been shown to release EVs that confer immune resistance [44, 45]. In addition to their role in cancer immunity, tumor cell-derived EVs also contain cargo proteins that mediate various tumor properties including cell proliferation, invasiveness, and metastasis via an autocrine/paracrine manner [46] [47]. To date, most studies have focused on the secretion of

cytokines and growth factors by MSCs. However, the above work in the fields of immunology and tumor biology suggest that MSC-derived EVs likely play similar roles in mediating many of the therapeutic mechanisms of action of MSCs. Early work in the field of MSC-derived EVs have shown therapeutic potential for their use in a number of disease processes including renal, cardiac, and neurologic injury [48] [49, 50].

3.2 Why Use Secretome?

The use of cell-free therapies in regenerative medicine holds several advantages over more conventional stem-cell based applications. Use of secretome could potentially bypass issues related to immune compatibility, tumorigenicity, and the transmission of infections associated with cell therapies. Secretome use could also greatly reduce the time and cost associated with the expansion and maintenance of clonal cell lines since secretome therapies could be prepared in advance in large quantities in an allogeneic or off-the-shelf fashion and be immediately available for treatment when desired, enabling their application to acute conditions such as myocardial infarction, cerebral ischemia, or military trauma. Furthermore, the protein milieu delivered for therapeutic application could be tailored to enhance or reduce certain cell-specific effects to produce different therapeutic outcomes using the same initial protein mix.

4. Mechanism of Action and Functional Role of Secretome

4.1 Angiogenesis and Revascularization

MSCs and their secretome play an important role in the regulation of angiogenesis that has been demonstrated in both *in vitro* and *in vivo* studies. There is great interest in the role of MSCs in angiogenesis given the wide spectrum of clinical diseases related to insufficient angiogenesis, including atherosclerotic diseases like coronary artery disease and peripheral vascular disease, and wound healing disorders, as well as the broad range of disorders related to pro-angiogenic factors such as chronic kidney disease, tumor growth and metastasis, and proliferative retinopathy. [51] Angiogenesis is defined as the process in which new vasculature sprouts from pre-existing blood vessels. While new vasculature is also derived by splitting or enlargement of existing vessels, the majority of new vessels are formed via angiogenesis. Normal angiogenesis is important not only during development but also during wound healing. A wide variety of molecules such as growth factors, chemokines, enzymes, matrix metalloproteases, and adhesion molecules tightly regulate this process [52].

A number of angiogenic stimulators and inhibitors have been identified in MSC secretome using ELISA, antibody-based assays, and immunohistochemistry. These include vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), interleukin-6 (IL-6), and placental growth factor, angiopoietin-1, mononcyte chemoattractant protein-1, and cysteine-rich angiogenic inducer 61 (Cyr61) [10, 53-57]. Studies have also found that the secretion of these angiogenic factors can be modified by a number of chemokines and hypoxic conditions. For instance, a study by De Luca and colleagues showed that transforming growth factor alpha (TGFα) had the ability to increase the level of several growth factors (VEGF, hepatocyte growth factor, platelet-derived growth factor BB, IL6-

and IL-8) in the MSC secretome [58]. Media conditioned by MSCs treated with TFGα induced greater blood vessel growth compared to control media in an *in vivo* assay. Multiple *in vitro* studies have demonstrated the effect of MSC secretome on each of the key steps in angiogenesis. For example, various MSC populations (including adipose-derived MSCs, Wharton jelly-derived umbilical vein MSCs amniotic MSCs, and bone marrow-derived MSCs) induce endothelial cell proliferation, migration, and tube formation, as well as prevent endothelial cell apoptosis *in vitro* [59-62]. Successful application of MSCs to promote angiogenesis has been demonstrated in animal models for peripheral artery disease, myocardial infarction, cerebral ischemia/stroke, stress urinary incontinence, and neurogenic bladder among other diseases [36, 63] [64, 65].

4.2 Immune and Inflammatory Modulation

While the innate immune response plays a key role as the body's first line of defense to infection or tissue damage, severe immune and inflammatory responses to tissue injury can often have detrimental effects. MSCs possess immunomodulatory and immunological tolerance-inducing characteristics that have been shown to ameliorate and modulate potentially damaging inflammatory reactions. Interestingly, these cells typically express MHC-I but lack expression of MHC-II, CD40, CD80, and CD86. Due to the lack of co-stimulatory cell surface molecules, MSCs often fail to induce an immune response by the transplant host [66]. Furthermore, MSCs have been shown to play a role in suppressing immune responses by three major mechanisms: 1) by direct cell to cell interaction, 2) via the action of soluble factors, and 3) by induction of regulatory T cells. The first report of suppression of cell-mediated immune responses by MSCs was by Di Nicola *et al.*, who found that MSCs inhibit proliferation of CD4+ and CD8+ T cells even in the absence of direct cell-cell contact [67]. Immunomodulatory properties of MSCs have since been identified to act in each of the three major stages of the immune response: 1) antigen recognition and presentation, 2) T cell activation, proliferation, and differentiation, and 3) and the T-cell effector stage [68].

