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Bone Marrow Mesenchymal Stem Cells: Aging and Tissue Engineering Applications to Enhance Bone Healing

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Abstract

Bone has well documented natural healing capacity that normally is sufficient to repair fractures and other common injuries. However, the properties of bone change throughout life, and aging is accompanied by increased incidence of bone diseases and compromised fracture healing capacity, which necessitate effective therapies capable of enhancing bone regeneration. The therapeutic potential of adult mesenchymal stem cells (MSCs) for bone repair has been long proposed and examined. Actions of MSCs may include direct differentiation to become bone cells, attraction and recruitment of other cells, or creation of a regenerative environment via production of trophic growth factors. With systemic aging, MSCs also undergo functional decline, which has been well investigated in a number of recent studies. In this review, we first describe the changes in MSCs during aging and discuss how these alterations can affect bone regeneration. We next review current research findings on bone tissue engineering, which is considered a promising and viable therapeutic solution for structural and functional restoration of bone. In particular, the importance of MSCs and bioscaffolds is highlighted. Finally, potential approaches for the prevention of MSC aging and the rejuvenation of aged MSC are discussed.

Keywords

MSC; aging; stem cell niche; bone healing; rejuvenation

1. Bone healing and aging

Annually, there are more than two million fragility-associated fractures with healthcare costs exceeding $20 billion in the United States [1]. There is a significant increase in the incidence of fractures as well as the associated morbidity with increasing age [2]. As an example, one-

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year mortality rates after hip fractures in the elderly have been reported as 33% [3]. FDA approved medical therapies for aging-related bone loss include estrogen and related agonists (e.g. raloxifene), bisphosphonates (e.g., alendronate, risendronate, ibandronate, zoledronic acid), recombinant parathyroid hormone (e.g. teriparatide, abaloparatide), and antibodies against receptor activator of NF-κβ (RANKL) (e.g. denosumab), as well as supplements such as vitamin D and calcium. Monoclonal antibodies targeting sclerostin (SOST) have recently shown promise in clinical trials [4]. Each of these therapies has its own limitations and side effects, including adverse cardiovascular events, risk of pathological fractures, increased incidence of bone tumors, and immune dysfunction, the majority of which are associated with the systemic administration of these agents [5].

To avoid these side effects, a variety of techniques have recently been developed that involve local delivery of combinations of cells, growth factors, and scaffolds to the site of fractures (Table 1). The Hernigou procedure involves application of bone marrow aspirate to the site of non-unions [6]. Recombinant bone morphogenetic protein-2 (BMP-2) is utilized in spine surgery to facilitate fusions [7], and recombinant human platelet-derived growth factor (rhPDGF) is used to help fill bony defects [8]. Finally, implantation of demineralized bone matrix, a frequent and long-practiced approach based on the well-known bone-inductive activity of bone tissue grafts, is used to facilitate fracture healing and spinal fusions, [9]. Thus, iliac crest bone autografts and fibula allograft are commonly utilized in clinical practice to facilitate spinal fusions, ankle arthrodesis, and the healing of long bone and pelvic fractures. Despite the availability of these approaches, the rate of delayed union and non-union for these procedures remains a significant 10–25% in elderly populations. [10].

While fracture healing is significantly delayed in older versus younger patients, the underlying mechanisms are not fully understood. Some aging-associated changes have been proposed, such as altered interaction between macrophages and MSCs [11], reduced level of estrogen in postmenopausal females [12], and transition of bone marrow, which changes from red marrow to fatty yellow marrow during aging [13]. For example, a recent report demonstrated that the accumulation of adipocytes in bone marrow significantly impairs the stem cell participation in bone healing [14]. In this article, we will review the current understanding of MSC aging and how aging-associated changes in MSCs may lead to compromised bone healing in the elderly. In particular, we will address MSC niche and discuss how niche aging affects MSC population density and functionality. An in-depth review of how tissue engineering strategies are being used to promote bone healing is also provided.

2. Aging of bone marrow-derived MSCs

In 1970, Friedenstein et al. first identified and described colony-forming fibroblasts (CFU-Fs) able to adhere to plastic culture substrate from bone marrow [15]. The term, mesenchymal stem cells (MSCs), was suggested for this cell population by Caplan in 1991 [16]. The first detailed description of the tri-lineage differentiation capacity of MSCs, including osteogenesis, chondrogenesis and adipogenesis, was reported in 1999 [17], which soon became a key criterion to identifying MSCs. It should be noted that MSCs are rare in bone marrow, constituting less than 0.01% of the overall mononucleated cells [17]. Under

normal conditions, they maintain a quiescent state. Upon stimulation by biological signals, e.g., as a result of tissue injuries, MSCs are activated and undergo symmetric or asymmetric division, and they are believed to be recruited to injured sites to replace or regenerate damaged tissues. Due to their robust reparative potential, MSCs have been applied in an expanding array of clinical indications, specifically for bone defects and cartilage loss [18]. The original rationale for the application of MSCs in tissue regeneration is the replenishment of damaged cells and their differentiation into tissue-specific cells for *de novo* tissue formation. Results from many animal studies have supported the clinical feasibility of MSC-based treatments, demonstrating the presence of MSCs in the newly-formed bone tissues. Whether the transplanted cells can directly engraft and become bone cells in the human body requires further investigation. In addition, MSC-produced agents, or the MSC secrectome, have recently been proposed as a more important biological mechanism than their differentiation capacity that contributes to the function of MSCs [19].

Currently, MSCs have been isolated from many different tissues, including from bone marrow, adipose, cartilage, muscle, bone, and umbilical cord and placenta. While cells derived from these different tissue sources all possess a tri-lineage (bone, cartilage, and adipose) differentiation capacity and display similar surface marker expression profiles [20, 21], differences in the biology of MSCs from different ontogenetic sources have also been extensively reported. For example, MSCs from human adipose tissue, and skin from both adults and newborns displayed significant differences in adipogenic and osteogenic potential [20]. Such origin-dependent characteristics of MSCs strongly implicate the critical role of the tissue microenvironment surrounding the MSCs, commonly referred to as the stem cell niche.

2.1 Contribution of MSCs to bone repair *in vivo*

The contribution of MSCs to bone repair has been long proposed. In particular, the crosstalk between MSCs and macrophages during the bone healing process has recently drawn investigative attention [22]. Studying the underlying mechanism has been facilitated by recent technical advancements in cell labeling and tracking, which allow the tracing of the lineage of a cell population *in vivo* to study their location and functions. For example, Mendez-Ferrer et al. exploited nestin as a marker of MSCs and tagged MSCs with a green fluorescent protein (GFP) transgene under regulation by the nestin promoter. Using this lineage-tracing method, they demonstrated that these GFP tagged MSCs directly contribute to bone remodeling by differentiating into osteoblasts [23]. Using a similar strategy, Park et al. showed Mx1-expressing bone marrow cells possessed all known MSC characteristics, and they could migrate to the injury site, supplying new osteoblasts during fracture healing [24]. Besides nestin and Mx1, leptin receptor (LepR) is another marker to identify MSCs. LepR positive cells were shown to be capable of giving rise to osteoblasts and adipocytes in bone marrow [25]. In a bone fracture model, 85% of the osteoblasts were shown to be derived from $LepR^+$ MSCs after 8 weeks. It should be noted that MSCs in bone marrow is by nature heterogeneous. Recently, gremlin-1-expressing cells were isolated from bone marrow, which are capable of osteogenesis, but not adipogenesis [26, 27]. However, these cells still contribute to 28% of the osteoblasts found in the facture callus. Collectively, these studies indicate the critical roles of MSCs during the bone healing process.

