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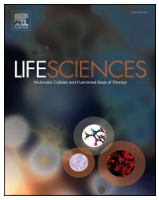

Review article

Osteoporosis treatment by mesenchymal stromal/stem cells and their exosomes: Emphasis on signaling pathways and mechanisms

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ABSTRACT

Osteoporosis is the loss of bone density, which is one of the main problems in developed and developing countries and is more common in the elderly. Because this disease is often not diagnosed until a bone fracture, it can become a life-threatening disease and cause hospitalization. With the increase of older people in a population, this disease's personal and social costs increase year by year and affect different communities. Most current treatments focus on pain relief and usually do not lead to bone tissue recovery and regeneration. But today, the use of stem cell therapy is recommended to treat and improve this disease recovery, which helps restore bone tissue by improving the imbalance in the osteoblast-osteoclast axis. Due to mesenchymal stromal/stem cells (MSCs) characteristics and their exosomes, these cells and vesicles are excellent sources for treating and preventing the progression and improvement of osteoporosis. Due to the ability of MSCs to differentiate into different cells and migrate to the site of injury, these cells are used in tissue regenerative medicine. Also, due to their contents, the exosomes of these cells help regenerate and treat various tissue injuries by affecting the injury site's cells. In this article, we attempted to review new studies in which MSCs and their exosomes were used to treat osteoporosis.

1. Introduction

Osteoporosis causes bone cohesion and density loss and is also associated with an increased risk of bone fractures [1]. Because MSCs differentiation in the bone marrow shifts to adipogenesis with age, osteoporosis becomes more common in the elderly [2]. The ability of bone to regenerate itself has been proven, but this ability is limited [3]. Deficiency in this ability leads to various discomforts such as osteoporosis, bone fractures, and osteonecrosis [4,5]. Therefore, this ability must be stimulated using various drugs and treatments to regenerate bone tissue in people with impaired repair. Osteoporosis is divided into primary and secondary categories, the primary type of which occurs in postmenopausal women (between the ages of 50 and 70) and in men in the absence of underlying and age-related disease. But the secondary type usually occurs in the presence of an underlying disease or the use of various drugs and is associated with increased bone

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fractures and decreased bone density [6,7]. The secondary type of osteoporosis is associated with inactivity, hyperthyroidism, hyperparathyroidism, hypercortisolism, and alcohol consumption, and usually occurs after the age of 70 and affects the trabecular and cortical bones [8]. Balance in osteoblast-mediated bone formation and osteoclast-mediated bone resorption in osteoporosis shifts toward increasing the number and function of osteoclasts [9]. This osteoblast/osteoclast axis imbalance is one of the factors that can lead to loss of proper bone density and osteoporosis [10]. One of the factors that can lead to this imbalance is mediated by unregulated immune system responses [11]. Improper immune system regulation leads to the onset of various inflammatory autoimmune diseases and adversely affects bone integrity [12]. Immune system cells and their secreted mediators play an important role in osteoporosis pathology, especially menopausal osteoporosis [13]. These cells interact with osteoblasts and osteoclasts through direct cell-to-cell communication and paracrine mechanisms and affect their function [14]. For example, T lymphocytes produced cytokines such as TNF-a can induce apoptosis in osteoblasts [15,16]. In addition, T lymphocytes indirectly stimulate osteoclastogenesis by stimulating the production of receptor-activator NF-κB ligand (RANKL) from B lymphocytes, thereby reducing bone density [17]. In general, balancing immune system responses in the bone microenvironment can play an important role in preventing osteoporosis and help tissue regeneration.

Over time, various treatments have been proposed to increase this ability and treat osteoporosis. In the 1940s, Fuller-Albright showed that estrogen therapy could reverse the negative calcium balance created in postmenopausal women or oophorectomy [20]. Pharmaceuticals including estrogen, calcitonin, teriparatide, bisphosphonates, denosumab, romosozumab, and odanacatib are used to treat osteoporosis [20]. Although these drugs' therapeutic benefits have been proven, they are controversial due to their side effects and limited effectiveness. Also, pharmacological treatments cannot restore bone tissue and usually prevent the disease from progressing [21]. Therefore, the development of new strategies to treat this disease can be helpful [22].

