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Mesenchymal stem cells: Identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stem cells. Although they were originally identified in bone marrow and described as 'marrow stromal cells', they have since been identified in many other anatomical locations in the body. MSCs can be isolated from bone marrow, adipose tissue, umbilical cord and other tissues but the richest tissue source of MSCs is fat. Since they are adherent to plastic, they may be expanded *in vitro*. MSCs have a distinct morphology and express a specific set of CD (cluster of differentiation) molecules. The phenotypic pattern for the identification of MSCs cells requires expression of CD73, CD90, and CD105 and lack of CD34, CD45, and HLA-DR antigens. Under appropriate micro-environmental conditions MSCs can proliferate and give rise to other cell types. Therefore, they are ideally suited for the treatment of systemic inflammatory and autoimmune conditions. They have also been implicated as key players in regenerating injured tissue following injury and trauma. MSC populations isolated from adipose tissue may also contain regulatory T (Treg) cells, which have the capacity for modulating the immune system. The immunoregulatory and regenerative properties of MSCs make them ideal for use as therapeutic agents *in vivo*. In this paper we review the literature on the identification, phenotypic characterization and biological properties of MSCs and discuss their potential for applications in cell therapy and regenerative medicine. We also discuss strategies for biomaterial micro-engineering of the stem cell niche.

1. Introduction

Stem cells are undifferentiated cells that can differentiate into more specialized cells. They can proliferate to produce more stem cells or differentiate into many different cell types in the body during development. In addition, in many tissues stem cells serve as an internal reservoir and repair system, dividing to replenish cells that have been lost as a result of injury or disease. Researchers have primarily worked with 3 types of stem cells: embryonically derived stem cells, fetal derived stem cells and non-embryonic 'somatic' or 'adult' stem cells. Embryonic stem cells are found in the blastocyst and epiblast while fetal stem cells are found in the fetal tissues of the developing fetus or new-born (e.g. umbilical cord), while adult stem cells can be found in adult tissue. Adult stem cells having

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a high intrinsic regenerative capacity maintain the normal turnover of organs. Blood, skin, fat and the intestinal epithelium are just a few examples of such tissues. Adult stem cells, generally unipotent or multipotent, have the ability to self-renew and differentiate towards different specialized cells. Adult stem cells are also present in children and adolescents. In contrast multipotent adult stem cells are rare and are generally present in small numbers in tissues and organs. Their basic role is to produce the specific cell types of the tissue wherein they reside. In this article we review the literature on the identification, phenotypic characterization and biological properties of mesenchymal stem cells (MSCs), a multipotent population of 'adult' stem cells. We discuss their potential for applications in cell therapy and regenerative medicine and also discuss strategies for biomaterial micro-engineering of their niche.

2. Identification of mesenchymal stem cells

When the first report of a 'fibroblast-like' osteogenic cell population isolated from the bone marrow (BM) [1] was published, the biological properties of these cells were largely unknown. Subsequent studies showed that bone marrow contains a population of cells capable of adherence to plastic culture dishes, a fibroblast-like morphology and the unique ability to differentiate towards different clonal subpopulations [2,3]. These properties are the major hallmarks of this cell type and have remained constant and undisputed features of their phenotype since their discovery. However, almost two more decades were needed to define and name these BM-derived cells as 'mesenchymal stem cells' (MSCs) [4] and describe their major biological characteristics.

In early studies MSCs were characterized as a cell population capable of differentiating into osteoblasts [5], adipocytes [2] and chondrocytes [6] *in vitro* [7], which was extended very soon with other tissues of mesodermal origin as tendon and ligament [8], cardiomyocytes [9] and muscle [10]. In parallel, an increasing number of studies reported a wider ecto- and endodermal differentiation potential of MSCs as well, including skin [11], retinal pigment epithelium [12], lung [13,14], hepatocytes [15], renal tubular cells [16], pancreatic islets [17], sebaceous duct cells [18] and neural cells [19,20]. These studies showed that the phenotypic potential of MSCs was wider than anticipated after their identification. MSCs have the capacity to differentiate towards all three lineages as ecto- meso- and endodermal tissues. It has long been known that MSCs are multipotent cells that can give rise to a wide range of cell types upon their differentiation which ends with a distinctive end-stage cell type [21]. However, they are considered to be multipotent cells from a 'mesenchymal' origin rather than true stem cells. As a consequence of the rapidly expanding information and published data, there was a need to unify the definition of the basic characteristics of MSCs, which was declared in 2006 by the International Society for Cellular Therapy (ISCT). The following criteria were proposed by the ISCT [7]:

MSCs are:

- plastic-adherent under standard culture conditions (α minimal essential medium plus 20% fetal bovine serum);
- express CD105, CD90, CD73 and CD44, and lack the expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR;
- and must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*.