These immunomodulating properties are currently being investigated for myriad applications including prevention of graft-versus-host disease following allogeneic transplantation, Crohn's disease, interstitial cystitis, and suppressing sepsis-induced severe inflammatory responses [9, 69, 70]. Clinical trials in cardiology have taken advantage of these properties of MSCs to investigate the efficacy of nontype-matched allogeneic MSC transplantation [71, 72]. In a study by Hare *et al.*, one year after intravenous administration of allogeneic human MSCs in reperfused myocardial infarction patients, recovery, as measured by global symptom score, ejection fraction, ambulatory electrocardiogram monitoring, and pulmonary function testing, was improved in treated patients compared to those that received a placebo [71]. Additionally, no signs of rejection were observed and adverse event rates were comparable between treated and placebo arms. Further investigations into the immunomodulatory effects of stem cell bioactive factors could someday obviate the need for cellular injections in future stem cell therapies.

4.3 Anti-apoptosis

MSCs play in role in the prevention of cell death not only via their restoration of the local microenvironment but also by specifically producing proteins that have been identified as classic inhibitors of apoptosis and by decreasing expression of anti-apoptotic proteins. A recent study by Li and colleagues supports this idea by demonstrating that bone marrow MSCs reduced apoptosis of alveolar macrophages when co-cultured at appropriate ratios [73]. The MSCs also decreased expression of the pro-apoptotic factors Bax and cleaved caspase-3 while increasing the levels of the anti-apoptotic protein Bcl-2. Tang and colleagues similarly found that Bax expression was decreased while expression of pro-angiogenic factors, including basic FGF and VEGF, and stem cell homing factor SDF-1 were increased in MSC-treated hearts compared to medium-treated hearts [74]. MSC-treated hearts subsequently demonstrated increased capillary density and improved left ventricular contractility 2 months after treatment, presumed to indicate improved function. Gnecchi and colleagues additionally found that Akt-overexpressing MSCs inhibit ventricular remodeling and restore cardiac function in less than 72 hours, an effect that they hypothesized was most likely attributable to the secretion of paracrine factors rather than myocardial regeneration [75][76]. They also demonstrated that the conditioned medium obtained from hypoxic Akt-MSCs markedly inhibited hypoxia-induced apoptosis in adult rat cardiomyocytes *in vitro*. Furthermore, the use of Akt-MSC conditioned medium *in vivo* led to significant reductions in infarct size and improved ventricular function compared to controls. In support of the paracrine hypothesis, they found that several genes coding for likely mediators of these beneficial effects (VEGF, FGF-2, HGF, IGF-1, and thymosin beta-4) were significantly upregulated in Akt-MSCs, especially in response to a hypoxic environment.

4.4 Wound Healing and Tissue Repair

As previously described, MSCs exert a beneficial therapeutic effect on tissue repair and wound healing without a significant degree of tissue engraftment at the site of injury [33]. This observation in numerous animal models of multiple pathophysiologic processes led to the hypothesis that paracrine/autocrine effects of MSCs rather than direct tissue differentiation and engraftment may play the key role in wound healing. This theory has been tested in a variety of clinical applications via the use of stem cell conditioned media. The investigation of these cell-specific proteins often begins in cell culture. While *in vitro* studies cannot fully capture and test the totality of MSC secretions in the *in vivo* microenvironment, investigators seek to replicate the effects of the MSC secretome via the use of media conditioned by the MSCs that contains their secretions an *in vitro* environment [15]. Research has demonstrated that the use of mesenchymal stem cell conditioned media alone can replicate the therapeutic benefits previously seen with the use of stem cells themselves [36, 77, 78]. Further examples of these clinical applications will be described in detail below.

5. The Study of Secretome

Challenges in the study of the MSC secretome include the difficulty of collection and preparation of very small quantities of secreted proteins and the analysis of the vast number of molecules that comprise this stem cell product. However, recent improvements in stem

cell culture techniques and technological strides in the field of proteomics have greatly facilitated secretome analysis.

5.1 Obtaining Conditioned Medium

A number of strategies have been utilized to improve MSC survival or to modify the MSC secretome to achieve greater therapeutic potential. Secretome is highly dependent on the local microenvironment. Preconditioning of MSCs and thus the modification of their secreted contents has been achieved with alterations in the microenvironment including: physiologic preconditioning via with exposure to hypoxic/anoxic conditions; molecular preconditioning via exposure to specific cytokines, chemokines or growth factors to alter MSC secretion; pharmacological preconditioning via exposure to small molecules available in large small molecule libraries; and preconditioning through cell-cell interactions [36, 78]. Additionally, genetic manipulation has also been used to selectively enhance gene expression and enrich the secretome in populations of advantageous trophic factors [36, 77].