Although many studies consider isolated MSCs from different tissues as stem cells papulations, there is still no study that proves the stemness of a heterogeneous population of these cells [23,24]. Considerably, the composition and number of stromal mesenchymal heterogeneous cells isolated from different individuals vary and may exhibit different characteristics during treatment. Comparison of mesenchymal cells isolated from the bone marrow of five different strains of mice showed significant differences in cell composition, alkaline phosphatase activities, and growth kinetics [25]. Three main features are considered for mesenchymal stromal cells: 1) During standard culture should be adherent to plastic surfaces. 2) expression of CD105, CD73, and CD90 markers and lack of expression of surface molecules of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR and 3) ability to differentiate into osteoblasts, adipocytes, and chondroblasts [26].

Nowadays, Many studies use from mesenchymal stromal/stem cells to treat osteoporosis. These cells are good sources for regenerating bone tissue due to their ability to self-renew, their ability to migrate to the site of injury, differentiate into osteoblasts, and modulate the immune system at the injury site. Factors such as oxygen concentration, mechanical stimuli, and cell culture conditions can affect the therapeutic potential of MSCs [27]. In addition, the tissue source in which MSCs are isolated, and the donor's age are other effective factors in the therapeutic potential of these cells [28]. Also, today, to overcome the limitations of cell therapy, exosomes of different cells are used to take advantage of cells' therapeutic properties. These vesicles transfer the mother cell's properties to the target cell and change their functions by transferring different cargos within them [29,30] (Fig. 1). In this review study, we first examine the properties of MSCs and then the mechanisms involved in their therapeutic potential, including the involvement of different signaling pathways, regulation of important transcription factor activity, and therapeutic applications of stem cell-derived exosomes.

2. MSC's immunomodulatory properties

MSCs are commonly found in all tissues and have the ability to regenerate and differentiate into various specialized cells [31]. These cells have less differentiative ability than embryonic stem cells (ES) and pluripotent stem cells (iPS) [32]. These cells' differentiation capacity has led to these cells' use in the regeneration and repair of various tissues. These cells look like fibroblasts and are closer to blood vessels in the body [33,34]. Mesenchymal stromal/stem cells are involved in many physiological and pathological processes, including maintaining cellular homeostasis, aging, tissue damage, inflammatory diseases, homeostasis

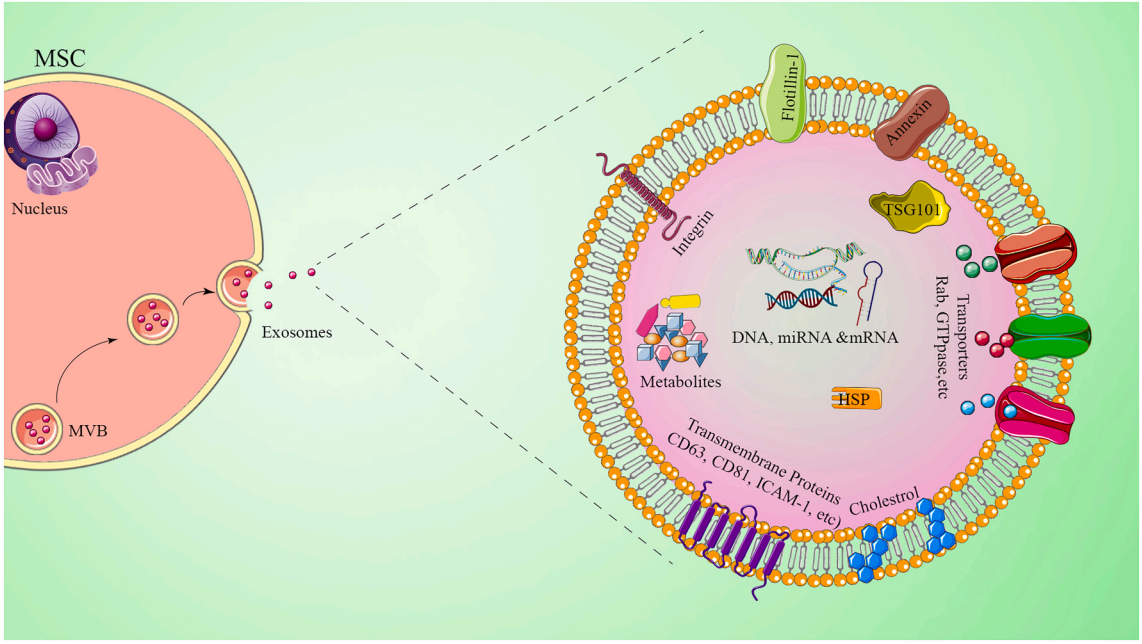

Fig. 1. Mesenchymal stem cells derived exosomes from have different contents that change their function by transferring them to the target cell.

