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REVIEW

Enhancing survival, engraftment, and osteogenic potential of mesenchymal stem cells

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Abstract

Mesenchymal stem cells (MSCs) are promising candidates for bone regeneration therapies due to their plasticity and easiness of sourcing. MSC-based treatments are generally considered a safe procedure, however, the long-term results obtained up to know are far from satisfactory. The main causes of these therapeutic limitations are inefficient homing, engraftment, and osteogenic differentiation. Many studies have proposed modifications to improve MSC engraftment and osteogenic differentiation of the transplanted cells. Several strategies are aimed to improve cell resistance to the hostile microenvironment found in the recipient tissue and increase cell survival once transplanted. These strategies could range from a simple modification of the culture conditions, known as cell-preconditioning, to the genetic modification of the cells to avoid cellular senescence. Many efforts have also been done in order to enhance the osteogenic potential of the transplanted cells and induce bone formation, mainly by the use of bioactive or biomimetic scaffolds, although alternative approaches will also be discussed. This review aims to summarize several of the most recent approaches, providing an up-to-date view of the main developments in MSC-based regenerative techniques.

Key words: Mesenchymal stem cells; Bone regeneration; Hypoxia; Anoikis; Preconditioning; Bioactive scaffolds; Senescence; Engraftment; Homing; Osteogenesis

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Core tip: Mesenchymal stem cells (MSCs) are important tools for a wide range of therapeutic applications, including the treatment of critical size fractures or bone defects. However, whereas early clinical studies showed great expectations, long-term benefits of MSC-based treatments are not entirely successful. Transplanted cells had to face a series of important challenges that greatly reduce their survival and engraftment, and thus, their capacity of regenerating the target tissue. Although there is solid data indicating that the paracrine actions exerted by MSCs are equally important in the outcome of the treatment, this review is based on the current strategies aimed to enhance the tissue regeneration directly occurring from the engraftment and differentiation of the transplanted MSCs.

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INTRODUCTION

First described by Friedenstein[1] in 1967, mesenchymal stem cells (MSCs) are adherent cells with a spindle shape, resembling fibroblasts, capable of self-renewal and differentiation into mesodermal lineages, such as osteocytes, adipocytes, or chondrocytes. This specific type of stem cells has unparalleled features that make them a unique and valuable tool for tissue repair and other cell-based therapies. In fact, nowadays, MSCs-based treatments are the experimental therapies drawing more attention in the generative medicine field, being the subject of nearly a thousand registered clinical trials, complete or on-going worldwide (www.clinicaltrials.gov)[2]. Due to their ability to differentiation towards the osteogenic lineage, in the last few years, there has been an increasing interest in using MSCs-based approaches to improve bone repair and regeneration. In particular, the use of MSC-based therapies would certainly benefit the treatment of non-union fractures or critical size bone defects resulting from direct trauma or from the removal of large bone areas through surgical procedures in patients with osteosarcoma, necrosis, or other pathologies. Due to the known drawbacks of autologous and allogeneic bone grafts, bone tissue engineering has emerged as an interesting alternative and the combination of MSCs with biocompatible scaffolds represents a promising strategy for treating critical size or non-union fractures.

Unlike other stem cells, MSCs are able to maintain a high degree of plasticity, expressing also ectodermal and endodermal genes[3]. This gives them the ability of trans-differentiating and producing cells from other germ layers, thus challenging the previous concept that tissue-derived adult stem cells could only give rise to cells and cell lineages found in the tissue of residence. In fact, MSCs have been found to produce, under specific circumstances, skin, neural, and hepatic cells[4].

Although the regenerative potential of MSCs was initially linked almost exclusively to their ability to differentiate into multiple cell lineages once engrafted in the recipient tissue, nowadays the extensive paracrine activity seems to be the focus of many studies since it appears to be directly related to the therapeutic action of MSCs. Besides being able to replace cells in damaged tissues, MSCs can also produce secretory factors that play critical roles in tissue repair and immune response modulation, and support both engraftment and trophic functions (autocrine and paracrine actions)[5]. Those secretory factors comprise cytokines, chemotactic factors, molecules involved in the remodelling of the extracellular matrix (ECM) and growth factors[6]. Thanks to the secretion of these molecules, after *in vitro* administration, MSCs can migrate to damaged tissue and promote the establishment of an anti-inflammatory environment that supports proliferation and avoids cell death, thus stimulating tissue remodelling and survival[7,8].

In addition to these properties, MSCs are generally easy to source from different adult tissues such as fat, blood, or dental pulp, using relatively simple, and minimally invasive procedures, making these cells very attractive for their use in the clinic. However, in relation to bone regeneration, MSC-based therapies, specifically bone marrow MSCs (BM-MSCs), which have associated a more complicated extraction method, seem to display the highest osteogenic potential when compared to MSCs

sourced from other tissues. Adipose derived stem cells (ASCs) seem to have similar osteogenic characteristics as BM-MSCs[9], but also possess the advantages of being easily isolated and of being present at a much higher concentration in the source tissue (500 times greater than that of the BM-MSCs)[10]. Although ASCs represent a good alternative to BM-MSCs due to these characteristics, the studies using these cells are still scarce and more information is needed referring their usefulness in bone repair.