While *in vivo* generation of MSC secretome would be ideal so as to most closely replicate the complex microenvironment in which these proteins are secreted, *in vivo* secretome profiling is not practical for a variety of reasons. The collection of the small quantities of secreted proteins is technically challenging. Furthermore, differentiation of stem cell secretome from proteins secreted by other tissues is virtually impossible as MSCs represent only a minute quantity (0.001-0.01%) of bone marrow cells [13]. Thus, the study of MSC secretome composition is currently conducted *in vitro.* Even so, a number of factors in stem cell culture continue to make the study of the stem cell secretome a challenging endeavor. Proteomic analysis of stem cell secretome involves a process of cell isolation and characterization, cell culture in serum-containing culture medium, and cell washing and culture in serum-free media to minimize the influence of serum proteins on the separation and detection of MSC-secreted proteins [13].

The methods used for cell isolation and cell culture strongly influence the quality of cells and the secreted proteins obtained for further study [13, 36]. Obtaining a pure cell population from donor tissue represents an initial hurdle. Subsequently, obtaining a secreted protein sample that is free of the serum typically present in culture media is difficult. However, it is ideal to obtain a serum-free sample as the presence of serum interferes with protein collection and analysis, especially of proteins that are present in small quantities. To circumvent this issue, investigators typically incubate cells in serum-free media for several hours prior to secretome collection and study. The time of incubation is a factor that needs to be carefully optimized to avoid obtaining samples with leaked intracellular contents from dead or apoptosed cells. Additionally the washing of cells and the flasks in which they are cultured during the switch from serum-containing to serum-free media is another key consideration to prevent contaminating serum proteins in the collected sample. In cell populations that rely on the presence of serum, the minimum quantity of serum necessary for normal cell function needs to be optimized and further studies need to control for the presence of serum proteins in the secretome. A final consideration in obtaining conditioned medium is the low quantities in which secreted proteins are produced and the degree of dilution of these proteins into the culture medium. Depending on the degree of dilution

present, reducing the sample volume and increasing the sample concentration may be necessary prior to protein study. Methods of protein concentration include lyophilisation or centrifugation, and/or protein precipitation by trichloroacetic acid or ultrafiltration.

5.2 Protein Characterization

Proteomics profiling is used extensively in the study of MSC secretome and allows for the large-scale investigation of secreted proteins and their function. The first proteomic analysis of human MSC secretome was published in 2003 and, since then, over 30 more studies have been published [13, 79]. Most of these works use either a targeted proteomic approach or a shotgun-based proteomic approach and include conventional techniques such as gel-based and chromatographic fractionation and protein identification as well as newer methodologies such as quantitative mass spectrometry, bioinformatics, and protein microarrays.

In a target-based proteomic approach, specific molecules with known, well-defined roles in physiological processes are identified and studied. For instance, proteins commonly involved in immune modulation and inflammation, or growth factors and hormones with well-established functions in angiogenesis are often targeted in MSC secretome studies. Enzyme-linked immunosorbent assay (ELISA) is the most commonly used method for targeted characterization of the MSC secretome. Such antibody-based techniques hold the advantage of having high sensitivity, specificity, and reproducibility. Furthermore, advances in these techniques to include high-throughput approaches have greatly increased the capability to detect and quantify large quantities of proteins simultaneously. Nguyen *et al* investigated the use of MSC secretome in a swine model of myocardial infarction and found that treatment with MSC conditioned media resulted in significantly reduced cardiac enzyme elevation and improved echocardiographic parameters. Protein array analysis was used to identify various angiogenic, anti-apoptotic, and anti-remodeling factors that may have contributed to this cardioprotective effect. The angiogenic properties of MSC secretome has also been investigated in various recent studies. Wu *et* al used real-time polymerase chain reaction and Western blot analysis to assess conditioned media obtained from bone marrow-derived MSCs and found high levels of VEGF and angiopoietin-1 [55]. These antibody-based techniques are limited, however, in that one can only explore known molecules since protein detection is limited to the antibody availability. Studies are thus more confirmatory in nature and mostly confirm the roles of these molecules in the MSC secretome as compared to their already-established roles from prior investigation. In this sense, studies are limited to only a few known molecules rather than exploring the vast array of soluble factors that actually comprise the secretome.