of inflammatory responses, and migration to the site of tissue injury [35–37]. But in the meantime, the most important therapeutic effect of MSCs is related to immunomodulatory properties and their differentiation into different cells [38].

Given that tissue damage is usually associated with inflammation, immune system cell proliferation, and immune system responses, controlling these responses to enhance the regeneration of damaged tissues is particularly important [39]. Tissue-resident or recruited MSCs, in coordination with fibroblasts, endothelial cells, and other immune system cells, modulate inflammatory responses at the site of tissue damage [40]. In the presence of inflammatory factors such as TNF-a, IL-1, IFN-g, toxins of infectious agents, and hypoxia, MSCs can secrete various factors such as fibroblast growth factor (EGF), platelet-derived growth factor (PDGF), angiopoietin-1 (Ang-1), keratinocyte growth factor (KGF), and stroma-derived factor (SDF-1) [41]. These growth factors help regenerate and repair tissue by increasing the fibroblasts, endothelial cells, and tissue progenitor cells differentiation [42]. Besides, these cells suppress naive and memory T lymphocyte's activity through cell-to-cell contact in the absence of antigen-presenting cells. MSCs also promote anti-inflammatory responses and induce tolerance by altering the cytokine profile of virgin and executive T cells, dendritic cells, and NK cells [43]. Table 1 lists some of MSC's immune response modulatory mechanisms in immune cells. These anti-inflammatory properties of MSCs can greatly affect their therapeutic applications in treating osteoporosis and help regenerate bone tissue by sealing the functions of osteoclasts [44].

3. Transcription factors in MSCs osteoblastic differentiation

During the development of osteoporosis, the osteogenic differentiation potential of bone marrow mesenchymal stromal/stem cells (BMMSCs) and their adipogenic potential increases [45]. Various signaling pathways stimulate osteogenic or adipogenic differentiation in MSCs by regulating the activation of two major transcription factors, Runx-related transcription factor (Runx) and peroxisome proliferator-activated receptor (PPARγ) [46]. Runx family members have different roles in determining stem cell commitment. This family consists of three members called Runx1–3, in which Runx2 plays a role in the osteogenic and chondrogenic differentiation of stem cells [47]. Runx2 regulates MSCs osteogenic differentiation and acts as a target transcription factor in many signaling pathways, including transforming growth factor beta-1 (TGF-β1), bone morphogenic protein (BMP), wingless-type (Wnt), Hedgehog (HH), and Nel-like protein type 1 (NELL-1) [48–50]. Studies on the PPARγ genetic manipulation in mice show that no other factor can stimulate adipogenesis when the PPARγ gene is knocked out [51]. Therefore, PPARγ is considered as the main stimulator of adipogenesis and an inhibitor of osteogenesis. Due to the role of PPARγ, fat stores are severely reduced in PPARγ$^{+/-}$ mice, while bone density and bone growth are increased in them [51]. In vitro analysis also shows that embryonic stem cells obtained from PPARγ-deficient mice cannot differentiate into adipocytes, and osteogenic differentiation increases [51,52].

Osterix is a transcription factor involved in the osteoblastic differentiation of MSCs [53]. As mentioned, Runx2 plays a role in the osteoblastic differentiation of MSCs and the maintenance of these cells in the pre-osteoblast stage [54]. However, an increase in the Osterix transcription factor leads to pre-osteoblast cells' maturation into osteoblasts [55]. Thus, the Osterix transcription factor complements the function of Runx2 and plays an important role in the osteoblastic differentiation of MSCs in osteoporosis treatment. Fig. 2 summarizes the interrelationship of receptors, internal signaling pathways, and transcription factors involved in the osteogenic and adipogenic differentiation of MSCs (Fig. 2).