Despite having been proven to have short-term benefits, the long-term benefits of MSC-based therapies are not currently clear, and the final outcome of the treatments involving MSCs show high inter-patient variability[11]. Importantly, the limited benefits seen in clinical trials are linked to the low engraftment and survival rate of the transplanted MSCs, regardless of the tissue of origin[12], and to ineffective osteogenic differentiation. At this point, it is important to highlight that different characteristics of the transplanted MSCs are required depending on their subsequent application, that is, whereas homing would be crucial for the treatment of systemic bone loss, such as that linked to osteoporosis, this has no relevance when MSCs are used to build bone grafts *in vitro*.

Current studies in the field of tissue engineering are now focused on finding the appropriate conditions that would lead to successful tissue regeneration. One possible strategy to increase the success rate of the MSC-based techniques is producing cells that are able to resist the hostile microenvironment through what is called cell-preconditioning, so they can increase their survival in the recipient tissue. This review will discuss the different approaches used for cell preconditioning, from the modification of culture conditions that promote cell survival and engraftment to the use of bioactive scaffolds that would increase the osteogenic capacity of the transplanted cells.

OPTIMIZING MSCs SURVIVAL AND ENGRAFTMENT

Cell survival, once transplanted in the recipient tissue, may be affected by length and culture conditions, such as the presence of serum or oxygen, mechanical stress during the implantation procedure, or cell death due to the lack of an anchorage among others. In the following sections, we will discuss the influence of all these factors on the success of the engraftment and the possible solutions proposed by different authors.

Optimizing in vitro culture conditions

Avoiding replicative senescence: The amount of MSCs that can be sourced from adult tissues is limited, thus, it is imperative to expand them *in vitro* in order to obtain the sufficient number of MSCs needed to achieve maximum therapeutic effect. However, clinical applications require that no differentiation potential is lost during the expansion process. This is particularly troublesome in the case of BM-MSCs, due to the low percentage of these cells present in the bone marrow, and therefore, the necessity of prolonged time in culture and an increased passage number. This need for a high number of MSCs brings up one of the first limitations to their clinical use: their limited replicative lifespan. In fact, it has been estimated that MSCs cultured *in vitro* can achieve a maximum of 15 to 30 population doublings, depending on donor age[13,14]. Although this restricted proliferative capacity would represent a safety advantage, since it ensures a low probability of malignant transformation, a large scale *in vitro* expansion also leads to the loss of proliferation and differentiation capacity, which would deem them unsuitable for several regenerative procedures[15,16].

Telomere shortening, one of the main hallmarks of aging[17], has been measured during culture of MSCs. Various studies clearly demonstrate that telomere attrition leads to BM-MSC senescence[13] and in fact, this shortening has been even established on 17 base pairs lost on each MSC division *in vitro*[13,14]. However, other works claim that no changes in telomere length are detected after 25 passages[18], therefore, the relevance of telomere shortening in the acquisition of a senescent phenotype after prolonged *in vitro* culture is currently controversial. Another hallmark of aging[17], the accumulation of free radicals or reactive oxygen species (ROS), has been linked to a decrease in adhesion of MSCs[19], something crucial for the engraftment of the transplanted cells, and also to an increased adipogenic potential[20] that would hamper their use to bone regeneration techniques. Oxidative stress is also a factor directly linked to a decreased cell survival[21]. At this point, it is interesting to mention that pretreatment of MCSs with vitamin E, done by Bhatti *et al*[22], seems to result in a protective effect against oxidative stress by increasing cell anabolism.

During prolonged cell culture, MSCs also suffer changes that result in an inability

to maintain the structure and function of chromatin, something indispensable for the correct execution of the gene transcription program[23,24]. Indeed, important changes in DNA methylation have been detected during *in vitro* expansion of MSCs[25]. These and other changes at the level of the epigenome (*i.e.*, histone methylation or acetylation) would have an important impact on gene expression. In fact, according to Wagner *et al*[26], more than 1000 transcripts were up-regulated at least two-fold in senescent MSCs whereas over 500 transcripts were down-regulated. Part of these changes in gene expression levels would lead to the acquisition of a senescent state[27], the subsequent decrease in MSCs viability[21], and the loss of potential.

To prevent cultured MSCs undergoing replicative senescence during *in vitro* expansion, different approaches have been analysed. Some of those methods were based on preventing telomere shortening, due to the putative link between this process and MSC aging. One way to achieve this goal is to express the catalytic subunit of telomerase so that the cells can further divide without losing telomere length[28,29]. However, these modifications so far have been done using viral-based vectors as vehicles, and therefore this could potentially transform the recipient cells, precluding their use in the clinic.