The shotgun-based proteomic approach is thus used to more broadly study the MSC secretome. Liquid chromatography with tandem mass spectrometry has proven to be an invaluable tool for the analysis of huge arrays of molecules and has been used to analyze serum, whole cells lysates, and stem cell conditioned media. Lee *et al* used this method to analyze the secretome of human adipose tissue-derived MSCs that were preconditioned by exposure to tumor necrosis factor alpha, an adipokine involved in systemic inflammation. [80] Using this proteomic analysis technique, they identified 187 secreted proteins in conditioned media obtained from treated MSCs, and found that 118 of these were secreted at

higher levels following tumor necrosis factor alpha exposure. One of the limitations of this technique, however, is dealing with the miniscule quantities involved in studying stem cell secretome. Recent work has circumvented this issue by using more integrated approaches – for instance, to allow protein purification and labeling prior to mass spectrometry and more robust data processing technologies downstream. Sze *et al* profiled the secretome of human embryonic stem cell-derived MSCs using not only mass spectrometry but also cytokine antibody array analysis, microarray, and quantitative real time-polymerase chain reaction assays and discovered 201 unique gene products [81]. They then used computational analysis to implicate these gene products in diverse signaling pathways including those involved with cellular metabolism, immunity, and tissue differentiation.

5.3 Advantages and Disadvantages of Current Techniques and Future Directions

Proteomic approaches to the study of secretome provide molecular evidence supporting MSC-mediated paracrine effects. However, potential molecular candidates identified via these approaches still require validation utilizing *in vivo* studies where one would expect different secretome profiles dependent upon exposure to varying cellular microenvironments. Furthermore, they are also limited by the significant heterogeneity of the MSC samples and of the conditioned media used for study. As previously described, different techniques for MSC culture and conditioned media isolation may result in significant differences in the study sample and may make it difficult to replicate results or to apply the results of one study to another. An area of future improvement would be standardization of methods of cell preparation as well as better quantification and characterization of secreted proteins. By its very nature, the complexity of the secretome makes it difficult to study but also leads to its significant potential for therapeutic application. Future work will likely increasingly recognize that, in most cases, it is not a few molecules that exert a certain therapeutic benefit but the totality of the soluble factors and their complex interplay that results in a desired effect. As such, further investigation will derive not just from a snapshot view of a few isolated molecules but also from technological advances that allow for the tandem analysis and utilization of a large array of molecules.

6. Paracrine Effects of Secretome

6.1 Cardiac

MSCs have been shown to aid in cardiomyocyte recovery after myocardial ischemia. Initially this was thought to be due to their ability to differentiate into cardiomyocytes to replace damaged or lost tissue. However, recent literature suggests that this tissue differentiation alone is not sufficient to account for the beneficial effects seen after MSC therapy. Furthermore, several authors have shown that the MSC secretome alone is adequate to promote functional recovery. In a study by Uemura *et al*, bone marrow-derived stem cells were injected into the left ventricle of mice following coronary artery ligation [82]. Four weeks after myocardial infarction, stem cells were detected in very low numbers in the cardiac tissue of treated mice. However, the beneficial functional effects of treatment were still seen at this timepoint including, decreased infarct area compared to control animals and increased left ventricular ejection fraction, suggesting that engraftment and differentiation of cells could not account for the observed functional improvements. The authors also

examined the role of preconditioning stem cells prior to delivery to further enhance therapeutic potential. A subset of mice received preconditioned bone marrow stem cells that had been exposed to hypoxic conditions to upregulate several survival factors; these mice subsequently achieved the greatest functional recovery. Their findings suggest an avenue of future study where secretome could be modified by cellular preconditioning to further enhance particular characteristics and to develop a more effective protein cocktail.

Another early work by Noiseux *et al* reinforced the idea that paracrine mechanisms are primarily responsible for the beneficial effects of MSC therapy in a rat model of myocardial infarction [83]. Despite only transient engraftment, low levels of cellular fusion, and low levels of differentiation into cardiomyocytic tissue, treatment with MSCs lead to functional recovery, again suggesting a paracrine role for MSC in functional cardiac recovery. A study of hamsters with heart failure suggests that MSCs act systemically as well as locally [84]. In this study MSCs injected into the hamstrings of affected animals were unable to migrate from the injection site; however, the authors found that treated animals still benefited from stem cell injection based on histologic and functional analysis of the myocardium.

MSCs likely have a therapeutic effect after myocardial infarction by exerting a proangiogenic effect in cardiac tissues. Timmers *et al* characterized human MSC secretions to identify a complement of various proangiogenic factors [85]. They then explored the effect of MSC conditioned media administered intravenously in a pig model of myocardial infarction. Pigs underwent left circumflex coronary artery ligation and were then administered intravenous conditioned media for 7 days. Animals that received MSC conditioned media had higher myocardial capillary density compared to control animals 3 weeks after myocardial infarction. These histologic findings were associated with functional benefit, including reduced infarct size and preserved systolic and diastolic function.

MSCs may also therapeutic benefit after myocardial infarction via their effect on the regulation of collagen biosynthesis and maturation after tissue injury via their secretions. [86]. Bone morphogenetic protein 1 (Bmp1)/tolloid-like metalloproteinase have been reported to be key regulators of collagen synthesis and maturation. A study by He and colleagues identified the role of secreted frizzled related protein 2 (Sfrp2) in reducing fibrosis and improving cardiac function in a rat model of myocardial infarction via its inhibition of Bmp1 activity [86]. Sfrp2 was demonstrated to inhibit Bmp1 activity *in vitro.* Subsequent *in vivo* studies then demonstrated that systemic administration of Sfrp2 could inhibit myocardial infarction-induced type 1 collagen deposition and significantly reduce myocardial fibrosis and improve myocardial function at 2 weeks after induced injury. Thus, it may be possible to determine the active factors of the MSC secretome in a variety of clinical applications and develop specific clinical therapies consisting of only those agents, reducing the potential for side effects.