2.2 MSC niches

The concept of stem cell niche was first described by Schofield et al. to refer to the specialized microenvironment around hematopoietic stem cells (HSCs) [28] and to propose a mechanism to maintain stem cells under a quiescent state [29]. Currently, it is generally accepted that the stem cell niche includes the supporting cells, extracellular matrix (ECM), vascular network, as well as biochemical and physical cues [30]. Under normal conditions, the niche maintains stem cells in a quiescent state to prevent them from being depleted. Upon stimulation by injury or other signals, changes in the niche will activate stem cells and promote stem cell proliferation, differentiation, and migration. These activated cells may either divide into daughter stem cells, which can maintain a stable stem cell pool, or divide into cells committed to differentiation, thereby decreasing the number of stem cells [31]. As mentioned above, MSC phenotypes and functions are distinct in accordance to their tissue origin, which suggests the presence of tissue-specific MSC niches. However, their specific nature and characteristics are incompletely understood.

In 2003, Gronthos et al. isolated a human MSC population from bone marrow using the antibodies targeting vascular cell adhesion molecule-1 (VCAM-1/CD106) and STRO-1 [32]. In that same year, Shi et al. performed the first study to identify the *in vivo* niche of MSCs, and the group suggested that MSCs may reside in the microvasculature of tissues [33]. In this study, expression of STRO-1 was confined to the vascular wall in both human bone marrow and dental pulp. Covas et al. performed a subsequent comparative study to investigate the difference between perivascular cells ($CD146^+$) and MSCs as well as between non-perivascular stromal cells and MSCs. They concluded that the gene expression profiles and differentiation capacities were similar between MSCs and perivascular stromal cells [34]. Again, in the same year, a landmark study by Crisan et al. demonstrated the perivascular origin of MSCs [35]. Prospectively, these investigators identified perivascular cells, principally pericytes, in multiple human organs. Long-term *in vitro* cultures of these cells expressed MSC markers and possessed tri-lineage, i.e., osteogenic, chondrogenic, and adipogenic differentiation potentials [35]. Additional subsequent studies by other groups confirmed the isolation of MSC-like cells from the vascular wall [36–38].

Based on this evidence, we elaborated on the perivascular location theory of the MSCs in 2010 [39]. In fact, residing in a perivascular niche improves the migration capability of MSC in response to injury or the pathogenic signals. It should be noted that the perivascular niche theory is challenged by the fact that MSCs have also been isolated from avascular tissues. For example, MSC have been isolated from cartilage [40], which also displayed clonogenicity and multi-lineage differentiation ability [41]. In addition, the precise location of MSCs within the vascular wall remains unknown, although there is increasing evidence suggesting that MSCs may stay in the adventitia. The potential adventitia nature of MSCs was first described by Hoshino et al. in 2008 [42]. They isolated vascular adventitial fibroblasts (hVAFs) from human pulmonary arteries and showed that these hVAFs were positive for MSC markers, but negative for hematopoietic and endothelial cell markers. hVAFs also showed osteogenic and adipogenic differentiation upon appropriate stimulation. Furthermore, Corselli et al. used surface markers to further isolate cells from middle and outmost layers of blood vessels [43]. In their work, $CD34^+$, $CD31^-$, $CD146^-$, and $CD45^-$

cells were sorted, which represented the cells in the outmost layer of blood vessels from adipose tissue. These cells expressed a surface marker prolife and differentiation potential identical to standard bone marrow-derived MSCs. In contrast, pericytes, defined based on the expression of CD34, CD31, and CD146, retained phenotypes and genotypes distinct from those of cells from adventitia. In addition, adventitia-derived cells treated with angiopoietin-2 upregulated the expression of pericyte markers, suggesting that MSCs may function as the precursors of pericytes. Based on the adventitial nature of MSCs and middle perivascular nature of pericytes, de Souza et al. proposed a model to describe the association between these two cell types [44]. In this model, MSCs serve as the progenitor cells of perivascular populations, including pericytes.

We have previously reviewed the potential interaction between MSCs and their niches, including supporting cells, ECM, soluble factors, etc. [39]. Next, we will briefly describe MSC aging, with special attention paid to how aging affects each niche component and the consequent influence on MSC characteristics.

2.3 Identification and characterization of MSC Aging

Aging is characterized by a global, gradual, and functional decline of the entire body. To date, there is no established method to indicate or monitor *in vivo* aging of cells. Unlike *in vitro* replicative senescence, which has been well characterized on the basis of chromosome telomere length, cell proliferative behavior, and differentiation potential [45], the debate of natural aging compromising MSC functionality *in vivo* is unsettled. To date, senescence-associated β-galactosidase (SA-β-gal) activity is the most widely used biomarker of aging cells, especially in combination with additional markers [46], although its role on the aging process is not known. In addition, p16 ^{INK4a} is a common marker of cell cycle arrest, which correlates with senescence both *in vitro* and *in vivo* [47, 48]. Based on these two markers, a protocol of quantitative analysis of cellular senescence has been described by Zhao et al. [49]. Recently, extracellular microvesicles (MVs) have been proposed as the potential biomarkers to identify MSC aging. Lei et al. reported that miR-146a-5p localized in MSC-MVs characterized the senescent state of late passage MSCs [50]. Expression of miR-335 was also reported to correlate with donor age of human MSCs (hMSCs), and overexpression of miR-335 resulted in a rapid senescent phenotype and abolished differentiation potential [51]. Furthermore, since the nuclear lamina undergoes aging-associated changes, prelamin A has emerged as a new marker to identify senescent MSCs [52]. McHugh et. al proposed that aging hallmarks should be divided into three categories: (1) primary, or the causes of age-associated damage; (2) antagonistic, or the responses to the damage; and (3) integrative, or the consequences of the responses and culprits of the aging phenotype [53]. In the future, more studies addressing these issues in MSCs are clearly needed.

MSC aging, as well as its effects on MSC properties such as telomere length, cell proliferation capacity, differentiation potential, epigenetics, and secretome (Figure 1), have been recently discussed in several review articles [54–57]. Depending on the criteria used to define young and old cells, the methods of cell isolation, cell culture, culture passage, sample size, and results are not consistent in practice. However, it is generally accepted that organismal aging reduces the density of MSCs in bone marrow and compromises their

osteogenic potential based on *in vitro* standard osteogenesis assay and *in vivo* bone repair experiments. Interestingly, a study by Brusnahan et al. indicated that the loss of stem cells started with those that possessed the lowest biological quality [58]. In addition, there is increasing evidence suggesting that MSC aging benefits adipogenesis at the expense of osteogenesis, resulting in impaired bone formation capacity [59]. This is consistent with the fact that, *in vivo*, bone marrow contained increased adipose tissue during aging. Such a shift may be due to reduced expression of transcriptional coactivator with PDZ binding motif, thus enhancing expression of peroxisome proliferator-activated receptor gamma (PPAR-γ). PPAR-γ is associated with adipogenesis, and it inhibits expression of Runt-related transcription factor 2 (RUNX2) expression, which is associated with osteogenesis [60].

To enable the function of MSCs to facilitate tissue repair, it is critical to direct MSCs to the site of injury, referred to as “homing”. When tissues are damaged, signaling factors are released into the body fluid and activate both residential and systemic MSCs, resulting in the elevation of the level of CD44 on the surface of MSCs. During this process, high expression of stromal derived factor-1 (SDF-1) at the injury site recruits MSCs and docks them to the right spot by binding with the cytokine receptor, CXCR4, on the surface of MSCs. Afterwards, integrin α4 and β1 combine together and form very late antigen-4 (VLA-4) in MSCs, further directing MSCs to endothelial cells by interacting with VCAM-1. Finally, lytic enzymes such as matrix metalloproteinases (MMPs) are produced to generate space to allow MSC migration [61, 62]. In a mouse study, expression of CXCR4 on the surface of MSCs was significantly reduced in old mice compared with young counterparts [63]. Interestingly, intravenous injection of old MSCs homed less to wound site with less neovascularization-promoting capacity than intravenous injections of young MSCs. MSCs isolated from mouse bone marrow were found to lose their homing ability rapidly after *in vitro* expansion [64], which may be associated with decreased expression of VCAM-1, an MSC surface marker that is known to regulate MSC interaction with endothelial cells [65]. Moreover, by measuring migration rates, Geibler et al. showed that young MSCs of each passage always demonstrated significantly higher migratory potential compared to old MSCs [66], suggesting an impaired response of aged MSCs to injury signals. Recently, we have collected young and aged MSCs from more than twenty donors and found similar decline of migration capacity (unpublished observation). Similar phenomenon is also observed in MSCs derived from adipose tissues, suggesting that this characteristic may be associated with aging-associated inferior microenvironment [67]. However, in a hMSC-based study, no difference in the migratory ability of MSC was reported with respect to the age of the donor, which could be due to the relatively “young” donors in the older group (38–58 years old) [68]. Similar to the findings from studies of natural aging, *in vitro* senescence was also found to severely impair the migratory capacity of human MSCs in response to proinflammatory signals [69].