More recently, a different approach, based on introducing variations in the culture media to avoid replicative senescence rather than on modifying the gene expression of the MSCs, has been tested. Grezella *et al*[30] tried to reduce the presence of senescent cells in MSCs cultures by using senolytic drugs. Although four different drugs were tested, only one of them (ABT-363_Navitoclax) seemed to have selectivity for senescent cells. The results, however, were not encouraging since this drug also affected non-senescent cells to some extent and no rejuvenation of MSCs was detected in terms of gene expression signature or telomere length. Other senolytic drugs are currently being investigated, which might, either on its own, or in combination with other compounds, have a clearer rejuvenating effect on MSCs in culture. Interestingly, other authors have managed to reduce the percentage of senescent MSC during *in vitro* expansion, by simply growing them in a defined xeno-free human plasma fraction[31] or in the presence of platelet lysate as a substitute of foetal bovine serum (FBS)[32]. This method has the additional advantages of avoiding the risk of transmission of zoonotic infections as well as immunological reaction to xenogenic supplements used in culture, such as FBS.

All in all, it seems clear that assaying MSCs aging "status" during their culture *in vitro* and positively selecting for non-senescent cells prior to their use in cell-based therapies would certainly improve the outcome of the procedures. This screening could be simply done by a variety of methods, with the easiest one being the observation of MSC size and morphology in culture, since MSCs rapidly loose their spindle shape and increase their size up to 10 times at later passages[26,33], a process associated with an increase in actin stress filaments[34]. This visual method, however, is highly subjective and does not allow accurate quantification of the percentage of senescent cells in culture. Bertolo *et al*[35] developed an *in vitro* expansion score to quantify the senescent state of MSCs and predict whether the cells would maintain their differentiation ability. By measuring population doubling time, senescence-associated β-galactosidase expression (SA-β-gal), cell size and telomere length, and assaying colony forming unit potential, these authors clearly demonstrated that whereas early passages of cells (from P1 to P3) maintained all their potential, at late passages (>P7) MSCs lost their osteogenic and chondrogenic potential while gained adipogenic potential. Another approach to increase the percentage of replicative active MSCs to increase chances of success in cell-based therapies would be to positively select cells free of senescence markers. Although scoring all the previous parameters will certainly help evaluate the state of MSCs, the various techniques involved made this process highly time-consuming. Interestingly, the same group recently published a fast and label-free flow cytometry-based approach to quantify the percentage of senescent cells in a given culture[36]. This method could be extremely useful to select MSCs with high regenerative abilities for subsequent applications.

Hypoxic preconditioning: Pathological conditions susceptible of being treated by MSCs transplantation are normally linked to the death of specialized cells in a particular tissue, as a result of toxic agents or autoimmune processes. The microenvironment surrounding this damaged tissue will have associated severe ischemic conditions (≤1% oxygen) that can also be the cause that triggered cell death. *In vitro* MSCs cultures are mainly maintained in normoxia (21% of oxygen), while the natural niche of the MSCs has a constant moderate hypoxia with concentrations ranging from 1% to 7% oxygen[37]. This restricted hypoxic microenvironment found in diverse pathological tissues also applies to bone defects, where the hypoxic conditions (<1% oxygen) close to the anoxia that can be found in the fracture microenvironment following bony injury, are favoured by the low vascularization at the implantation

site.

The dramatic transition suffered by the transplanted cells going from normoxia to hypoxia or anoxia, could be alleviated by cell preconditioning[38,39]. Since transplanted MSCs are likely to be placed in a hypoxic environment, culturing the cells in hypoxic conditions might improve their survival. Besides reducing the percentage of oxygen in the culture settings, addition of other drugs might help the engraftment process. In fact, Zhang *et al*[40] showed that BM-MSCs that have been preconditioned in hypoxia (0.1% oxygen) in serum free medium and in the presence of 0.5 mmol/L dimethyloxaloylglycine (DMOG) had an improved angiogenic capacity. This improvement was related to the upregulation of hypoxia inducible factor-1α (Hif-1α), which enables cells to survive in oxygen deprivation conditions by providing oxygen-independent adenosine triphosphate (ATP) production or by inhibiting apoptosis induced by hypoxia. Importantly, after being cultured in those conditions, MSCs also showed a greater osteogenic and regenerative potential even in aged animals, where the MSCs potency is known to be already very limited[40-42].