Table 1
Some of the mechanisms that mesenchymal stem cells use to inhibit the immune system.

Involved factors	Example	Mechanism
lipid intermediates	ProstaglandinE2 (PGE2) [179]	Suppresses T-cell proliferation [43] Increase M2 macrophage differentiation [180] Inhibition of NK-cell function and proliferation [181]
Tryptophan metabolism related enzyme	Indoleamine 2, deoxygenase (IDO) [182]	Induce IL-10 production Suppresses T-cell proliferation and DC maturation [183] Increase M2 macrophage differentiation
Other soluble factors production	TGF-β, HGF [184]	Suppresses T-cell proliferation [185]
Cytokines production	IL-6 [186] and IL-10 [187]	Positively regulates COX2 and EP2/EP4 Suppress DC maturation [188] Inhibition of bone marrow progenitors conversion to DC [189]
Chemotaxis proteins production	CCR5, CCR10, CXCR3, CXCL9, and CXCL10 [190]	Inhibit proliferation of the immune System effector cells [191]
HLA nonclassical class I production	Soluble HLA-G5 [192]	Decreasing the expression NKp44, NKp30, NKG2D, and CD132 in Nk cell [193,194]

4. Signaling pathway in MSCs osteoblastic differentiation

4.1. Wingless-type (Wnt) signaling pathway

The Wnt signaling pathway is an important pathway involved in regulating mesenchymal stromal/stem cell differentiation [56]. This signaling pathway regulates organogenesis, cell polarity, cell migration, and stem cell regeneration during embryonic development [57]. Many studies show that Wnt signaling activation stimulates osteogenic differentiation in MSCs and inhibits adipogenic differentiation [58]. Gain of function mutations in the Wnt pathway leads to bone overgrowth. Also, loss of function Mutations in this signaling pathway is associated with decreased bone growth and osteoporosis [59].

Some proteins, such as Secreted frizzled-related protein 1 (SFRP1), Dickkopf proteins (DKK), Wnt inhibitor factor I (Wifl), and sclerostin (SOST), block Wnt signaling and inhibit osteogenic differentiation in mesenchymal stromal/stem cells [60]. For example, preadipocyte-produced DKK1 inhibits in vitro osteogenesis of stem cells by increasing adipogenic differentiation [61].

In vitro studies show an inverse theoretical relationship between the osteogenic and adipogenic differentiation of mesenchymal stromal/stem cells [62]. It has been demonstrated that these mechanisms are regulated by various transcription factors [63].

4.2. Hedgehog (HH) signaling pathway

The HH signaling pathway plays an important role in organogenesis, embryonic pattern formation, tissue maintenance, and adult stem cell homeostasis [67]. The HH protein family has been identified in all vertebrates [68] and, when activated and signaling in MSCs, stimulates osteoblastogenesis in them through the Gli transcription factor [46,69].

In vitro studies show that osteoblastic differentiation increases by activation of the HH signaling pathway in C3H10T1/2 (murine MSC cell line) and KS483 cell lines [70–72]. This pathway reduces insulin sensitivity in the cell and lowering C/EBPα and PPARγ transcription factors level leads to specific markers of adipocytes such as fatty acid-binding proteins of adipocytes, adipsin, CD36, adiponectin, and leptin in the cell [73,74]. Also, when MSCs differentiate into adipocytes, the amount of factors involved in HH signaling, such as Gli1, Gli2, Gli3, and PTCH, decreases [74].