Although this definition was precise enough to combine the majority of the existing knowledge at that time, other published reports suggested that MSCs were biologically more divergent. Many subsequent studies have shown that MSCs are residual cells in almost all organs, located in the connective tissue compartment of a given organ and can be easily isolated due to their 'adhesive' nature and phenotypic characterization using specified cell surface markers. MCSs were identified and successfully isolated from a wide range of tissues such as adipose tissue [22], lung [23], liver and bone marrow [24], umbilical cord (Wharton's jelly) [25], synovium [26], amniotic fluid [27], fetal blood [28], dental pulp [29], skeletal muscle [30] or even from the circulatory system [31] (Fig. 1).

These studies raised another important question: are BM-derived MSCs equivalent to MSCs obtained from other tissues/organs source derived or are they divergent from them? It is now clear that there is ongoing controversy concerning the definition of these cells. Moreover, new nomenclature and classification criteria were also suggested by different laboratories: e.g. multipotent stromal cells, multipotent adult cells, mesodermal progenitor cells, marrow-isolated adult multi-lineage inducible (MIAMI) cells [32], or 'mesenchymal-like cells'. More and more studies were published to suggest that BM-derived MSCs might be different from adult organ derived MSC-like populations and the similarity is 'just' a stem cell marker expression rather than the same ontogeny of cell population. Accordingly, to give a precise definition seems complicated, if at all possible [33,34].

2.1. Where do MSCs come from? – the origin and physiological roles of MSCs in the body

To better understand the functional roles of MSCs, we must first review several aspects of their basic cell biology and developmental attributes. First, we should start with their ontogeny and determine where and how they arise. Bone marrow (BM) is situated in bones, as a soft and flexible tissue. As we now know, the basic role of bone marrow cells is to repopulate the blood by providing a multipotent cell population (also known as hematopoietic stem cells, HSCs) capable of differentiating towards different cell types of blood during hematopoiesis. BM is also responsible for the production of the elements of the lymphatic system (e.g. lymphocytes). This means the hematopoietic compartment of BM is a mixed population of (i) multipotent stem cells which are able of self-renewal, (ii) committed progenitor cells (both myeloid and lymphoid lineage) and (iii) maturing cells [34]. However, BM also contains other cell types, which are not directly involved in hematopoiesis. However, these other cell types are still relevant to the hematopoietic microenvironment (HME); these cells are called the marrow stromal compartment or marrow stromal cells. BM stromal cells include fibroblasts, stromal cells, macrophages, adipocytes, osteoblasts, osteoclasts, vascular endothelial cells and endothelial stem cells, interspersed in trabecular bone. In a more complex manner, these cells establish and organize the hematopoietic niche *in vivo* through a multistep cell lineage [35]. This very heterogeneous cell population is responsible for maintaining the diverse distribution of cell morphologies, gene expression and tissue growth rates.

Developmentally, hematopoietic cells arise from the 'islands of hematopoiesis', located in the wall of the yolk sac (an extra-embryonic tissue of the developing embryo), stemming from mesenchymal cells named hemangioblasts. During fetal development the developing liver begins to play a role in hematopoiesis and subsequently the spleen then the newly formed red bone marrow – or rather 'primitive marrow' – becomes the major location for hematopoiesis [36]. At this time, the role of fetal liver and spleen in the production of blood cells significantly diminishes. The process of hematopoiesis is constantly ongoing through the life of humans, producing about 100 billion cells daily.