Other pharmacological agents are also able to improve cell survival when MSCs face a hypoxic microenvironment. Pretreatment of MSCs with trimetazidine enhanced cell viability when cells are re-oxygenated after being exposed to hypoxic conditions. The effect of trimetazidine might also be mediated by HIF-1α *via* upregulation of the anti-apoptotic gene Bcl-2, and downregulation of Bax, an apoptotic gene[43]. Kheirandish *et al*[44] developed a system of preconditioning consisting of culturing the cells during 15 min in 2.5% O_2, re-oxygenation for 30 min in 21% O_2, and hypoxia preconditioning in 2.5% O_2 during 72 h. This system seems to significantly improve the proliferation and migration abilities of MSCs *in vitro*. According to these authors, the re-oxygenation after a few minutes of hypoxia improves the expression of pro-survival genes as well as the expression of various trophic factors, angiogenic factors, VEGF, and basic fibroblasts growth factor (bFGF) in MSCs[45,46]. Moreover, this re-oxygenation process also results in a decrease of caspase-3/7 activity and lactate dehydrogenase release, decreasing the sensitivity of the cells to the ischemic microenvironment[47,48]. Another important point in favor of MSC preconditioning in hypoxic conditions is the evidence that this procedure seems to inhibit the malignant transformation of MSCs after transplantation[49].

Regarding specifically to the effect of hypoxic preconditioning in bone regeneration, in animal models, hypoxic conditioning seems to lead to an enhanced angiogenic and osteogenic potential[50,51]. Also, in human MSCs, there are data indicating that culturing MSCs in 2% and 5% O_2 highly favors their proliferation and increases their osteogenic differentiation[52,53]. In addition, one of the factors that reduces cell survival when MSCs reach the target tissue is oxidative stress[21]. Interestingly, pretreatment of MCSs with vitamin E, as described by Bhatti *et al*[22], results in a protective effect against oxidative stress by increasing anabolism of the cells.

It is important to highlight that when MSCs are re-implanted, they not only have to face hypoxia to the point that they can become apoptotic, but they also have to face a lack of nutrients[54,55]. Wang *et al*[54] observed that MSCs preconditioning with a low dose of lipopolysaccharides reduced the apoptosis induced by hypoxia and nutrient depravation by inhibiting the downregulation of CX43, a process apparently related with the Erk signaling pathway. Sun *et al*[56] demonstrated that preconditioning of MSCs with sevoflurane not only minimized cell apoptosis when exposed to hypoxic-serum depraved media but also enhanced MSC migration, suggesting that this improvement in the therapeutic potential of MSCs might be related to the upregulation of HIF-1α, HIF-2α, VEGF, and pAkt/Akt.

Three-dimensional (3D) cultures: 3D cultures of MSCs, called spheroids, have been shown to increase the expression of homing-related genes[57], angiogenic and growth factors[58,59], and anti-inflammatory and immune-modulator compounds[60-63]. Besides, 3D cultures also improve cell survival, promoting the expression of anti-apoptotic genes and inhibiting the expression of pro-apoptotic genes[60,64]. MSCs cultured in spheroids present higher expression of pluripotency-related genes, leading to an increased potency and trans-differentiating capacity[65,66]. Importantly, MSCs obtained from spheroids present a smaller size, which may improve intra-venal administration by avoiding lung-trapping[60], something to take into account if intravascular delivery is involved in the procedure. Although these enhanced capabilities are related to 3D culture, their acquisition also depends on the culture conditions of the spheroids[60,61,64]. Despite the fact that the concentration of oxygen in core of spheroids is reduced, Murphy *et al*[67] observed that changes in MSCs expression pattern are not oxygen mediated, which might induce to think that the improvement associated to this culture method should be due cell-to-cell interactions. Regarding the effect of spheroids in bone regeneration, Ma *et al*[68] observed a significant improvement in

bone formation after implantation of MSCs spheroids, with a high rate of survival and retention at the injection site in murine models. It is important to note that the bone tissue formed from MSCs spheroids presents similar histological characteristics to native bone, as well as a good mechanical strength[69].

Administration and implantation procedure

Selecting the appropriate administration route for MSCs delivery: In the field of MSC-based bone regeneration, different delivery approaches have been tested for the transfer of MSCs to the site of damage. There are mainly three different ways of administering MSCs: local injection directly to the site of damage, systemic injection, and *via* the use of biocompatible scaffolds.

Use of local or systemic injection for MSC delivery: There is mounting evidence indicating that both the correct route of administration and the proper dose can increase the success rate of MSCs therapies[70,71]. More clinical studies are necessary to determine these two parameters in order to achieve the maximum therapeutic effect in different diseases.