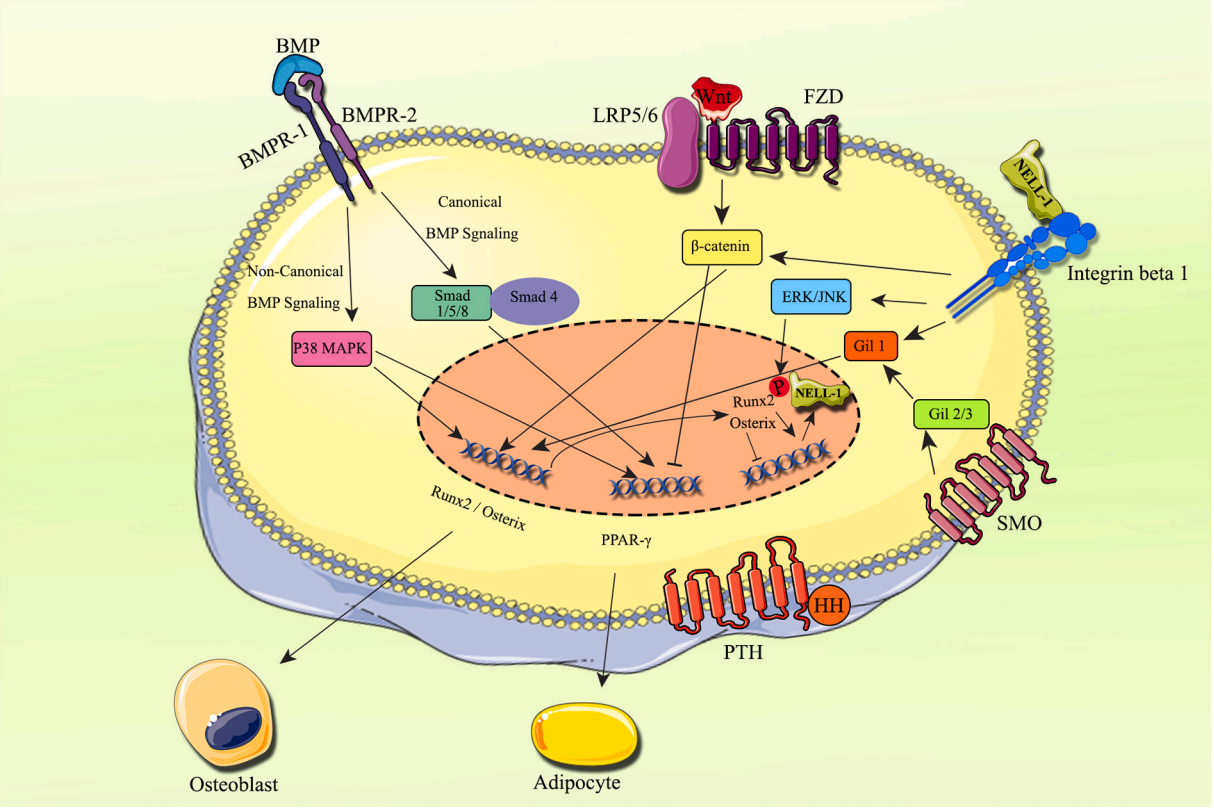

Fig. 2. Different receptors and transcription factors control the osteogenic and adipogenic differentiation of mesenchymal stromal/stem cells. These transcription factors alter MSCs' function by increasing the production of proteins involved in their differentiation. Activation of RunX2 and osterix genes transcription stimulates osteogenic differentiation, and transcription of PPARγ gene promotes adipogenic differentiation in this cell.

In a study by Gillian P. Johnson et al., signal transduction from primary cilia stimulates osteoblastogenic differentiation in MSCs via increasing HH pathway activation by IFT88, Gpr161 AC6, and cAMP-dependent manner. Gpr161 is a mechanoresponsive GPCR in the primary cilia and, after activation, regulates the intracellular cAMP concentration through AC6. So the cAMP concentration increases and leads to PKA activation [75]. During Gpr161 signaling, Smo-to-cilia trafficking increases through PKA activation, thereby activating the HH pathway and causing osteoblastogenic differentiation in MSCs [76].

4.3. NELL-1 signaling pathway

NELL-1 Is a secretory protein that binds to integrin B1 [77] (integrin a3 also reported) and activates the intracellular signaling cascade, increasing MSCs cell adhesion, osteogenic differentiation, and proliferation [77]. This signaling pathway increases B-catenin's nuclear localization, Gli1 expression, Runx transcription, and Runx2 phosphorylation and decreases PPARγ expression [78,79]. This function of NELL-1 distinguishes it from other secretory factors that induce bone differentiation, such as BMP-2 and BMP-9 because it permanently inhibits adipogenic differentiation in MSCs [80].

In summary, this protein induces osteogenic differentiation in MSCs by increasing the efficiency of the BMP, HH, Wnt, and mitogen-activated protein kinase (MAPK) signaling pathways. Therefore, it is suggested that Nell-1 can be used to increase osteogenic differentiation and proliferation in MSCs in the treatment of osteoporosis [81]. In various in vitro and in vivo studies, the combined use of BMP2 with NELL-1 synergistic effects has been proven [82,83].