The BM (*medulla ossium*) is located in the marrow cavity and in spongy bone. There are two types of BM: red (*medulla ossium rubra*) and yellow (*medulla ossium flava*) also known as 'fatty

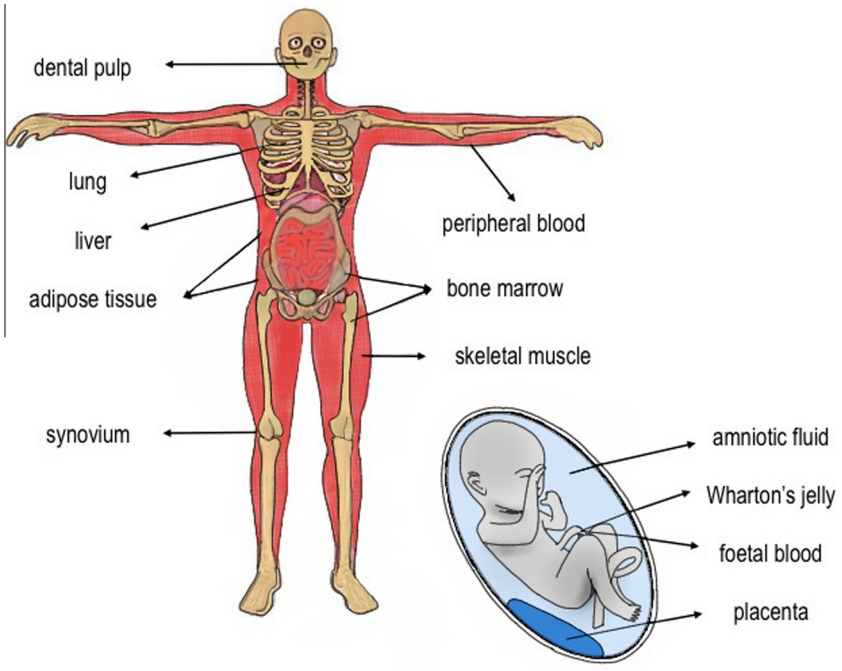

Fig. 1. Adult and fetal/neonatal tissue sources of mesenchymal stem cells in the body. Mesenchymal stem cells can be isolated from several tissue sources of the adult but fetal and neonatal birth-associated tissues contain MSCs in different quantities.

marrow'. Red BM is rich in vessels, a very soft substance of cells, which is responsible for hematopoiesis. In adulthood, red marrow is predominately located in the flat bones such as pelvis, vertebrae, cranium sternum, scapulae and ribs and also in the cancellous ('spongy') material at the epiphyseal ends of long bones such as humerus and femur [36]. Remarkably, the capacity of the hematopoietic activity decreases in long bones like the tibia and femur with aging, as they provide less than 5% of the total cell release when we reach our twenties. Interestingly, in cases of severe blood loss, when it is necessary, yellow marrow can revert back to red marrow to increase blood cell production.

The non-hematopoietic stem cells of the primitive marrow stroma are established in the same developmental processes through a series of events and developmental episodes. This cluster of cells is less than 0.01% of the overall cell population resident in the BM [37]. They are actively replacing the 'used' cells but also, upon injury, they are activated and able to replace, regenerate or rejuvenate compromised or damaged adult tissues. Upon receipt of appropriate signals and environmental clues, the progenitors begin to divide and are committed to a given lineage which terminates with a specific end-stage cell type (e.g. cartilage, bone, intervertebral disc, tendon or ligament; Fig. 2) [33].

Based on their developmental origin and structure within bones the cell replacement and rejuvenation function of MSCs is clear. The other major function of MSCs is to secrete bioactive molecules. Bone marrow MSCs produce growth factors, cytokines, which are able to regulate the production of blood cells. This trophic effect is important in the maintenance and regulation of the local microenvironment [38]. Nonetheless, the hematopoietic compartment is strongly vascularized. Hematopoiesis takes place around the specialized sinusoids that drain into the central vein. Mature cells translocate from the site of their growth and maturation through the wall of the sinusoids by active trans-endothelial migration. The sinusoids are lined with specialized endothelial cells and sub-endothelial pericytes [39] which have a very active phagocytosis and able to produce growth factors (mainly hematopoietic cytokines) [36]. In this niche stromal cells are involved in the maintenance and regulation of the microenvironment called as