Systemic administration of MSCs has been widely studied. Intra-vascular delivery of MSCs is the less invasive route for MSC delivery and thus, the more interesting from the clinical point of view. Although this route has shown some benefits avoiding intervertebral disc degeneration in a murine model,[72]intra-venous delivery of MSCs has important drawbacks. The main downside of this delivery route is the fact that a high percentage of the administered cells could be entrapped in the lungs, something known as pulmonary first-pass effect[73], and in other organs such as the liver[74], forming microemboli that can have severe consequences to the functionality of those organs. Intra-arterial administration was considered a good alternative to avoid MSCs retention in the lungs and increase the homing rate of the cells[75,76]. However, despite this apparent benefit, Cui *et al*[75] also detected the formation of micro-occlusions in a cell dose-dependent manner in murine models, bringing important safety concerns to the use of the intra-arterial route. In animal models, systemic administration to treat generalized bone loss associated with osteoporosis has been tested with unclear results. Whereas one study demonstrated that systemic administration of allogenic MSCs had no obvious effect on osteoporotic bone loss in ovariectomized rats, another group reported that repeated injection of allogeneic MSCs might promote fracture healing when combined with local administration[77]. However, a few studies have shown the usefulness of MSC intra-arterial administration of MSCs in humans. Direct injection of BM-MSCs into the defect is widely used to treat non-union fractures with a high percentage of patients achieving union one year after the treatment[78]. Treatments of steroid-induced osteonecrosis using MSCs delivered *via* the medial circumflex femoral artery have also proven satisfactory after 5 years[79]. An alternative route for administering MSCs in osteonecrosis treatment is the administration with core decompression[80]. This has also given good results although it has normally been performed with bone marrow concentrate and not with *in vitro* expanded autologous MSCs[81].

Intramuscular administration of MSCs has been recently suggested as a better alternative to intravenous administration[82]. Different MSC administration routes, including intravenous, intraperitoneal, and subcutaneous, were compared to intramuscular administration. Whereas intravenously infused MSCs were not detectable just a few days after administration, and intraperitoneally and subcutaneously delivered cells were detected up to 3-4 weeks, intramuscularly delivered MSCs achieved more than 5 months of survival *in situ*. In spite of these results, it is still not clear whether this administration route can be effective to treat bone related diseases or other kind of pathologies.

Use of biocompatible scaffolds for MSCs delivery: In an attempt to increase the retention rate of MSCs, cells can also be applied in association with certain biocompatible scaffolds. The use of scaffolds responds to the increasing evidence indicating that MSCs prefer 3D culture conditions and that, after seeding, cells are able to survive in the scaffolds, probably because these conditions are closer to their natural environment than the monolayer two dimensional (2D) culture[83]. When cells reach a high confluence after culture in monolayer, it is necessary to detach them from the dish, which leads to a down-regulation of important cell adhesion genes[84], and the subsequent decrease in engraftment efficiency after cell infusion. Thus, culturing the cells onto a support or scaffold may improve the engraftment of cells and the result of certain cell-based therapies.

Plenty of different scaffolds have been designed to improve different parameters such as cell survival, proliferation, and differentiation. Although initially scaffolds were mainly based on the use of hydroxyapatite and tricalcium phosphate, the latest

generation of scaffolds trying to resemble the properties of bone microenvironment are based on natural polymers, such as alginate, collagen, chitosan, or cellulose, which are subjected to a biomimetic mineralization process[85]. Of those, collagen is probably one of the main materials used because of the high biodegradability and biocompatibility, although it is commonly used in combination with other biomaterials, such as ceramic coated collagen nanofibers[86,87]. As we will discuss later, it is also important to highlight that scaffolds could be modified to create bioactive structures that favour engraftment or elicit appropriate responses for specific applications, such as promoting bone formation.

Bioprinting has also emerged as an alternative for artificial bone generation. This technique aims to produce a construct with a pre-defined 3D architecture resembling the original tissue. Bioprinting holds a great potential for producing tissues in a patient-specific manner[88]. The development of biocompatible inks that can undergo a transformation from a liquid to a gel-like structure is crucial for the success of bone bioprinting. In this sense, hydrogel based bioinks seem to be a good choice[89,90]. Despite the promising future of bioprinting, this is a rather new technique and more work needs to be done to overcome some challenges and limitations of the current techniques besides the choice of bioink, such as the optimal cell source or the best bioprinting method for replicating heterogeneous tissues and organs[91].

Another approach uses what is called ECM powder to improve biocompatibility of different materials[92]. Cells can be directly cultured over the powder and injected into the patient or alternatively, ECM powder can be used to coat poor biocompatibility biomaterials improving cell engraftment after transplantation[92]. Interestingly, an osteoblast-derived ECM has proven to stimulate osteogenesis and promote bone formation[93,94]. Mao *et al*[95] cultured fibroblasts, chondrocytes, and osteoblast over microfibers of tyrosine-derived polycarbonates (pDTEC) until the cells released ECM over all scaffold surface and then decellularized the scaffolds to preserve the ECM. This pDTEC-ECM showed enhanced chondrogenic and osteogenic differentiation[95]. Some authors have proposed that the osteoblast-derived ECM could be used to coat titanium scaffolds[96], which have shown unparalell mechanical properties and are routinely used in bone tissue engineering for orthopaedic implants in load-bearing areas. Despite these positive aspects, the difficult production of the ECM-powder currently hinders its application[92]