4.4. BMP signaling pathway

The role of BMPs in the osteogenic differentiation of MSCs is indispensable. RUNX2 and Osterix are downstream targets of the BMP pathway and play a role in bone and cartilage development [84]. BMP2,9 (FDA approved for spinal fusion surgery) and BMP9 are among the most important members of this family whose role in the osteogenic differentiation of MSCs has been proven. Since BMP is a member of the TGF-B family [85], this signaling pathway is initiated by binding BMP to BMP type II receptors (BMPR), a serine-threonine kinase receptor, and then BMPR type II Phosphorylate and activate BMPR I receptors [86]. BMPR I then activate receptor-regulated Smads (R-Smads) by phosphorylating and stimulating their complexation with Co-smads [87]. This complex migrates to the nucleus and changes cell functions in interaction with transcription factors [88].

Two types of BMP receptors, called BMPR-IA and BMPR-IB, are involved in the differentiation of MSCs [89]. During BMPR-IA signaling and low BMP2 concentrations, Smads form a complex with CEB/Pa and stimulate PPARγ expression and adipogenic differentiation in MSCs [90,91]. However, BMPR-IB signaling and high doses of BMP2 lead to the physical association of Smads with Runx2, which induces osteogenic differentiation and expression of Runx2 in MSCs [92,93].

5. Mesenchymal stromal/stem cell transplantation in osteoporosis treatment

There are two methods for MSCs cell transplanting, which include systemic and local transplantation. During local transplantation, cell survival is reduced due to the lack of permanent oxygen and nutrients at the injection site. During systemic transplantation, MSCs are injected intravenously (IV) and intra-arterially (IA); using this method, exposed injected cells constantly to nutrients and oxygen [94]. But this systemic transplantation leads to the accumulation of injected cells in the lungs and inflammatory areas [95,96]. Another disadvantage of systemic injection of MSCs is the accumulation of these cells in abnormal

proliferation areas, such as breast or ovarian cancer tissues [97]. Therefore, to increase the efficiency of MSCs transplantation in osteoporosis treatment, a delivery system should be designed that leads to the migration of transplanted cells to the site of osteoporosis and bone. The use of effective scaffolds and hydrogel carriers are of these ways. Osteoblast progenitor cells seeding in scaffolds represent a promising bone repair method in osteoporosis [98]. However, interactions between scaffolds and MSCs affect these cells' survival, differentiation, and proliferation [98]. As mentioned in the local injection, lack of oxygen and nutrients supply in the cell-scaffold structures due to angiogenesis's slow process is one of these methods' disadvantages [99]. Genetic modifications of MSC, such as activating the secretion of cytokines, increasing the expression of cell surface receptors involved in migration to the target tissue, and activating transcription factors, are other ways to improve their effectiveness in osteoporosis treating [100]. It is also possible to increase the MSCs' therapeutic potential in treating osteoporosis by using osteogenic differentiation-induced drugs [101]. Table 2 shows the MSCs based clinical trials in osteoporosis treatment (Table 2).

The use of bone marrow-derived and adipose-derived mesenchymal stromal/stem cells [102,103] in ovariectomized rabbits leads to the formation of new bone and the improvement of osteoporosis symptoms in them. Also, the use of human umbilical cord blood MSCs (hUCB-MSC) in the mouse model of osteoporosis increases bone formation and thickness and is associated with improved trabecular parameters [104]. hUCB-MSCs have a higher potential for proliferation and osteogenic differentiation than other MSCs [105]. Also, due to the immunomodulatory properties of hUCB-MSC, the simple method of isolating MSCs from the umbilical cord, and the high potential for osteogenic differentiation, umbilical cord-derived MSCs can be used to treat osteoporosis and tissue engineering [106]. A study by Boohwi Hong shows that local injection of hUCB-MSC induces bone formation and treatment of osteoporosis in ovariectomized rats. Micro-CT analysis (which shows a 3D reconstructed image of the distal femur at eight weeks postoperatively) showed an increase in the density of trabecular bone, epiphyses, and distal femur compared to the control group. Also, a higher rate of new bone formation is seen in the peripheral and central areas of the osteoporotic bone in the MSCs treated group [107].