As discussed above, MSC may function via the production and secretion of paracrine factors [70]. Autocrine and paracrine properties of senescent MSCs have been recently reviewed by Lunyak et al [71]. To date, how natural aging affects MSC secrectome has not received sufficient attention. In one study on adipose derived MSCs, adult MSCs (derived from >40 years old individuals) produced significantly higher interleukin-6 (IL-6) and IL-8 than those isolated from donors of <16 years of age [72]. The difference was further confirmed by a

recent study [73]. Such enhanced pro-inflammatory secretome was likely to significantly diminished the immunomodulation capacity of MSCs [73]. In comparison, there have been a number of studies analyzing the secretome of early and late passage MSCs *in vitro*. Secretion of senescence-associated secretory phenotype (SASP) products from senescent MSCs has also been characterized. By generating senescent human bone marrow-derived MSCs with gamma irradiation, Sepúlveda et al. identified more than 27 SASP components, such as IL-17F, leptin, IL-8, Eotaxin, VCAM1, interferon β, IL4, and MCP1. In particular, IL-6 was robustly generated by these senescent cells, representing the most prominent SASP factors [69]. Severino et al. showed that conditioned media (CM) from passage 10 (P10) senescent MSCs could directly induce a senescent phenotype in P1 cells in association with insulin-like growth factor binding proteins (IGFBP) 4 and 7 [74]. Ozcan et al. used four different methods to generate *in vitro* senescence in human MSCs, including oxidative stress, doxorubicin treatment, X-ray irradiation, and replicative exhaustion, which were all confirmed by positive staining of SA-β-gal [75]. To date, how SASP from MSC affects bone healing has not yet been reported. However, IL-6, one of the most recognized SASP factors, has been shown to drive osteoclastogenesis [76] and negatively regulate osteoblast differentiation [77]. Knockdown of IL-6 significantly enhanced Runx2 and collagen type I gene expression in osteoblasts while decreasing the expression of osteoclast related genes such as tartrate resistant alkaline phosphatase (TRAP), MMP9, and CTSK [78]. Therefore, it can be extrapolated that SASP production would not be beneficial to bone regeneration.

Finally, it is noteworthy that most MSCs studied were isolated from surgical waste from patients with bone or osteochondral diseases, such as osteoarthritis. How important these disease states are as a variable that affects the experimental outcomes is unknown, and more rigorous and standardized methods to collect MSCs from “healthy” or “abnormal” tissues are clearly needed. Also, it is important to point out that aging or senescence does not always imply a negative physiological function. For example, acute or chronic injury is found to lead to the accumulation of senescent muscle stem cells, which could initiate *in vivo* reprogramming, leading to the repair of muscle injury [79].

2.4 Mechanisms Underlying MSC Aging

To date, the mechanism of *in vivo* natural MSC aging in humans is still not clear, as there is no feasible way to trace the in-body changes of MSCs. However, several factors have been proposed to be associated with this process, including diseases, over-division, and exposure to multiple stresses such as reactive oxygen species (ROS) [55]. Since MSCs reside in unique tissue niches within the body, their aging is expected to be associated with systemic and local factors. For example, natural aging is accompanied by increased levels of pro-inflammatory cytokines, called inflammaging [80], in particular IL-6 [81–83]. Although it is clear that IL-6 may disrupt bone formation, its effect on MSCs is apparently opposite. Previously, we, for the first time, showed that IL-6 gene expression is significantly higher in undifferentiated MSCs than those exposed to an osteogenic differentiation medium. Interestingly, treatment with IL-6 enhances MSC proliferation rates and protects MSCs from apoptosis, which may function through the activation of ERK1/2 pathway [84]. Moreover, IL-6 and the IL-6 receptor (IL-6R) complex activate STAT3 signaling pathway, which promotes osteogenic differentiation in BM-MSCs [85]. Therefore, whether elevated IL-6,

which is a natural response to aging, is in fact acting to activate or rejuvenate the functionality of MSCs requires further investigation.

These potential risk factors then function on cell phenotype or functions through signaling pathways. For example, canonical Wnts and members of the forkhead family were shown to be significantly involved in the MSC aging process (see review in [55]). Recently, forkhead box P1 (FOXP1) has been shown to play a major role in transcriptional control of MSC senescence. The expression of FOXP1in bone marrow MSCs decreased with the aging progress, and the conditional depletion of FOXP1 significantly accelerated MSCs aging *in vivo* [86]. In fact, overexpression of FOXP1 increased the proliferation rate and resulted in higher osteogenic potential over adipogenesis of MSCs, which may function through the inhibition of p16.

2.5 Effect of niche aging on MSC

2.5.1 Effect of aged supporting cells—As shown in Figure 2, MSCs reside together with many other cell types, including osteoblasts, osteoclasts, fibroblasts, endothelial cells, adipocytes, and within bone marrow compartments [39]. The interactions between MSCs and niche supporting cells may be through direct physical contact, secreted soluble factors, or ECM proteins [30]. Due to well-recognized technical challenges, analysis and understanding of the cellular interactions within bone marrow *in vivo* are difficult. Therefore, most of the current studies have used *in vitro* culture system in an attempt to investigate the interactions of cells during the aging process, which may not mirror the situation *in vivo*.

Since aging represents a decline in systematic functions, all cells in bone marrow should undergo a similar “aging stress”, affecting neighboring cells. For example, conditioned media (CM) from passage 10 senescent MSCs could directly induce a senescent phenotype in P1 cells by secreting IGFBP 4 and 7 [74]. Additionally, aging decreased the expression level of fibroblast growth factor-2 (FGF-2) in most cells, which may cause the diminution of proliferative capacity of MSCs [87]. Furthermore, data from our laboratory showed that canonical and non-canonical Wnts, Wnt3a and Wnt5a, respectively, act in opposite ways for modulating the behavior of MSCs, i.e., Wnt3a maintains stemness, while Wnt5a induces osteogenesis [88]. With aging, increased Wnt/β-catenin signaling was seen in old mice, the function of which, however, requires further investigation [89].

Currently, the supporting function of MSCs to HSCs has been well demonstrated. Given their very close location in bone marrow, how HSCs affect MSCs, in particular during the aging process, requires further investigation. A study from June et al. suggests that HSCs do not rest passively in the bone marrow [90]. Instead, HSCs directly induce MSC osteogenesis through the secretion of BMP-2 and −6. In addition, aging causes the dysregulation of BMP expression by HSCs, which may contribute to the pathogenesis of osteoporosis.

Although the mechanism of generation of inflammaging is unclear, adipose tissue has been proposed as the major generator of systemic proinflammatory cytokines with advancing age [91–93]. After the discovery of leptin in 1994, adipose endocrine function and involvement in many physiologic and pathologic processes are now well recognized [94]. Aging

significantly alters adipose tissues in both lean and obese individuals, including progenitor cell function decline, cellular senescence, adipose-derived hormone changes, and reduced miRNA processing [95]. In particular, through a senescence-associated secretory phenotype, senescent cells themselves generate many pro-inflammatory cytokines and chemokines [96, 97]. Accumulation of fat tissues and adipocytes has been observed in the bone marrow of older human [98] and mice subjects [14]. These cells impair the bone healing process through the excessive generation of dipeptidyl peptidase-4. It is noteworthy that the increased number of adipocytes may be due to the bias of aged MSC towards adipogenic differentiation [99], which increases the complexity in understanding the interaction between adipocytes and MSCs during aging.