Avoiding mechanical stress during administration: As previously stated, the administration of cells by injection has the advantage of being less invasive and therefore it is gaining popularity for clinical applications. However, during the procedure of injection, if cells are resuspended in low viscosity solutions, the mechanical stress can cause cell membrane disruption and subsequent cell death in a high percentage of the population[97]. This importantly limits the successful use of MSCs injection for regenerative approaches. In order to optimize delivery protocols and avoid mechanical stress during MSCs injection, cells could be suspended in a hydrogel that will encapsulate and protect them from membrane disruption. Although these hydrogels can lose viscosity due to stress in the syringe, they can still protect, to some extent, the cells from mechanical stress, slightly improving the survival rate. The most common hydrogels used for this procedure are alginate hydrogels[97], hyaluronic acid-based hydrogels[98-100], supramolecular beta-hairpin hydrogels[101], and protein-assembled hydrogel[102,103]. However, the microenvironment provided by the hydrogel implies a limited interfacial interaction between the cells and the hydrogel material, allowing only weak dynamic interactions between them, such as hydrogen bonds or hydrophobic and electrostatic interactions. These weak associations are lost during injection when the hydrogel is exposed to shear-stress, leading to restricted tissue regeneration. In order to improve this delivery method, Zhao *et al*[104] designed a strategy based on the use of microfluidics-assisted technology to encapsulate bone marrow-derived MSCs (BMSCs) and growth factors in photo-crosslinkable gelatin methacryloyl (GelMA) microspheres. This type of encapsulations, known as microcarriers, offers mechanical stress protection and also allows a high-cell density administration, which improves cell secretion of paracrine factors and enhances cell differentiation, improving the therapeutic effect of the cells[105]. Also, using four different microcarriers (Cytodex, Cytodex3, SphereCol, and Clutispher-S) and a reduced number of cells, Lin *et al*[106] were able to demonstrate improved cell proliferation and also better chondrogenic differentiation. Alginate microcarriers covered with silk are also a good alternative, as they can be used to culture MSCs *in vitro* with good rates of cell adhesion, proliferation, and differentiation, reducing cell manipulation, as these microcarriers can be directly transplanted into the patient[107].

Promoting cell homing: According to Karp *et al*[108], MSC homing can be defined as

"the arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium". We have already discussed how systemic infusion of MSCs is an interesting approach because of the minimally invasive procedure associated. An important barrier for achieving successful regeneration of the target tissue when this delivery method is used, is the inability of targeting the exogenously infused MSCs to the tissues of interest with a high efficiency, that is, the inability of the cells to accomplish homing.

Despite the fact that the exact mechanism of MSC homing to the injury site is not completely elucidated, we know that homing is a multistep process, where chemotactic factors released at the site of damage play an essential role[109]. Chemoattraction of MSCs into the target tissue appears to be mainly mediated by the stromal derived factor (SDF-1)/CXCR4 axis[110-112], but in MSCs migration there might also be a contribution from monocyte chemoattractant protein/CCR2 and the hepatocyte growth factor/c-met signaling pathways, and from cytokines such as TGF-β1, IL-1β, TNF-α, or G-CSF[109,110,113]. Circulating MSCs are attracted by the SDF-1 secreted by the injured tissue; subsequently, interaction of MSCs with the endothelial cells through the P- and E-selectins leads to MSCs rolling over the endothelium. Afterwards the attachment of MSCs is mediated not only by SDF-1 but also by ligands, such as VCAM-1 and ICAM-1, β1 integrin, and very late antigens-4[109]. During transmigration, MSCs also need to cross the basement membranes located between the endothelial cells and the targeted tissue. In this step, matrix metalloproteinases (MMPs) have a crucial role. The secretion of MMP-2 and MMP-9, the main MMPs involved in MSC migration, is stimulated by the CXCR4 receptor activation and also by inflammatory cytokines such as TGF-β1, IL-1β, and TNF-α. MMP-2 is released as a pro-enzyme, proMMP-2, which will be activated by the tissue inhibitor of metalloproteinases-2 (TIMP-2) and membrane type1 (MT1)-MMP, also released constitutively by MSCs[113].