As mentioned, the use of scaffolds can increase the therapeutic efficacy of MSCs in the treatment of osteoporosis. In a study by Yamini Chandramohan et al., to evaluate the effectiveness of human ovarian follicular fluid (OFF) MSCs for use in bone regeneration, these cells grew in a biocompatible scaffold composed of chitosan (CS) And polycaprolactone (PCL) (scaffold coated with Zn2 + ion to obtain osteogenic properties) [108]. Cellular and molecular analyses indicate an increase in OFF-MSCs potential for osteogenic differentiation using these scaffolds. At the cellular level, alizarin red staining and ALP activity indicate an increase in calcium deposition in these cells, and at the molecular level, the expression of osteogenic differentiation-related genes such as collagen type 1, Runx2, osteonectin (ON), and osteocalcin (OC) is increased. Therefore, this scaffold can increase the osteogenic differentiation of MSCs and transform them into better cells in the treatment of osteoporosis [108].

Recent studies show that chemical chaperone tauroursodeoxycholic acid (TUDCA) reduces lipid levels in adipocytes and body mass in obese individuals [109]. In vitro injection of TUDCA into MSCs culture medium significantly decreases their adipogenic differentiation [110]. It can prevent pro-apoptotic proteins' binding to mitochondria and release cytochrome C by modulating endoplasmic reticulum-induced apoptosis [111]. Due to macrophages' and monocytes' role in bone tissue regeneration [112], minimizing pro-inflammatory responses is of particular importance. A study conducted by Byung-Hyun Cha et al. shows that the use of TUDCA increases the osteogenic differentiation of mouse BMMSCs in vitro by stimulating Integrin α5 (ITGA5) and the ERK1/2-MAPK and PI3K signaling pathways. The combined use of mouse BMMSCs and TUDCA in vivo is associated with decreased apoptosis and inflammatory responses in the implantation site cells [113]. Table 3 lists some of the MSCs modifications in regenerative medicine to increase their therapeutic potential (Table 3).

Lack of bone marrow donation, invasive isolation methods for donors and patients, low ratio of MSCs to other cells in different tissues, and reduced multipotency ability of MSCs after multiple passages limit the use of MSCs in large commercial applications. Therefore, using methods that do not have the above limitations can help develop osteoporosis treatment.

6. Standardization of MSC for use in clinical trials

Examination and characterization of cells isolated from different donors for use in therapy are of particular importance [114]. A systematic review published in 2021 by Wilson et al. shows that 28 studies (33 %) have no characteristic data. Forty-five studies (53.6 %) reported average values per marker for all lots used in the experiment, and 11 (13.1 %) studies included individual values per lot. In addition, the differentiation of extracted cells is of great importance. During this study, it was reported that osteogenic differentiation was studied in 29 % of studies, adipogenic in 27 % of studies, and chondrogenic in 20 % of studies [115].

Determining a donor for MSC is just as important as for other tissue and cell-based transplants. It is difficult to find an allogeneic donor with the desired characteristics to transplant these cells. For this reason, accurate criteria are required for donor selection. Donor age is one of the main criteria. It seems that younger donors are better due to having a higher concentration of CFU-F, high potential for proliferation and differentiation, and low probability of harmful damage to these cells [28].

After selecting a suitable donor, the process of culturing MSCs for therapeutic uses is of great importance. Because MSCs are adherent cells, their cell-plating density is an important parameter to ensure a reasonable growth rate and maintain the required function of these cells. Cell-plating is very different in different studies. For example, some studies have used 170×10^3 cells/cm^2 for high-density culture, and some use 50×10^3 cells/cm^2 for low-density culture [116]. The selection of the appropriate density during these studies is important. Based on published studies, the use of low density can lead to maintaining the potential for proliferation and differentiation in MSCs. But on a clinical scale, the use of low cell density is very difficult and may lead to the production of non-optimal cells [117]. After the first phase of culture and to passage the cells, it seems that using a density of 1000 cells/cm^2 is a suitable density and leads to the production of a suitable number of

Table 2
Mesenchymal stem cell-based clinical trials for osteoporosis treatment.