MSCs also have direct physical contact with osteoblasts. In monolayer co-cultures, MSCs and osteoblasts were observed to actively establish cell-cell contact, which was sufficient to drive MSC osteogenesis [100]. Similar results were reported by Glueck et al. [101]. Based on next- generation sequencing and bioinformatics analysis, differentially expressed microRNAs and genes were identified between normal and senescent osteoblasts. In particular, miR-204–5p was identified as an upstream regulator, inhibiting the expression of Runx2 [102]. Aged osteoblasts showed compromised capacity of generating cell junction upon stimulation; in fact, connexin 43 was shown to be critical for MSC survival and migration [104, 105]. As discussed above, MSCs reside in a perivascular environment. By co-culturing MSCs with human umbilical vein endothelial cells (HUVEC), there was not only a significant increase of MSC proliferation, but also the promotion of osteogenic differentiation of MSCs [106]. In fact, we have previously shown that ECM generated from HUVECs directs differentiation of MSCs into vascular lineages [107]. Since endothelial cell dysfunction was shown to be associated with sedentary aging, such as the robust production of proinflammatory cytokines [108, 109], they may have the potential to change the functionality of MSCs as well.

2.5.2 Effect of aged ECM—The composition, mechanical properties, and topography of ECM have all been shown to affect the phenotype of MSCs to varying extents. We have previously reviewed the function of ECM on MSCs [39]. Recently, our results, drawn together with others, showed that the ECM derived from MSCs could promote MSC proliferation and maintain their potential [110, 111]. MSCs expanded on this ECM regenerated significantly higher bone *in vivo* than those maintained on tissue culture plastic. Interestingly, ECM generated by aged MSCs showed significantly decreased capacity in preserving normal MSC function, suggesting the critical role of ECM in MSC aging [112]. However, whether the amount of ECM generated by MSCs representing a major determining factor in bone marrow is not clear. In addition to stem cell-derived ECM, the aging of tissue matrix also affects stem cell function. Our recent study showed that aged muscle ECM displayed decreased collagen tortuosity and stiffness with aging, which increased the fibrogenic marker expression in muscle stem cells at the expense of myogenesis [113]. In 2006, a landmark work by Engler et al. showed that MSCs could sense the stiffness of culture substrate and differentiate into different lineages according to the stiffness of substrate, including neurogenesis on the soft surface and osteogenesis on the hard surface [114]. Afterwards, Winer et al. cultured MSC on a soft gel with 250Pa stiffness and found

that the cells maintained a quiescent state without division. However, they were activated by being switched to a stiff substrate [115]. Similar results were reported in another independent study [116]. Taken together, these results suggest the effects of mechanical cues derived from the ECM on MSC aging. Unfortunately, how aging may change these aspects of the ECM within bone marrow is still unknown.

2.5.3 Effect of aged systemic factors—As mentioned, aging represents a systemic dysfunction. In addition, MSCs are physically located around the blood vessels. Therefore, the aging of MSCs must be associated with the circulating systemic factors. Zhang et al. showed that serum from old rats caused significantly higher senescent cell ratio in MSCs compared with young serum, which may functionally relate to enhanced β-catenin level [117]. By inhibiting canonical Wnt/β-catenin, the negative effect of old serum was mitigated. Similar inhibition by aged serum on MSC proliferation was also reported [118]. Interestingly, serum from aged rats treated with NT-020 did not show such inhibitory effects. Utilizing parabiotic pairing method to allow the transfer of blood borne cells, bone marrow stem cells from old animals significantly enhanced osteogenic differentiation upon stimulation, which also resulted in enhanced fracture repair [119]. Results from these studies also suggest that this effect is partially mediated through β-catenin. Recently, Rebo et al. has reported a new method, which allows heterochronic blood exchange between young and old mice without sharing other organs. They also showed that blood from old mice significantly inhibited the proliferation of stem cells in young mice [120]. Taken together, these results suggest that the old serum/plasma contains inhibitory factors that impede MSC function. Murphy et al. reviewed the potential mechanisms and proposed that CCL11, GDF11, mTOR, and insulin/IGF1 signaling pathways may significantly participate as the systematic factors on stem cell function [121].

3. Stem cell therapy in bone repair and regeneration

3.1. Current strategies for stem cell-based therapy

As discussed previously in this review article, bone has a unique and well-documented natural healing process that is normally sufficient to repair fractures and other common injuries [122, 123]. However, with aging, bone healing capacity is decreased, partially due to functional decline of MSCs. Therefore, an intervention is usually required to treat delayed union or nonunion. The current surgical methods of repairing bone defects are highly invasive and not always successful due to poor vascularization, soft tissue conditions, infection, and pre-existing bone malignancy [124]. Stem cell-based therapy is a viable alternative with promising therapeutic advantages in restoring both the structure and function of damaged bone [125]. In general, stem cells can be therapeutically used in one of three ways. First, freshly isolated stem cells can be transplanted directly into tissue and undergo *in vivo* differentiation to become a desired cell type. Second, stem cells can be manipulated *in vitro* before implantation. In many cases, stem cells are genetically engineered to express specific types of genes or pre-differentiated into a particular cell type prior to implantation to enhance lineage-specific differentiation [126]. Third, administration of specific cytokines can recruit circulating endogenous stem cells into injury sites and further facilitate cell proliferation, migration, adhesion, and differentiation [126, 127].

A review of the literature from the past fifteen years shows that various types of stem cells, including bone marrow-derived MSCs (BM-MSCs), adipose-derived stem cells (ADSCs), muscle- derived stem cells (MDSCs), umbilical cord blood-derived MSCs (UCB-MSCs), and dental pulp stem cells (DPSCs), have been used to enhance bone regeneration and repair (Table 2). These studies have utilized strategies to transplant different types of stem cells in animal models, with or without scaffolds; however, they all report similar stem cell implantation effects. Stem cell-based therapy has been shown to facilitate bone or blood vessel formations and/or decrease inflammation and lower immunogenicity. A good amount of literature has reported that the direct injection of autologous MSCs from different tissue sources were able to engraft and regenerate bone to repair critical-size bone defects after implantation in different types of animals [128–134]. Among these, some recent studies have reported that engrafted stem cells play a crucial role in improving the process of bone regeneration, primarily through the secretion of paracrine factors to create a pro-osteogenic microenvironment at the defect site [135–138]. For example, Gao et al., reported that MDSC implantation enhanced angiogenesis and bone regeneration in a critical size calvarial defect by promoting endothelial cell proliferation via secreting multiple growth factors. Moreover, the MDSCs were shown to suppress initial immune responses in the host animal by secretion of monocyte chemotactic protein 1 and attract macrophages [135]. Another group from Japan recently reported that secretomes in MSCs include various cytokines that are important in regulation of osteoclast differentiation and the recruitment and proliferation of osteogenic- and angiogenic- cells [136]. These studies suggest that investigating and developing drugs that can deliver the above-mentioned secreting factors from stem cells may lead to effective therapeutic modality for the treatment of bone fractures and defects.

In addition to direct stem cell injection, researchers have used tissue engineering approaches by delivering stem cells on biodegradable scaffolds [139–144]. In this case, several groups show that pre-differentiating or pre-manipulating stem cells *in vitro* prior to implantation could be beneficial to generate a particular cell phenotype [143, 145, 146]. Various genes, such as BMP-2 and BMP-4, have been introduced in stem cells to enhance osteogenic-specific differentiation. Additionally, US2/US3 genes, which can decrease the expression of MHC I protein in cells and reduce the activation of T-cells of the recipient animals, have been utilized for this goal as well [147–150]. Interestingly, in 2010, Lyons et al. reported that cell-free collagen-based scaffolds developed for bone repair showed excellent healing, relative to MSC-seeded constructs. Their results suggested that the matrix deposited by MSCs during the differentiation process may adversely affect healing by forming a barrier to healthy macrophage activity and thus preventing remodeling of the implanted tissue and formation of new bone by the host. This barrier prevents vascularization of the implanted tissue, resulting in the initiation of avascular necrosis at the center of the implanted scaffold [151]. From a bone healing and repair perspective, this study may provide a simpler solution, which is the development of a biomimetic scaffold with a composition similar to native bone tissue and without requiring any cell compartments. However, further studies will be needed to accurately access the effect of stem cells on scaffolds. Utilization and effects of different types of scaffolds and biomaterials for bone tissue engineering will be discussed in detail, in a later section of this review.