6.2 Oncology

There is a lot of interest in the development of tissue regenerative strategies to restore function in the wake of cancer treatment and remission [87]. However, a crucial concern is the oncologic potential surrounding stem cell therapy and the possibility of triggering a cancer recurrence. Unfortunately, many of the properties unique to stem cells such as tissue

revascularization, multipotentiality, immunomodulatory effects, and cell homing and migration are also properties that characterize many neoplasias and could aid in tumor progression and metastasis. However, use of a cell-free therapy such as stem cell secretome could obviate these concerns.

A number of models have been developed for the study of MSC-cancer cell interactions. The exact effects of MSCs on tumor growth remains controversial and is a vibrant and complex area of current study. Conflicting data have been obtained such that MSCs have been shown to have both pro- and anti- tumorigenic effects, even in the same cancer model, and, sometimes, even utilizing the same cancer cell lines [88, 89]. Some potentially concerning oncogenic effects of MSCs include modulation of paracrine activities resulting in local immunosuppression, angiogenesis, promotion of tumor growth and invasion via remodeling of the extracellular matrix, promotion of the epithelial to mesenchymal transition of tumor cells necessary for invasion and later metastasis, and inhibition of tumor necrosis/apoptosis. Cytokines such as VEGF, TGFb, and IL-6 which are normally secreted by MSCs are expressed in increased quantities by MSCs that have been recruited by tumor cells, supporting neoplastic growth and invasion [87]. Release of factors such as Dickkopf-related protein 1, however, have also been found to be secreted by MSCs with subsequent antitumorigenic effects in MSC-leukemia and multiple myeloma models [90].

Stem cells have been demonstrated to have immunomodulatory effects that promote the proliferation of tumor cells. For example, the signal transducer and activation of transcription 3 (STAT3) pathway is a key mediator in glioma immunosuppression [91]. Glioma stem-like cells have been found to possess a constitutively active STAT3 pathway leading to inhibition of T cell proliferation and activation, induction of regulatory T cells, and triggering of T cell apoptosis [92]. Interestingly, the secretome of this cell population was found to be as effective as the cells themselves in inhibition of the normal immune response. Factors were subsequently identified in the stem cell secretome including transforming growth factor beta 1, prostaglandin E2, and galectin-3 – all molecules with known immunosuppressive effects.

Stem cells have also been found to promote angiogenesis and tumor growth and invasion in a variety of cancers. In human renal cancer, stem-like cells expressing the mesenchymal stem cell marker CD105 were found to trigger angiogenesis by the secretion of EVs which induced blood vessel growth and formation in normal human endothelial cells [93]. Metastatic growth of human renal carcinoma cells in SCID mice was enhanced when mice were treated with EVs in the absence of stem cells. Molecular analysis of EV contents identified a set of proangiogenic mRNAs and microRNAs with a likely role in tumor progression and metastasis. In prostate cancer, both human MSCs and their conditioned medium have been shown to promote the proliferation, migration, and invasion of prostate cancer cells *in vitro* and *in vivo* [94].

6.3 Urologic Applications of Secretome

While secretome is only beginning to be studied for urologic applications, it holds great potential as a noncellular regenerative therapy. For example, stem cell secretome may play an important role in recovery from renal injury. Bi *et al* investigated the mechanism by

which bone marrow-derived stem cells and adipose-derived stem cells facilitate recovery in a cisplatin-induced model of acute renal injury in female mice [95]. While they found that intraperitoneal injection of these two stem cell populations resulted in enhanced tubular cell proliferation and decreased tubular cell apoptosis following injury, histologic examination of treated kidneys at one and four days post-stem cell infusion revealed no engraftment in the tubules and only rare engraftment in the renal interstitium. Stem cell conditioned medium replicated the above results in both *in vitro* and *in vivo* experiments, suggesting that transplantation of stem cells themselves may not be necessary to mediate renal recovery. A subsequent study by Van Koppen *et al.* used a rat model of established chronic kidney disease to also demonstrate that human MSC secretome contributes to renal recovery following kidney injury [96]. Treatment with the secretome decreased progression of chronic kidney disease and reduced prevalence of both glomerular injury and hypertension.