To improve MSC homing, it is possible to perform a preconditioning during cell culture. In fact, Kim *et al*[114] observed that pretreatment of MSCs with a glycogen synthase kinase-3 inhibitor increased cell migration after transfusion by promoting cell expression of CXCR4. The presence of HIF-1α also improves MSC migration *via* a similar mechanism, leading to the enhancement of CXCR4 and CCR2 expression, which recognizes damaged-tissue signals. In this case, there is also an enhancement of proteolytic enzymes, such as MMP-2 and MMP-9, necessary for the cells to reach the damaged region by degrading ECM[115]. Cell migration can also be promoted by enhancing the expression of MMP2 through the exposition of cells to erythropoietin and G-CSF, as confirmed by Yu et *al*[116]. Other strategies of tissue pretreatment have been tested. Zhang *et al*[86] saw increased capillary permeability and expression of VCAM-1 in the renal interstitial after ultrasound-targeted microbubble destruction (UTMD), improving MSCs migration and retention in the kidney. Li *et al*[117] also noticed an increase in SDF-1 and CXCR4 expression after UTMD as intravenous infusion of MSCs was performed in ischemic myocardium, which led to a higher retention of MSCs in the tissue. Najafi *et al*[115] demonstrated that pharmacological pretreatment with deferoxamine leads to an accumulation of HIF-1α in the cells. Liu *et al*[118] noticed that pretreatment of MSCs with SDF-1, secreted by the injured tissue, activates the signaling pathways Akt and Erk, leading to an increased ratio of Bcl-2/Bax with pro-survival consequences, concluding that through CRCX4 receptor, pretreatment with SDF-1 increase MSC migration, survival, proliferation, and secretions. Despite that some studies observed that cytokines such as IL-1β impair bone formation by inhibiting MSC proliferation, migration, and differentiation[119], Carrero *et al*[120] described an increase in cell migration and adhesion due to the secretion of chemokines and growth factors induced by pretreatment with IL-1β. Other tissue pretreatments were also observed to enhance MSCs migration and homing. Hepatic radiation prior to MSCs transplantation ameliorates hepatic fibrosis in an animal model[121] and extracorporeal shock wave positively modifies the microenvironment to favor MSC homing for spinal cord injury[122].

Avoiding anoikis: All mammalian cells forming part of a tissue are surrounded by an ECM, which function goes far beyond offering structural support. The ECM also provides biochemical and biomechanical signals that have an important role in cell function regulation. Anoikis (greek word for homelessness) is the name given to the induction of cell apoptosis that occurs in anchorage-dependent cells in response to inappropriate interaction between the cell and the ECM[123,124]. Together with the harsh environment found on the recipient tissue, anoikis is one of the important barriers to a successful engraftment. It is important to note that using microarray and proteomic screening, Copland *et al*[125] identified plasminogen activator inhibitor-1 (PAI-1), a protein that inhibits cell migration as up-regulated in mouse and human MSCs under hypoxic conditions. The MSCs isolated from PAI-1 knockout mice showed more

survival and adhesiveness than wild-type MSCs after transplantation on Matrigel. These findings corroborate that PAI-1 negatively regulates transplanted MSC survival and adhesiveness *via* promoting anoikis[125], establishing a link between this process and hypoxia.

Anoikis is highly important when the cells are delivered through local or systemic injection. To promote cell survival in these conditions, integrins need to be bound to immobilized ligands. An approach that has already been discussed in the previous section is to mimic the adhesive response of these cells in suspension by encapsulating the cells in a provisional hydrogel matrix[126] that will preserve cell adhesion in what can be called a portable microenvironment, while the cells are travelling through the vasculature until they can engraft in the recipient tissue.

An interesting report by García *et al*[127], used protease-degradable polyethylene glycol hydrogels functionalized with an $\alpha_2\beta_1$ integrin-specific peptide (GFOGER) or an $\alpha_v\beta_3$ integrin-targeting peptide and loaded with VEGF to study whether the integrin-specific biomaterials modulate the effect of VEGF on bone regeneration. These authors demonstrated that both types of scaffolds have different effects when applied to critical size segmental defects in a murine model, highlighting the importance of integrin specificity in engineering constructs for vascularization and associated bone regeneration.

Another valid approach to improve endothelial adhesion, avoid anoikis, and thus enhance the survival of transplanted cells, would be regulating the levels of integrins and conexins, the main molecules involved in cell adhesion to the ECM. In agreement to this, MSCs homing is enhanced, *via* the SDF-1 axis[128,129], by the transfection of these cells with a vector expressing CXCR4. Also, ectopic expression of $\alpha_1\beta_1$ integrin in MSCs ameliorates homing to the bone in mice. The aforementioned approaches enhance gene expression by using viral vectors, which ensures long-term expression of the transgene, but clearly precludes their use in clinical practise.

ENHANCING OSTEOGENIC DIFFERENTIATION

Since MSCs have the potency to produce different cell types, a successful bone tissue engineering technique requires a way of preferentially inducing bone formation over the formation of other possible tissues. The osteogenic and adipogenic differentiation of MSCs are carefully balanced and, more important, mutually exclusive processes. This is highlighted by the fact that inhibition of adipogenesis seems to improve bone development and repair[130,131].

Although it is possible to genetically engineer the MSCs to promote cell differentiation, the viral nature of the vectors normally used to achieve this end could lead to unregulated cell growth and a markedly increased risk of tumour formation. An alternative approach to promote osteogenic differentiation consists in modifying the microenvironment surrounding the cell.