3.2. Effect of aging on stem cell therapy

3.2.1. Intrinsic and extrinsic factors affecting stem cell behavior and their niche—

Although MSC-based therapy offers the possibility of a renewable source of replacement cells and tissues to treat various types of bone defects and injuries [152], aging and the aging-associated processes can significantly impact the beneficial effect of stem cell-based therapy. The active stem and progenitor cells in young adult tissues persist into old age; however, these populations are different from their younger counterparts. In old age, resident stem cells are affected by both intrinsic and extrinsic factors, which often compromise their functions [153]. For example, Baster et al. showed that the number of BM-MSCs with osteogenic potential decreases early during aging in humans, which may be responsible for the age-related reduction in bone formation and in the mechanical properties, and integrity of bone [154]. In addition to MSCs, neural stem cells (NSCs), satellite cells, and HSCs have also been reported to show significant age-related decrease in the proliferation and differentiation potentials both *in vitro* and *in vivo* [155–157]. The loss of a number of functional stem cells with age can lead to profound consequences on tissue viability. The mechanism of stem cell exhaustion/depletion is unclear; however, it may be caused by a combination of a number of both intrinsic and extrinsic factors, including a change in growth factors activity, accumulation of DNA damage, and decline in progenitor cell responsiveness.

Intrinsic factors: Independent lines of evidence suggest that forms of cell cycle regulators, cell DNA damage, and telomere shortening lead to the activation of the tumor-suppressor mechanism, such as senescence, and cause stem cell behavior change in old age [158, 159]. As with all dividing cells, tight regulation of cell cycle is important for regulating the rate of cell division and cell kinetics [158]. Some studies have shown that the inhibitor of cyclin-dependent kinase, $p16^{Ink4a}$, which regulates the G1-S phase cell cycle transition, restricts stem cell self-renewal in aging systems, including NSCs [160] and HSCs [161]. Stem cell aging is also due to accumulation of heritable intrinsic events such as DNA damage. This damage is associated with exposure to ROS, ionizing radiation, chemical mutagens, and repeated DNA replication [158, 159]. Moreover, recent studies suggest that stem cell exhaustion, decreased proliferation, differentiation, and homing capabilities may be due to the shortening of telomere length in stem cells [162, 163]. Several studies have reported that there is a strong correlation between MSCs self-renewal capacity and telomere length in culture with donor age [162, 163].

Recently, the best strategy suggested to cure aging due to cell damage is a rapid and effective elimination of the damaged stem cells by apoptosis [164]. When the DNA repair mechanism falls apart due to the aging process, cells respond to innate changes and enter independent stress-response mechanisms including apoptosis and cellular senescence. Senescent cells (SCs) are permanently withdrawn from the cell cycle; however, unlike apoptotic cells, which are permanently eliminated, they are viable for prolonged periods of time, and accumulate with age. These persistent SCs are thought to accelerate aging and the onset of age-related diseases [164, 165]. Recently, a group of researchers have identified a molecular mechanism of SC viability and have reported that disruption of FOXO4-p53 interactions in SCs selectively induce cell-intrinsic apoptosis. Furthermore, targeted

apoptosis of SCs restored fitness, hair density, and renal function in fast and naturally aged mice models [164]. Similarly, another group has reported that the clearance of SCs in a progeroid mouse model delays several age-associated disorders. They have identified a specific inhibitor of the anti-apoptotic proteins Bcl-2 and Bcl-xl, ABT263, as a potent drug and showed that ABT263 selectively kills senescent HSCs, and muscle stem cells (MuSCs) both *in vitro* and *in vivo* [166]. Taken these together, therapeutic targeting of SCs could counteract the loss of tissue homeostasis in response to aging.

Extrinsic factors: In addition to the intrinsic factors, the function and regenerative potential of MSCs are impacted by extrinsic factors in aging. Studies report that the age-associated impairment of stem cell function is induced to a significant extent by the molecular composition of the surrounding niche rather than by cell intrinsic changes alone [167]. Additionally, many studies show that morphological and functional changes within different tissues and organs due to aging result in deleterious changes in the stem cell niche and their microenvironments, which further inhibit their regenerative potential [55, 158, 168]. For example, in old age, the status of stem cells is altered with decreased niche cells, fragmented ECM, increased genomic, mitochondrial DNA damage, disruption in a hypoxic microenvironment, and increased ROS [169]. Additionally, stem cells require growth factors, cytokines, and mitogens for maintaining self-renewal and multipotency; however, changes in ECM components and structure will further cause subsequent modification on the availability of these factors [158, 159, 169].

A concrete example of the influence of the local and systemic environment on stem cell function during aging has been well demonstrated by Conboy et al. This group has shown that the rejuvenation of aged progenitor cells in old animals is possible by exposing the old to a young, systemic environment [156]. In their experiments, they have used strategies such as heterochronic parabiosis to expose cells in an aged mouse to the systemic environment of the young mouse. Their results clearly indicated that aged murine MuSCs and hepatocytes showed significant increase in their proliferation and regenerative capacities after heterochronic parabiosis with a young mouse [156]. More recently, using the same parabiosis technique, Villeda et al. has shown that when young stem cells were subjected to an aged systemic milieu, they exhibited functional decline in neurogenesis, which further negatively affects cognitive function in mice [170]. Lastly, a group of researchers have also shown that systemic inhibition of TGFβ2 signaling pathways, responsible for increased fibrosis and inhibit muscle regeneration in dystrophic skeletal muscles, rescue the satellite cell phenotypes and significantly reduce fibrogenesis of satellite cells in dystrophic mice [171]. Defective regeneration and accumulation of fibrotic tissue also characterize aging muscles [172]. Therefore, this study suggests that changes in stem cell milieu definitely have a vital effect on changing stem cell behavior and fate. These studies, taken together, clearly indicate that changes in the stem cell microenvironment that occur with aging affect stem cell behaviors.

The vast majority of stem cell-based therapies are currently being explored for cell replacement. However, the above studies strongly suggest that stem cell-based therapy might be less effective when the cells are derived from older individuals or when therapy is

performed in older patients. Therefore, for any stem cell transplantation-based strategy, it may be crucial to consider the age of both the donor tissue and the recipient environment.

3.3. Stem cell rejuvenation for bone repair in aging

Similar to other stem cell types, bone marrow-derived MSCs are affected by aging, and MSCs from old patients show significantly limited fracture repair and osteoblast differentiation potential compared to MSCs from younger patients [154]. The cause of this difference in MSC bone repair and differentiation potential with age is still unknown; however, this phenomenon is irreversible and similar in mice [173]. Recently, Baht et al. has reported that exposure to youthful circulation by heterochronic parabiosis reverses the aged fracture repair phenotype and the diminished MSC-medicated osteoblastic differentiation capacity of old animals. They have also demonstrated that the engraftment of young bone marrow, particularly $CD45^+$ hematopoietic cells, was able to rejuvenate bone repair and osteoblast differentiation. This rejuvenation was driven by a factor that was able to modulate Wnt/β-catenin signaling pathways through the downregulation of β-catenin expression in old fracture calluses early in fracture repair and subsequent revitalizion of fracture repair processes in mice [119]. It will be interesting to further investigate if rejuvenation affects the levels of any growth factors related to bone regeneration or inflammatory response during bone repair in these parabiosis experiments. Considering all these results together, this study strongly suggests that aged cells exposed to a youthful systemic milieu display more youthful characteristics, promising that it may be possible to mitigate certain aging features that hinder bone repair and regeneration in old patients. Furthermore, rejuvenating MSCs prior to the cell therapy for bone repair may be a possible option to consider.