Novel methods for administration of secretome have also been explored in the setting of renal disease. In a study by Wang *et al*, ESC secretome preconditioned nanofibers were used for delivery of secretome to renal cells [97]. This method could reverse issues with cell hyperpermeablity and apoptosis that were encountered with use of stem cells in *in vitro* experiments. Additionally, they observed renoprotective effects against acute kidney injury in *in vivo* experiments in which renal protection was found to be related to a decrease in Rho kinase activity and to the combined effects of follistatin, adiponectin, and secretory leukoprotease. In another study by Bakota and colleagues, ESC secretome was delivered via an injectable nanofiber hydrogel for treatment of diabetes-induced kidney injury [98]. Human ESC secretome-infused nanofiber hydrogels were found to significantly reduce protein permeability in treated glomerular epithelial cells *in vitro*. The feasibility of future *in vivo* use of this drug delivery platform for sustained drug release was also demonstrated in preliminary studies that demonstrated preserved hydrogel localization in the abdominal cavities of treated mice over 24 hours [98].

The use of stem cell secretome has also been explored in the area of voiding dysfunction. Lin *et al*. investigated the effects of intraurethral injection of adipose-derived stem cells on recovery of continence in rats subjected to vaginal distension and ovariectomy to simulate childbirth-related injuries to the continence mechanism [31]. Despite limited cell engraftment on histologic analysis, these rats demonstrated significant functional recovery. These findings were suggestive of a potential paracrine/autocrine role of stem cells. A more recent study by Dissaranan *et al* further delved into potential paracrine/autocrine effects of stem cells by investigating therapeutic MSC secretome administration in a rat model of stress urinary incontinence [99]. They found that rats treated locally with MSC secretome administered in the form of conditioned media had recovery of continence as measured by leak point pressure. Furthermore, histologic examination of the urethra revealed an increase in elastin fibers and urethral smooth muscle suggestive of a potential mechanism by which rats regained continence. These findings have also been borne out in studies of other forms of voiding dysfunction. Song *et al*. utilized bladder wall injections of MSCs to demonstrate functional recovery in a rat model of overactive bladder from partial bladder outlet obstruction despite limited engraftment 4 weeks post-treatment [34].

Bladder dysfunction may also result from diabetes mellitus and manifest as urothelial dysfunction, alteration in smooth muscle, and neuropathy. In a study by Zhang *et al.*, diabetes was induced in rats via administration of a high fat diet and treatment with low-dose streptozotocin [100]. Rats then received injections of adipose-derived stem cells either intravenously or directly into the detrusor muscle. Cystometry demonstrated improved bladder function compared to untreated animals, and histologic analysis showed that only a small fraction of transplanted adipose-derived stem cells differentiated into smooth muscle cells at one month post-treatment. These findings were again suggestive that cellular engraftment and differentiation only plays a minor role in the therapeutic benefit of adipose-derived stem cells. The authors hypothesized the positive effects of stem cell transplantation may, in part, be due to the paracrine release of cytokines and growth factors [100].

Preliminary investigations into the use of stem cell secretome for erectile dysfunction, a major complication following radical prostatectomy, have also been conducted. Albersen *et al* studied the effects of intracavernous injection of adipose-derived stem cells or stem cell lysate in a rat model of erectile dysfunction via bilateral cavernous nerve crush injury [101]. Both administration of stem cells and of lysate alone resulted in significant recovery of function as assessed by cavernous nerve electrostimulation at 4 weeks post-treatment. Histologic analysis revealed less fibrosis and preserved corpus cavernosum smooth muscle content in treated animals. The authors postulate that the functional effects of lysate administration could result from either intracellular preformed substances or from secretion of bioactive molecules.

While urologic investigations involving stem cell secretome is only in its nascent stages, the studies thus far are promising and additional investigations into the use of stem cell bioactive factors could someday obviate the need for cellular injections in future stem cell-derived therapies.

7. Future Directions

7.1 Improving Upon the Stem Cell Secretome

A number of current approaches are aimed at investigating methods to augment stem cell paracrine, autocrine, or endocrine activity. Two primary methods have been used thus far: 1) preconditioning of stem cells, and 2) modification of stem cells via gene expression approaches [36, 102].

Stem cell preconditioning can be achieved using a variety of different strategies [36]. These include exposure of cells to hypoxic environments, exposure to different cytokine cocktails, and exposure to other cells. Multiple studies have demonstrated augmented therapeutic efficacy of MSCs exposed to hypoxic conditions that mimic the environment following ischemic injury *in vivo*. These cells upregulate expression of several pro-survival and pro-angiogenic factors, including VEGF, IGF-1, HGF, and angiopoietins [53, 59, 103]. Hypoxic preconditioning can enhance the therapeutic potential of secretome obtained from human MSCs administered for traumatic brain injury [104]. Preconditioned cells expressed enhanced levels of bioactive factors, such as HGF and VEGF, which are thought to account for the decreased brain damage and improved performance on motor and cognitive

functional testing in rats receiving conditioned media from preconditioned cells compared to those that received conditioned media from non-preconditioned MSCs. Hypoxic preconditioning has also been shown to enhance the therapeutic benefit of cardiac stem cell therapy for the treatment of myocardial infarction [105]. Hypoxic preconditioning was found to increase CXCR4 expression in cardiac stem cells and significantly increase their migration and recruitment to ischemic myocardium in a murine model of myocardial infarction. Four weeks after injury, mice that were administered hypoxic-preconditioned cells fared significantly better in terms of infarct size and heart function compared to mice administered cells grown under normoxic conditions. Furthermore, these functional benefits were largely negated upon administration of a CXCR4 inhibitor, strongly suggesting the role of hypoxia in the mediation of the CXCR4-SDF-1 axis.