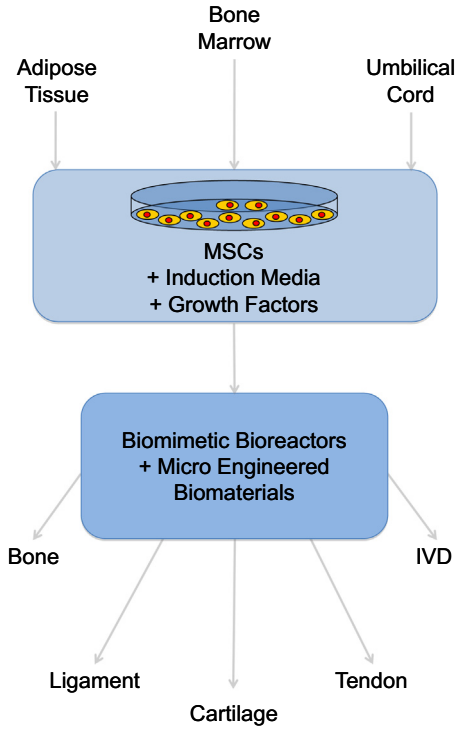

Fig. 2. Mesenchymal stem cells isolated from bone marrow, adipose tissue or umbilical cord may be treated with induction media, growth factors and then combined with microengineered biomaterials in biomimetic bioreactors to generate musculoskeletal tissues for regenerative medicine applications.

hematopoietic niche. This effect can be direct or indirect: in the first case secreted biomolecules activate intracellular signaling pathways, while in the second case signaling is initiated in a neighboring cell, resulting in the release bioactive molecules (paracrine signaling). Examples include G-CSF, M-CSF [40] or RANK ligand [41]. Furthermore, they are also involved in inflammatory

processes. Therefore, it seems MSCs may play specific roles as immunomodulators in several processes such as transplantation tolerance, autoimmunity, tumor evasion, and importantly in fetal-maternal tolerance in case of a pregnancy [42,43].

3. MSCs as residual stem cells in organs

Until now it has widely been accepted that almost all adult tissues have reservoirs of specific stem cells that influence turnover and regenerative processes. Examples include satellite cells of muscles [44], epithelial stem cells [45] or neural stem cells [46].

MSC-like cells have been identified and isolated from a variety of organs/tissues. These cells are similar in their molecular expression profiles (including cell surface marker expression) and biological functions but maintain a distinctive differentiation process depending on their tissue origin. These stem cells play a major role in the repair of damaged tissue in two different ways: first, by directly differentiating to different resident cell types and second, by secreting trophic factors which can trigger the tissue repair processes. It is also widely accepted that they have a role not just in the repair mechanism but also more prominently in tissue cell turnover.

'Mesenchyme' is the derivative of the embryonal mesodermal connective tissue. In this respect MSCs are the residuals of the embryonal mesoderm compartment, remaining undifferentiated in the connective tissue of different organs and the bone marrow as well. If we accept the mesenchyme theory and draw a parallel with BM-derived MSCs, their role in organs will become obvious: (i) cell turnover, (ii) cell replacement or repair, (iii) rejuvenation and (iv) immunomodulation. Again, in this line the effect can be direct through differentiation or secreting trophic factors, or indirect through trophic factors which trigger other cells in the vicinity. It is an open question how these cells of 'mesenchyme' origin can be located in different germ layer-derived organs and became a residential stem cell pool of a given organ/tissue. Although there are several theories of how and why the connective tissue is the major source of the adult stem cells, none of them are fully convincing. There is controversial data about how MSCs might be related to pericytes, although they are closely situated in the hematopoietic niche and in several organs in the perivascular niches [47]. However, it is clear that naive MSCs reside in the perivascular region in a quiescent state [48]. Despite the publication of hundreds of papers on the subject, the origin of MSC-like cells residing in adult organs is uncertain. Moreover, the relation and ontogeny between BM-derived MSC and MSC-like cells isolated from different organs/tissues is still unclear.

4. Sources of MSCs

As we discussed above, BM stromal cells are widespread in trabecular bones and due to their accessibility, expandability, and multipotent nature BM-derived MSCs hold significant promise for applications in tissue engineering and regenerative medicine. However, there are several well-defined sources for MSCs in the body besides the BM. Probably the most primitive MSC population can be obtained from fetal tissues such as the umbilical cord tissue, the Wharton's jelly [49] and the umbilical cord blood [25] (see Fig. 1). We have to note that although umbilical cord blood contains MSC cells as well, it is rather a good HSC source, while Wharton's jelly contains mainly primitive MSCs. This primitive nature of MSCs derived from umbilical tissue increases their potential in therapeutic applications [49]. Furthermore, as a fetal cell source, the amniotic fluid has been shown to contain MSCs which can be obtained with amniocentesis or at the time of the birth [27]. Other birth-associated tissues like placenta and amnion contains MSCs as well, however due to their amount and to the fact that they contain a mixed population of endothelial stem/progenitor cells (EPC, ECFC) and hematopoietic stem cells (CD34+, CD133+), their potential usage is different from pure MSCs [50].