4. Scaffolds for bone regeneration

In addition to growth factors and stem cells, bioscaffolds are also needed. Often, bioscaffolds can function as the reservoir for multiple factors, the carrier for cells, the filler for the void space, and the template for bone regeneration. The requirements of an ideal scaffold for bone tissue engineering are as follows: (1) showing no local and systematic toxic effects to the host tissue; (2) supporting normal cellular activity; (3) allowing cell adhesion, proliferation, extracellular matrix deposition, and inducing new bone formation; (4) prompting the formation of blood vessels after several weeks implantation; and (5) appropriate mechanical properties, pore size, and *in vivo* biodegradation rate [174–177]. Many researchers have investigated the development of biomimetic scaffolds with osteogenic microenvironment to facilitate the ossification process and to improve clinical therapy (Figure 3).

4.1 Decellularized extracellular matrix scaffolds

Bone tissue ECM retains multiple bioactive molecules, such as pro-inflammatory cytokines and growth factors (e.g. BMPs and vascular endothelial growth factor (VEGF)) that will be beneficial for bone repair without adverse immune responses. In addition, decellularized ECM can be combined with growth factors or calcium phosphates (CaP) (e.g. β-tricalcium phosphate, β-TCP, and hydroxyapatite, HA) to enhance osteoinductivity [178, 179].

Decellularized native ECM (nECM), which is obtained from a mature organ (allograft or xenograft) after the removal of cells and cellular antigens, retains the structure and architecture of the original tissue and has the ability to facilitate bone repair [180]. Moreover, nECM is also considered a promising candidate structure for stem cell delivery system due to its functionality of growth factor regulation [181]. Another widely used bone ECM is demineralized bone matrix (DBM), which is obtained from allogeneic cortical bone after demineralization by acid [182]. DBM contains collagenous proteins and ECM-associated growth factors (BMPs and FGFs), which act to increase bone healing efficiency. Decellularized engineered ECM (eECM), which is a cell-free matrix generated by stem cells *in vitro* containing various components of the native ECM, has also been demonstrated to improve *in vitro* MSC expansion and osteogenic differentiation [183]. Additionally, combining bioactive eECM with synthetic scaffolds has been found to increase the osteoblastic hMSC differentiation, calcium deposition, and osteoid tissue formation, as compared to eECM-free scaffolds [184].

4.2 Synthetic scaffolds

Calcium phosphate based bioactive ceramic scaffolds—Calcium phosphate ceramics (CaP), such as HA and β-TCP, major components of bone, have good biocompatibility, high bioactivity, and osteoconductivity, and they are widely applied in bone tissue engineering. Zhang et al. found that composite porous scaffolds containing β-TCP as a matrix and HA nanofibers of different concentrations showed improved mechanical properties. The compressive strength of the β-TCP porous scaffold with the presence of 5% of HA nanofibers was 9.8 ± 0.3 MPa, which is comparable to that of human cancellous bone (2–10 MPa) [185]. A porous collagen-calcium phosphate scaffold fabricated via three-dimensional (3D) printing showed suitable mechanical properties and osteoconductivity for non-loading bone defect implantation and new bone formation [186].

Metallic scaffolds—Metallic materials, such as stainless steel and cobalt- and titanium-based alloys, are widely used in clinical orthopedics and demonstrate good biocompatibility and mechanical properties. However, the lack of bio-specific recognition epitopes on the metallic material surface makes these metallic scaffolds biologically less active. To improve cell-scaffold interaction and tissue repair/regeneration efficiency, growth factors and other bioactive factors have been coated onto the surface of the scaffold [187]. Moreover, development of magnesium and its alloy scaffolds has attracted great research interest, as magnesium-based scaffolds are bioresorbable and osteoconductive, and they do not elicit an inflammatory response [188]. Hybrid scaffolds, which combine two or more materials, endow the benefits of each type of material and therefore exhibit novel properties. For example, metal–ceramic–polymer hybrid scaffolds with porous structure have increased implant interface with preservation of the titanium implant lifetime, and they show improved tissue formation through load-sharing and stress distribution compared with fully dense titanium [189].

Polymeric scaffolds—Both natural and synthetic polymer-based (e.g. collagen and polycaprolactone (PCL)) scaffolds have been widely used in the bone tissue engineering field because the porosity, mechanical properties, and degradation behavior can be designed

and controlled. Hutmacher [190] and Liu et al. [191] have provided extensively reviewed polymer materials for bone repair applications, including polymeric materials and the design, fabrication methods, and modification of scaffolds.

Hydrogels, formed by highly hydrated polymers, can provide an appropriate microenvironment for cell culture and are actively being explored as promising substrates for bone repair. Hydrogel scaffolds can be modified or bio-activated with growth factors (such BMP and FGF), gene constructs, and small inductive molecules to enhance the proliferative and osteogenic activities of the seeded stem cells [192]. Hydrogels can also be combined with calcium phosphate ceramics to improve the mechanical properties [193]. Additionally, 3D hydrogel scaffold itself also induces osteoblast differentiation and mineralization by virtue of the mechanical properties of the scaffold. For example, poly (ethylene glycol) dimethacrylate hydrogel with a gradient in compressive modulus (~10–300 kPa) and encapsulated osteoblasts resulted in gradient-dependent osteoblastic differentiation [194]. Shen et al. also found that methylcellulose-based scaffolds with different cross-linking densities and controlled Young's modulus had a stimulatory effect on inducing osteogenic differentiation of seeded MSCs in the absence of inductive agents [195].

The development of 3D printed synthetic material-based scaffolds fabricated via computer aided design (CAD) using ceramic, metallic, polymeric, and composite materials has shown great potential for regenerative bone application due to controllable chemistry, shape, and porosity [196]. The precise, designed, 3D printed scaffold can mimic the structure and mechanical properties of trabecular bone, as well as support vessel formation [197].

4.3 Nanomaterial-based scaffolds

With a hierarchical structure ranging from nanoscale to macroscale by virtue of its composition of organic (e.g. collagen) and inorganic (e.g. nano-hydroxyapatite) materials, bone can be considered a "nanomaterial". To overcome limitations in traditional therapies, nanomaterials are being explored in bone tissue engineering, as they provide a closer structural support approximation to native bone architecture and can also regulate cell fate [198]. Scaffolds composed of nanofibers, nanotubes, nanoparticles, and hydrogels with nanostructures/nanopatterns have recently attracted increasing research interest.

Nanopatterned scaffold—To mimic the native tissue, nanogrooved matrices were investigated. It has been reported that the body and nucleus of hMSCs cultured on substrate with sparser nanogrooved pattern were elongated and orientated along the direction of nanogrooves [199]. The nano-topographical density of the nanopattern scaffold also played an important role on the osteogenesis of hMSCs by regulating the formation of cytoskeleton, which is necessary for tension effects on cell morphology and stem cell differentiation mediated via the Rho-associated protein kinase (ROCK)-pathway. In addition, multi-scale hierarchical topography-based substrates could provide native ECM-like topographical signals to influence substrate adhesion and differentiation of hMSCs. For example, the poly (lactic-co-glycolic acid) (PLGA) patches with nanopatterned hierarchical topography showed enhancement of hMSC osteogenesis and *in vivo* bone regeneration [200]. The symmetry and order of the nanopits also present the capability of regulating the expression

of bone-specific ECM proteins (osteopontin and osteocalcin) [201]. Interestingly, a nanopatterned substrate with surface immobilization of osteoinductive BMP-2 peptides exhibited additive effects of BMP-2 induction and nanotopographical stimulation on osteogenic differentiation of hMSCs [202].