Stem cell modulation via exposure to different cytokine cocktails also has been shown to attenuate negative host immune responses and promote angiogenesis. Anti-inflammatory activity of MSCs may be induced by incubation in a pro-inflammatory cytokine cocktail [106]. In a study by Yagi *et al* using a rat model of systemic inflammation, MSCs exposed to lipopolysaccharide-stimulated rat serum led to a responsive secretion of soluble tumor necrosis factor receptor 1 and decrease in nuclear factor kappa-B activation, a molecule previously implicated in inflammatory organ damage and mortality. Furthermore, preconditioned MSCs were found to attenuate a number of inflammatory cytokines and to decrease the inflammatory infiltration of neutrophils and macrophages in various organ systems as compared to non-preconditioned cells [106]. The use of cytokine preconditioning has been studied in models of ischemic stroke. For example, in a study by Sakata *et al*, IL-6 preconditioned neural stem cells were investigated in a mouse model of cerebral ischemia since IL-6 is known to promote a cell survival pathway via activation of STAT-3 [107]. IL-6 preconditioned cells expressed increased levels of VEGF and administration of pretreated cells lead to significant decreases in infarct size and improvements in neurocognitive functional testing.

Recent work has also shown that cell to cell interactions have great impact on the stem cell secretome. MSCs are often cultured as monolayers; however, these cells can lose stem cell-specific properties due to growth conditions that poorly reflect the *in vivo* microenvironment. In contrast, aggregation and growth of MSCs in 3 dimensional spheroids improves their ability for multilineage differentiation [108, 109]. Subsequent microarray analysis of these cells found many differences in gene expression compared to cells grown using traditional 2 dimensional culture techniques [110]. Upregulation of a number of genes was observed in this study including increased expression of CXCR4, anti-cancer proteins (TRAIL, IL-24, and CD82), the anti-apoptotic protein stanniocalcin-1, and the anti-inflammatory protein TSG-6. The observed upregulation in IL-24 was subsequently shown to selectively impair the viability of prostate cancer cells when exposed to the conditioned media of stem cells grown in this 3 dimensional configuration [108].

Genetic modification of stem cells has been described using both viral and non-viral genetic delivery to augment the production of specific tissue trophic factors [111, 112]. Not only have stem cells been modified to upregulate the expression of cytokines and growth factors such as VEGF, GDNF, galectin-1, and IL-10 but they have also been altered to produce

increased levels of therapeutic enzymes important in correcting pathophysiologic processes related to lack of a crucial enzyme [113-116]. Neri *et al*. genetically modified murine neural stem cells to express supranormal levels of beta-galactocerebrase (GALC) for therapeutic administration in a murine model of globoid cell leukodystrophy, a lysosomal storage disorder characterized by GALC deficiency. Modified neural stem cells were found to stably express GALC *in vivo* but overall improvements in symptomatology and lifespan were attributed not just to improved enzyme levels but also to alternative mechanisms, including cellular rescue through trophic support, immunomodulation, and neuroprotection. While the aforementioned studies have all involved therapeutic administration of the modified stem cells themselves, future avenues of study could involve administration of stem cell conditioned media alone or could involve the combination of multiple techniques to enhance secretome composition.

7.2 Hurdles in Clinical Translation

While the use of secretome represents a promising alternative to stem cell-based therapies, a number of challenges remain to be addressed prior to clinical translation. The study of stem cell secretome is still in its nascent stages. One of the greatest challenges is the determination of a therapeutic regimen taking into account the myriad complex interactions of paracrine factors during the acute and chronic phases of injury [117]. A better understanding of how cytokines are expressed during injury and wound healing and how they modulate the therapeutic effects of stem cells will aid in the development of more effective protein-based conditioning approaches [78]. Further study is required to better characterize the secretome and to define which factors are responsible for its therapeutic effects. Additionally, not all components of the secretome are beneficial and molecules such as tumor necrosis factor alpha or IL-6 may be harmful [68]. Improved definition of both the molecular pathways that control secretome expression and of the secretome composition itself will allow for improved control and regulation of its production, a key hurdle in translating secretome into a clinically useful product. Furthermore, modification of the secretion profile to augment the therapeutic effects of the secretome may be achieved with a better understanding of the effects of stem cell preconditioning or genetic manipulation strategies.