With aging, the next potential but still 'young' cell source is the developing tooth bud of the mandibular third molar, which is very simple to collect between the ages of 8–10 in children [29]. There is a debate if these cells are multi- or might be pluripotent, however, they might be a major source for cell banking in the future and might be able to replace umbilical cord cell banks [51].

Perhaps the richest source of MSCs in adults is adipose tissue (AT), which is easily accessible and a well-characterized methodology is available for the isolation of cells from this source. As it is estimated, about 500 times more AT-MSCs can be isolated from fat tissue than from the same amount of BM [52]. Of course, MSCs can also be isolated from the peripheral blood as well, however, their quantity is very limited, compromised by several factors relating to the donor (e.g. age, sex, daytime, feeding habit, health status, administered drugs, etc.) [53]. Furthermore, a new potential cell source for MSCs can be obtained by *in vitro* differentiation of pluripotent cells such as ESCs or iPSCs [54,55].

5. Potential clinical applications of MSCs

An increasing number of publications have highlighted the importance and potential of MSCs in regenerative therapy. While a study from 2012 mentioned about 200 active clinical trials [56] a fresh search in the same public database (http://clinicaltrials.gov), for 'mesenchymal stem cells' identified 524 studies in a wide variety of different diseases. The most commonly studied areas are cardiovascular diseases (e.g. myocardial ischemia, acute myocardial infarction, heart failure, cardiomyopathy); graft-versus-host disease (GVHD); liver diseases (liver cirrhosis, liver failure, liver transplantation), anemia, Alzheimer's disease, spinal cord injury, ischemic stroke.

An interesting and important area is the treatment of autoimmune diseases, which relies on the immune-modulatory effect of MSCs. The inhibition of innate immune activation by MSCs can occur through hampering the maturation of dendritic cell, or impairing the activation of macrophages or directly blocking the inflammatory signal (e.g. producing IL-1 receptor antagonist or IL-10), reviewed in [57,58]. The inhibition of allogeneic T cell proliferation by MSCs can induce generation of regulatory T (Treg) cells [59], which can modulate the immune system and cause immunosuppression (reviewed in [60]). As we mentioned above, this effect can be used in the treatment of GVHD, but might be applicable in autoimmune diseases, like multiple sclerosis, rheumatoid arthritis (RA), lupus, neuromyelitis optica or Type 1 diabetes mellitus, and represents a very dynamically developing field and holds a great potential in the treatment of severe diseases. In fact the only approved treatment using MSCs, and at the same time the first 'off-the-self stem cell drug' *Prochymal*, is used in special infant cases of GVHD [61].

The list of potential applications is promising and impressive. Indeed, when we narrowed our search, 44 'stage 3' and 'stage 4' studies were found. This suggests that the progression of these studies is likely to be important for the development of clinical applications for MSC therapy. Even though many of these studies may fail, the knowledge gained will be important for therapeutic innovation in the coming decades.

6. Biomaterial micro-engineering of the stem cell niche

Guiding stem cell fate decisions towards a specific lineage is governed by a variety of factors [62–64]. These cellular processes are tightly regulated within their three-dimensional (3D) microenvironments and intricate interactions. Therefore, in order to mimic their native microenvironment, it is critical to modulate and recapitulate these complex cellular architectures, properties

and signaling pathways [65,66]. For example, biomaterials that mimic the native extracellular matrix (ECM) could provide the necessary biophysical and bioactive cues with specific niches required for guided cellular remodeling [67]. Previous studies have assessed the role of biochemical cues that include direct cell–cell contacts or soluble factor signaling in guiding stem cell fate decisions [68–70]. On the other hand, others have concentrated on how biophysical properties affect cell behavior [63,71,72]. Depending on the biomaterial synthetic strategy a variety of biophysical parameters can be modulated such as swelling, porosity and degradation where each variable can significantly influence scaffold properties and ultimately stem cell behavior [67,73,74]. More complex systems can be built by controlling the release of bioactive cues as a function of biomaterial degradation to induce changes in cell spreading, migration and viability. However, most studies have been unable to fully recapitulate the stem cell niche and only offer a glimpse into stem cell linage commitment.