Nanofibrous scaffold—Nanofibers fabricated via electrospinning technique have a wide range of diameter from tens of nanometers to microns. The diameter of the polymeric nanofibers can be affected by controlling the fabrication parameters, including polymer properties, solvent properties, solution flow rate, voltage, distance from the needle to the collector, and polymer concentration. Therefore, by tuning the parameters of electrospinning, the nanofibers produced are able to mimic the intricate fibrillar architecture of natural ECM components [203]. Synthetic polymers, such as poly-L-lactic acid (PLLA), polyglycolic acid (PGA), PLGA and PCL, and natural polymers, such as collagen, gelatin, alginate and chitosan, are used to design nanofibrous scaffolds. The synthetic polymers are more flexible in terms of synthesis, processing, and modification, while the natural polymers are more bioactive for stimulating cell adhesion [204, 205].

Our group has previously found that 3D PCL nanofibrous scaffolds supported and maintained osteogenic, chondrogenic, and adipogenic differentiation of hMSCs *in vitro* [205]. To endow the nanofibers with desired functionalities, hybrid nanofibers containing multiple components were explored. For example, chitosan/PCL nanofibers showed improved cell adhesion and osteogenic differentiation compared to pure PCL nanofibers [206]. PCL–$CaCO_3$ hybrid nanofibers improved mechanical tensile properties and water affinity [207]. Furthermore, nanofibrous scaffolds with surface-functionalization or internal incorporation of growth factors, gene constructs, and small molecules were widely investigated as controlled delivery systems for the acceleration of bone regeneration [208]. The silk/polyethylene oxide nanofibrous substrate with BMP-2 encapsulation showed enhancement of osteogenesis and calcium deposition [209]. PLGA nanofibers with DNA encapsulated within polylactide–poly (ethylene glycol) incorporation were able to gene transfection of the cultures and subsequently encode protein β-galactosidase [210].

Nanoparticles—Nanomaterials were explored as factors/genes nanocarriers during bone regeneration. For example, gold nanoparticles promoted osteogenic differentiation and inhibited the adipogenic differentiation of MSCs by causing mechanical stress on the MSCs to activate p38 mitogen-activated protein kinase pathway (MAPK) signaling pathway through the interaction with cell membrane and cytosolic proteins [211]. Interestingly, Henstock et al. found that functionalized magnetic nanoparticles directly targeted cell-surface mechanosensors and transduced forces from an external magnetic field, thus providing mechanical stimuli to hMSCs. The functionalized magnetic nanoparticles binding to the mechanically gated TREK1 K(+) channel of hMSCs increased mineralization of the cells as a result of mechanotransduction. This study presented the potential of using nanoparticles to enhance bone formation via stimulation of mechanotransduction [212]. Besides metal nanoparticles, carbon nanoparticles, including carbon nanotube and graphene, have also been explored in acceleration osteogenesis. Due to the unique physicochemical properties of graphene and its derivatives, graphene-based nanomaterials not only acted as a

nanoplatform for growth factor adsorption and delivery, but also provided mechanical support [213]. For example, we found that PLGA nanofibrous scaffold with graphene oxide incorporation accelerated hMSC osteogenesis as a result of preconcentration of protein and inductive agents by graphene oxide [214]. In addition, graphene-based nanomaterials have the capability of biomineralization under simulated body fluid environment [215], likely contributing to the enhancement of osteogenic differentiation of MSCs by graphene–biomineral materials [216].

4.4 Vascularization in engineered bone

Bone has a rich vascular supply. Thus, providing an artificial environment rich in functional vascular networks may achieve efficient osseointegration and accelerate bone repair after implantation. VEGF and FGF-2 are two key factors involved in angiogenesis [217]. A combination delivery and release of angiogenic and osteogenic factors (such as VEGF and BMP-2) by a PLGA scaffold combined with condensed plasmid DNA encoding for BMP-4 and VEGF [218], or a scaffold encapsulated with BMP and VEGF proteins [219], were shown to significantly promote bone formation after implantation. In addition, co-culturing MSCs and HUVECs on a 3D β-TCP scaffold also resulted in the formation of vessel-like structure and osteogenesis, which can be considered as a cell-based strategy for vascularization in the biomaterial scaffolds [220]. Interestingly, nanomaterials have shown the potential of promoting vascularization even without the use of growth factors. For instance, a self-assembling peptide amphiphile (PA) molecule, functionalized with bioactive groups mimicking heparin, was designed and synthesized for angiogenesis induction. Both *in vitro* and *in vivo* experiments demonstrated that the 3D nanofibers formed as a result of PA-mediated self-assembly have the capacity of angiogenesis and robust vascularization [221].

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Future perspectives

Because aging is accompanied by increased incidence of bone diseases and compromised fracture healing capacity, the successful development of strategies that maintain healthy or functional MSCs *in vivo* should be of great benefit. The strategy to maintain MSC functionality during the host aging process includes two major avenues: maintaining cell quantity within the bone marrow or rejuvenating aged cells. The second method, rejuvenating MSCs of declining functions, is as described above. Since aging causes inevitable stem cell exhaustion, the success of the first method may rely on the supplementation of functional MSCs. In fact, direct injection of MSCs into the bone marrow has been performed, and these cells were found to stay in the injection area and home to bone fracture site after injury occurred [222, 223]. Twenty-four hours after transplantation of uncultured MSC into sublethally irradiated mice, more than half of the injected cells were found in the bone marrow [64]. However, whether hMSCs also have such capacity and how long these transplanted cells could require further investigation. In addition, the compromised homing capacity of MSCs during aging may not be solely due to the aging of MSCs, and additional guiding signals may be required. For example, a synthetic high-affinity and specific peptidomimetic ligand (LLP2A), against integrin α4β1 on the MSC surface, was attached to a bisphosphonate (alendronate, Ale) with high affinity for bone. Interestingly, the introduction of LLP2A-Ale was able to increase the homing of transplanted MSCs and augment bone formation [224]. Finally, to completely overcome the aging-associated issues of MSCs, an unlimited supply of young and functional MSCs from other resources is required, such as induced pluripotent stem cell (iPSCs). Our previous study had generated MSC-like cells from iPSCs, which displayed a robust osteogenic potential similar to primary MSCs [225].

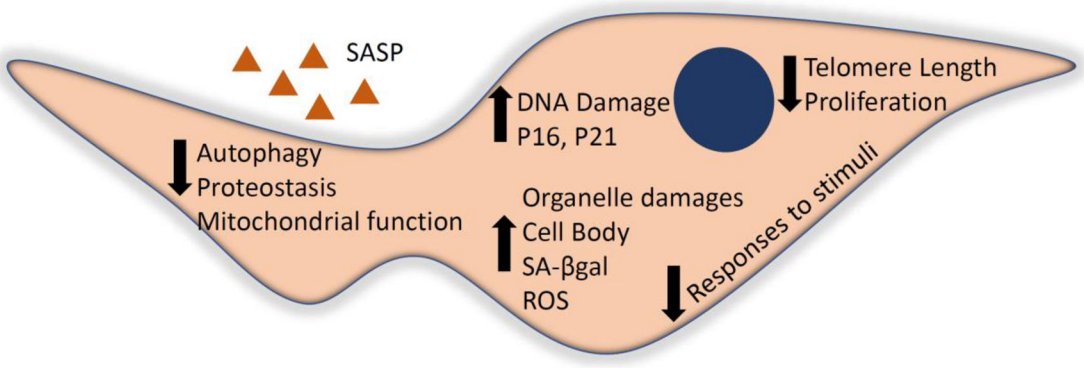

Figure 1.

Major changes during cell aging. Compared to young cells, old cells display reduced autophagy, proteostasis, and altered mitochondrial function. The proliferation capacity also declines with aging, due to the increase of P16 and P21 level. In addition, old cells produced SASP factors, which cause adverse effect on other cells.