The use of conditioned media as a proxy for the administration of stem cell secretome also presents several limitations. Firstly, stem cells possess a dynamic expression profile that is difficult to capture with the use of conditioned medium which has a static composition. Additionally, conditioned medium is not a fully accurate representation of secretome in that it also contains proteins that are released during cell death. The preparation of conditioned media thus requires careful optimization for each cell type to avoid leakage of intracellular proteins from dead cells or from cells undergoing apoptosis [13]. Finally, the production and concentration of secreted molecules in quantities sufficient for clinical administration is also challenging.

Limitations of secretome therapy also include tissue transport, pharmacokinetics, and protein stability [78]. The delivery of these bioactive molecules may need to be coupled with bioengineered materials to achieve controlled release of stem cell conditioned media. The

timing of therapy is another factor that must be considered during clinical application. For example, granulocyte colony stimulating factor (GCSF) therapy administered at different time points and for different durations following myocardial infarction yielded different patterns of efficacy in a clinical trial for GCSF therapy [118]. This and similar studies highlight the need for a clearer definition of the role of secretome in stem cell recruitment and wound repair at different timepoints following injury. Some studies have suggested that early delivery of stem cell-based thearpies may be required for therapeutic benefit which could present a challenge in patient identification, recruitment, and availability and administration of therapy [119].

Furthermore, thus far we have only tested the use of single cytokines for clinical application. Unfortunately, many of these early clinical trials with single cytokine use have failed to meet expectations [120]. Safety, tolerability and efficacy have been a matter of significant concern and debate in these early clinical trials regarding single cytokine/growth factor therapies. Similar issues will likely be encountered in moving toward the use of stem cell conditioned media which encompasses a wide range of molecules, each with different effects on the growth and survival of host cells [78] [121]. Additionally, there are long-term theoretical risks associated with the administration of potentially angiogenic factors including an increased risk for development of neoplasia or retinopathy. Thus far, no link between angiogenic cytokine administration and the above pathologic processes have been reported in clinical trials. Nonetheless, candidates for therapy will need to be adequately screened and monitored for these conditions [120].

8. Conclusions

Since the discovery of MSCs in the bone marrow in the early 1970s, many other sources of MSCs have been identified and applied for potential use in a variety of clinical disorders including cardiovascular, neurodegenerative, autoimmune, and urologic applications. The therapeutic potential of stem cells has been associated with three main functions: 1) the ability of systemically delivered cells to home to sites of acute injury, 2) the ability for multipotent differentiation, and 3) the paracrine/autocrine action of stem cell secretome including its imunomodulatory and anti-inflammatory effects and ability to initiate or assist in tissue regeneration. The importance of the paracrine/autocrine function of stem cells via the stem cell secretome is being increasingly recognized and a number of studies have identified stem cell-specific factors as key players in the outcomes of many disease processes. However, exploration of the stem cell secretome is only in its nascent stages, particularly as applied to Urologic diseases and disorders. Advances in high-throughput technologies, protein microarrays, and bioinformatics have already facilitated analysis of the myriad soluble factors that comprise the secretome and will continue to aid in identification of secretome contents of multiple stem cell types under different conditions. To date, individual components of the secretome have been implicated in a number of essential cellular processes including angiogenesis and revascularization, immune and inflammatory modulation, and wound healing and tissue repair. Stem cell secretome has been studied in aggregate as conditioned media and shown efficacy in early animal experiments for diverse pathologic processes including those in cardiac, neurologic, and urologic fields, including renal injury. A number of hurdles remain to be overcome, however, prior to making

secretome a clinically viable option for stem cell-based regenerative therapies. More robust *in vitro* and *in vivo* models to study the effect of stem cell secretome are required to elucidate the complex pathways involved in stem cell-tissue interaction and to translate these findings into clinically relevant results. In addition, practical considerations such as the timing and mode of drug delivery after injury, the regulation and dosing of a complex set of bioactive molecules, and safety will need to be carefully addressed prior to clinical utilization. Stem cell secretome represents a novel, promising alternative to cell-based regenerative medicine therapies – one that could obviate many of the practical and oncogenic/immunologic concerns associated with stem cell use and with greater flexibility to provide a tailored individualized therapy either by modification of the secretome contents or the mode of delivery.

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Abbreviations

Akt	Protein kinase B
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bmp1	Bone morphogenetic protein 1
CXCR4	C-X-C chemokine receptor type 4
CXCL12	C-X-C motif ligand 12
Cyr61	Cysteine-rich angiogenic inducer 61
CCR1	C-C chemokine receptor type 1
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular vesicle
FGF-2	Fibroblast growth factor 2
GALC	Beta-galactocerebrase
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
MSC	Mesenchymal stem cell
SDF-1	Stromal-cell-derived factor 1
Sfrp2	Secreted frizzled related protein 2
STAT3	Signal transducer and activation of transcription 3

TGFα	Transforming growth factor alpha
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

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