Many treatments have tried to induce osteogenesis while preventing adipogenesis to improve cell therapy for bone regeneration. Luo *et al*[132] observed that in canine MSCs, pharmacological pretreatment with icariside II may promote osteogenic differentiation *via* PI3K/Akt/mTOR/S6K1 signaling pathways. Wan *et al*[133] noticed that preconditioning of MSCs with rapamycin promoted cell osteogenesis by activation of autophagy. On the other hand, Lu *et al*[134] demonstrated an improvement of ASCs mobilization, proliferation, and osteogenic differentiation after pretreatment with TNF-α. Bisphosphonates are commonly used as treatments against bone diseases related to exacerbated bone resorption, such as osteoporosis. Hu *et al*[135] observed an interesting dose-dependent effect in MSCs pretreated with zoledronic acid (ZA), a commercial bisphosphonate. When MSCs were exposed to high doses of ZA, there was an inhibition of cell proliferation, while low dose pretreatments would induce the upregulation of osteogenic-related genes, such as alkaline phosphatase (*Alp*), osterix (*Osx*), and bone sialoprotein (*Bsp1*), which translated into an induction of osteogenic differentiation. Although TGF-β was initially described to inhibit both osteogenic and adipogenic differentiation, Van Zoelen *et al*[136] described an inhibitory effect of this cytokine over adipogenic differentiation of MSCs, and an enhancement of osteogenic differentiation. Some pathologies linked to bone lose are characterized by low magnesium concentration[137]. In relation to this, magnesium supplementation has been shown to improve osteogenesis and tissue mineralization in a dose-dependent manner, although the mechanisms involved in this process are not well understood[138].

Bioactive scaffolds represent a valuable alternative to provide molecular cues for the seeded cells and not only physical support. These molecular cues can drive the activation of intracellular signalling pathways promoting osteogenic

differentiation[139]. The most common way to currently promote MSC osteogenic regeneration in the clinic is the concomitant administration of bone morphogenetic proteins (BMPs). Binding of a BMP homo- or hetero-dimer to a BMP receptor, activates intracellular downstream Smad proteins that translocate into the nucleus, where they interact with *Runx2*, the master osteogenic regulator, to activate the expression of osteogenic genes[140]. One recurring problem observed in the regenerative treatments that are accompanied by the administration of BMPs, is the presence of important dose-dependent side effects. One possible solution is the use of scaffolds that locally and sustainably release low dose BMPs, alone or in combination with other osteogenic factors, such as TGF-β-1 o MMP10[141,142], to the bone defect microenvironment. Such scaffolds have proven to be very successful in animal models. Although these bioactive scaffolds were mainly alginate based, it has also been possible to increase bone formation by a dual delivery of MSCs and BMP-2 in a coral scaffold as Decambron *et al*[143] were able to observe.

Although silk fibroin-chitosan (SF-CS) scaffolds are able to improve cell adhesion without the need of adding any molecules, Tong *et al*[144] were able to increase the benefits of this scaffolds by adding TGF-β1, leading to enhanced proliferation and osteogenic potential in an animal model. Scaffolds made of poly(L-lactic acid) nanofibers have been seen to be a good support for MSC proliferation and osteogenic differentiation[145], but it is possible to improve these properties by coating these scaffolds with baghdadite[146], nanobioactive glass[145], nanohydrohyapatite[147], or willemite[148].

Lee *et al*[149] developed a bio-ink consisting in a hybrid hydrogel with a base of hyaluronic acid complemented with different peptides. Their objective was to improve angiogenesis and osteogenesis, two processes that are crucial in bone regeneration. With this in mind, bio-ink complemented with (1) substance P (SP) was able to promote not only angiogenesis but also enhanced expression of osteogenic genes such as RUNX2 and ALP; or (2) BMP-7D led to high osteogenic differentiation with also an angiogenic effect over MSCs.

As hypoxic stress is an important factor to decrease cell engraftment, Alemdar *et al*[150] developed a calcium peroxide (CPO) laden GelMA able to produce oxygen under hypoxic conditions during 5 days, reducing the possible tissue necrosis that can appear due to the hypoxic conditions. Another important fact reducing cell survival once in injured tissue is the oxidative stress due to high amount of ROS. To avoid this, Dollinger *et al*[151] developed a triblock polymer with protective properties against ROS.

CONCLUSION

Bone microenvironment shows high complexity in terms of composition and geometry and therefore, designing a scaffold that can imitate those particular conditions is a challenging process. A relatively recent advance in tissue engineering involves the use of biomimetic scaffolds. These scaffolds do not need any morphogens or biomolecules to activate osteogenesis. Instead, they have a matrix that per se initiates bone formation[152]. It seems that scaffold geometry has a critical role in initiating bone formation and this process is known as geometric induction of bone formation. Cells are able to sense surface roughness through differences in focal adhesion that are translated into morphological changes[153]. It has been proposed that this feeling of the geometry of the scaffold occurs through actin-myosin contractions[153-155]. This is indeed an interesting approach to eliminate all side effects that could rise as a consequence of the presence of different molecules or factors.

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