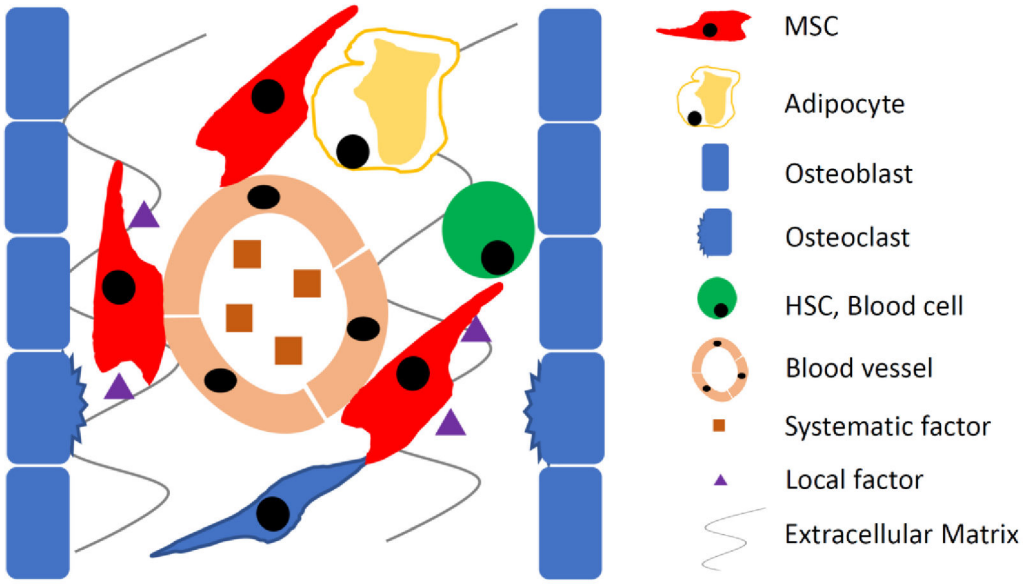

Figure 2.
MSC Niche in bone marrow. In quiescent state, MSCs reside in a homeostatic microenvironment, which contains different cells, soluble factors and extracellular matrix.

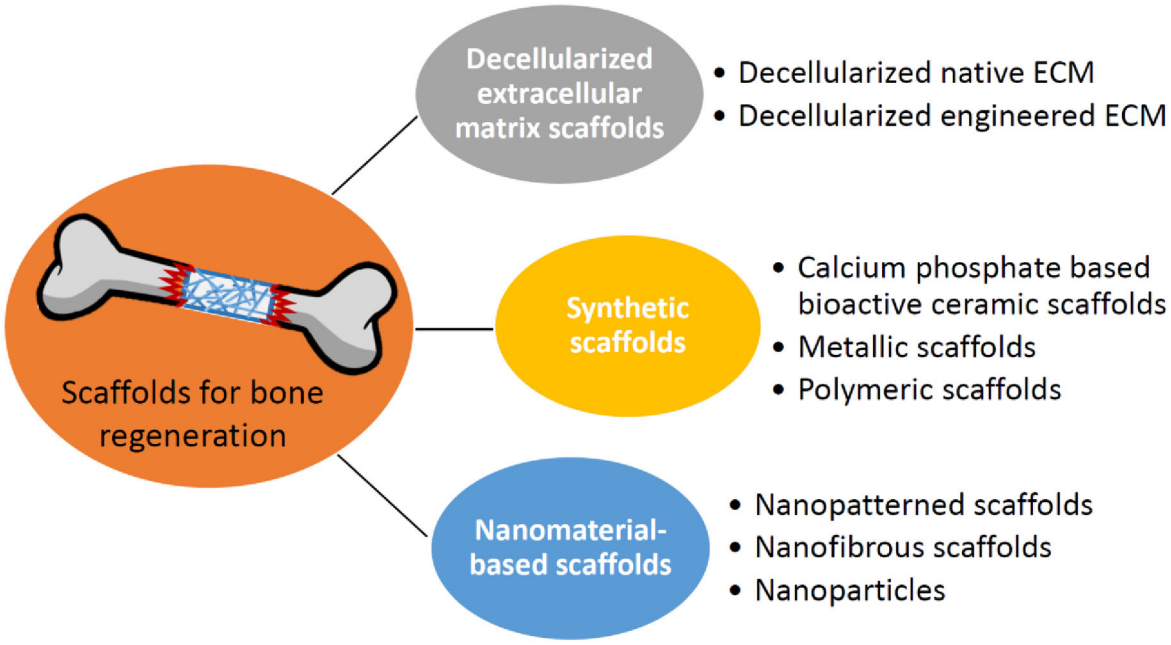

Figure 3.
Application of engineered scaffolds to augment MSC-based bone repair.

Table 1

FDA approved therapies for bone healing

Systemic	Local
Bisphosphonates	INFUSE (rhBMP-2)
Recombinant parathyroid hormone	Regranex (rhPDGF-BB)
RANKL inhibitors	rhBMP-7 *
SOST inhibitors (pending)	Healos (GDF-5) (pending)
	Demineralized bone matrix
	Fibula allograft
	Iliac crest autograft

* FDA humanitarian device exemption in 2003, failed to pass FDA approval in 2009 (https://www.fda.gov/)

Table 2

Characteristics of studies involve stem cell therapy in bone repair and regeneration

Cell Type	Source	Animal Model	Type of Defect	Study Design	Ref.
MSCs	Bone Marrow	Dog	Mandibular	MSCs + PRP injection	[142]
MSCs	Bone Marrow	Sheep	Metatarsal	MSC seeded PRP-based scaffold implantation	[144]
MDSCs	Skeletal Muscle	Mouse	Skull	BMP-2. VEGF. sFlt1 expressing MDSC transplantation	[147]
ASCs	Adipose Tissue	Dog	Parietal bones	MSCs seeded coral scaffold implantation	[141]
MSCs	Bone Marrow	Dog	Mandibular	MSCs seeded β-TCP scaffold implantation	[143]
ASCs	Fat Tissue	Dog	Ulna	BMP-2 expressing ASC transplantation	[149]
MSCs	Umbilical cord blood	Dog	Radial	MSC injection	[226]
MSCs	Bone Marrow	Dog	ONFH	MSC injection	[131]
DPSCs	Teeth	Pig	Mandibular	DPSC transplantation	[133]
MDSCs	Orbicular oris muscle	Rat	Cranial defect	hMDSC transplantation	[134]
MDSCs	Skeletal muscle	Mouse	Calvarial	BMP4 expressing MDSC transplantation	[148]
MSCs	Bone Marrow	Dog	Femoral head	MSC-seeded BCP scaffold implantation	[140]
MSCs	Bone Marrow	Rabbit	Femurs	Pre-osteogenically differentiated MSC transplantation	[146]
MSCs	Bone Marrow	Sheep	Tibial	hMSC transplantation	[132]
MPCs	Bone Marrow	Sheep	Tibial diaphyseal defect	MPC-seeded scaffold implantation	[128]
MSCs	Bone Marrow	Dog	Craniofacial	PRFG-MSCs injection	[139]
MSCs	Bone Marrow	Dog	Inferior orbital rim bone	Pre-osteogenically differentiated MSC-seeded β-TCP scaffold implantation	[145]
MSCs	Bone Marrow	Rabbit	Tibial	MSC-seeded bone scaffold implantation	[130]
ASCs	Adipose Tissue	Pig	Ulna	US2/US3 gene transfected ASC transplantation	[150]
MSCs	Bone Marrow	Sheep	Mandibular	MSC injection	[129]
MSCs	Adipose Tissue	Rabbit	Jaw bone	hMSC transplantation	[137]
MDSCs	Skeletal muscle	Mouse	Calvarial	BMP-4 expressing MDSC transplantation	[135]
ASCs	Adipose Tissue	Rabbit	OA-like damage	hASC injection	[138]
MSCs	Bone Marrow	Rabbits	Jaw bone loss	MSC injection	[136]

MSCs, mesenchymal stem cells; MDSCs, muscle-derived stem cells; ASCs, adipose-derived stem cells; DPSCs, dental pulp stem cells; MPCs, mesenchymal progenitor cells; h, human; PRP, platelet rich plasma; β-TCP, beta-tricalcium phosphate; BCP, biphasic calcium phosphate ceramic.