Initial studies on the spatiotemporal control of stem cell differentiation were performed on two dimensional (2D) biomaterial substrates. These 2D approaches can be configured for high-throughput screening (HTS) that lower cost and support studying multiple and often synergistic parameters at once [65,75,76]. Even though major achievements have been made in stem cell culture and directed differentiation, 2D approaches are intrinsically limited and are ultimately unable to fully capture the native tissue architecture. Furthermore, several reports have already demonstrated that cell behavior significantly differs in 2D versus 3D microenvironments [77–80]. Ultimately, native tissues provide dynamic presentation of both biophysical and biochemical signals that are able to regulate stem cell fate [81–83]. These soluble (i.e. growth factors) and insoluble (i.e. ECM proteins) spatiotemporal signals are the guiding features present throughout both embryonic development and tissues morphogenesis. Such dynamic cell signaling and organization could potentially be simulated within innovative micro-engineered biomaterials that could exhibit a physiologically relevant cell microenvironment [65,75]. Currently, at least four clinical trials are ongoing with biomaterials, all of them related to bone tissue engineering (source: ClinicalTrials.gov).

To generate the next generation of micro-engineered biomaterials powerful tools stemming from micro- and nanoscale technologies are being applied [84]. These micro- and nanofabrication techniques are based on a 'bottom-up' and a 'top-down' method [67,74]. Top-down approaches aim to control cell–cell interactions within mesoscale biomaterials with the use of miniaturization techniques such as nanotopography thereby providing biophysical cues for stem cell differentiation. Alternatively bottom-up approaches can be used that include micro- and nanopatterning techniques based on photolithography, micromolding, and microfluidic-based methods. More recently, rapid, additive processes such as bioprinting have revolutionized the field and were able to produce 3D cell-laden biomaterials. These methods are able to readily produce combinatorial 3D microenvironments that allow for systematic analysis of multiple insoluble (i.e. biophysical properties including ECM protein composition) and soluble (i.e. biochemical cues including growth factors) signals on stem cell fate [65,67]. Similarly, recent approaches utilize self-assembly to produce cell-laden biomaterial blocks with controlled shape and size of unique micro-environmental niches.

For improved spatiotemporal control of biomolecular cues several critical challenges remain. For example, perfusable systems under dynamic culturing conditions within micro-engineered biomaterial niches could advance temporal control of micro-environmental cues, be able to sustain long-term culturing and limit unwanted cellular crosstalk during HTS screens [85,86]. When coupling controlled biomaterial biophysical properties and spatiotemporal control of biochemical cues these platforms could significantly advance manipulation of stem cell behavior [65–67]. Recent reports have demonstrated that novel micro-engineering approaches can ultimately have translational impact and be able to replicate these microenvironments to the macroscale constructs. We hope that soon many platforms will probe the complexity of the stem cell microenvironment in great detail that goes well beyond individual tissue engineering applications. When combined these and other improved designs we can imagine broad studies that could support intricate cell manipulation. Ultimately such systems could be the stepping-stone to personalized and precision medicine where an individual's stem cell could be used for generating customized tissue engineered constructs. We envision that such platforms have the potential to go beyond tissue engineering and accelerate the next generation of novel biomaterials for variety of applications that include regenerative medicine.

7. Conclusions

The objective of regenerative medicine is to develop novel therapies to replace or restore function to tissues and organs within the human body. By combining cell biology and materials science translational technologies are being developed for clinical applications. MSCs are multipotent stem cells with significant potential in regenerative medicine and as such are currently the subject of more than 200 clinical trials aimed at treating a broad range of degenerative conditions [87]. Many of these clinical studies have reported on immediate or medium term improvements of clinical symptoms. Furthermore, systemic application of MSCs has shown benefits in different pre-clinical disease models including acute lung injury, myocardial infarction, diabetes as well as renal and hepatic failure. The immunoregulatory properties of MSCs highly complement their regenerative properties. The production and secretion of immunomodulatory and cytoprotective factors contributes to the regeneration of injured tissues. The release of paracrine factors by MSCs also provides protective micro-environmental cues and promotes the activation of local tissue-resident progenitor populations. These properties make MSCs very suitable for use as therapeutic agents *in vivo*, especially for regenerating damaged or diseases musculoskeletal tissues (Fig. 1).

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Contributors

The authors researched, discussed and approved the concept, drafted and submitted the commissioned paper. All co-authors

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Conflict of interest statement

The authors wrote this paper within the scope of their academic and affiliated research positions. The authors declare no conflict of interests.

Competing interests

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