Study title	Source of MSC	Status	Intervention model	Phase	NCT number
Evaluation of Clinical and Bone Density Improvement After Implantation of Allogenic Mesenchymal Stem Cell From Umbilical Cord on Osteoporosis Patients	UCB-MSC	Recruiting	Single group assignment	Phase 2	NCT04501354
Clinical Trial of Intravenous Infusion of Fucosylated Bone Marrow Mesenchyme Cells in Patients With Osteoporosis	BM-MSC	Completed	Single group assignment	Phase 1	NCT02566655
Effectiveness of Adipose Tissue-Derived Mesenchymal Stem Cells as Osteogenic Component in Composite Grafts (ROBUST)	AT-MSC	Terminated	Parallel assignment	Phase 2	NCT01532076

MSC (mesenchymal stem cell), UCB-MSC (umbilical cord blood-derived MSCs), BM-MSC (bone marrow-derived MSCs), AT-MSC (adipose tissue-derived MSCs).

Table 3
Mesenchymal stem cells modifications for enhanced their regenerative potential.

Method	Example	Function
Scaffolds application	Ceramic scaffolds Hydroxyapatite ceramic scaffolds [195] β-tricalcium phosphate scaffolds [196]	Mechanical support for the MSCs to proliferate, differentiate into osteoblasts [100]
Hydrogels application	Bone marrow-derived ECM scaffolds [197,198]	Contain pro-inflammatory cytokines, BMPs, and various growth factors, including VEGF and mechanical support of the cells [198]
Genetic modifications	Activation of cytokines' secretion increasing expression of the cell surface receptors activate osteogenic differentiation-related of transcription factors [100]	Increase MSCs osteogenic differential potential [199]
Targeted modification	Synthetic high affinity and specific peptidomimetic ligand (LLP2A) [200]	Bind to integrin α4β1 on the MSC surface Increasing the homing and retention of the MSCs to bone [200]

cells for therapeutic use [118]. It should be noted that successive passages of these cells lead to a decrease in the proliferation rate of these cells and loss of their differentiative ability [119]. For this reason, the number of doublings should be given special attention (the number of times they proliferate is less than 20 times) [120].

The culture medium used in these cell cultures is also very important during the preparation and propagation of MSCs for therapeutic applications. There are different protocols for culturing mesenchymal cells isolated from autograft or allograft sources, including the use of fetal calf serum [121], human platelet lysates, and culture under normoxic or hypoxic conditions [122], and growth factor-containing media. It can affect the results of the study. There are several growth factors and cytokines that are beneficial for the growth of these cells. Still, the concentration of these cytokines depends on the number of cells and the required goal of the application of these cells (differentiated or not differentiated) [120].

All preclinical studies of MSCs in mouse models mimicking human diseases use live MSCs in the growth phase to inject, transplant, or implant. Almost no published clinical reports examine the effects of injections on animals model immediately after MSCs' sources freeze-thawing [123]. Therefore, since cryopreservation can affect the efficiency of cells, it is recommended that these cells be used in the growth phase for therapeutic applications [124].

After collecting and culturing these cells in vitro, a series of surface markers are used to characterize them, such as those defined in 2006 by the ISCT [26]. Although these markers help confirm and characterize these cells, they do not affect predicting their function or ability to suppress or modulate the immune system-related responses [125]. To evaluate the function of isolated MSCs in different individuals, it is very important to assess their response to interferon-gamma and activation of IDO transcription factors [126,127]. The product of this transcription factor is an enzyme that plays an important role in suppressing the activity of T cells and the immune system modulation. In patients who received MSCs from a low interferon-gamma responsive donor, the treatment may not be as good as patients who received a high-interferon-gamma responding MSCs donor. Therefore, study the ability of isolated cells' to respond to inflammation and inflammatory conditions is significant to standardize MSCs based therapy [123,127].

7. MSC-derived exosomes and their application in osteoporosis treatment

Cells can release different membrane-surrounded vesicles (whose size ranges from a few nanometers to a few microns) into their extracellular environment, collectively called extracellular vesicles (EVs) [128]. Apoptotic body, microvesicles (MVs), and exosomes are three types of EVs and are divided based on their size, formation model, and content. Apoptotic bodies' sizes range from 50 to 4000 nm and are typically released from apoptotic cells in the last stage of apoptosis [129]. These EVs are heterogeneous and contain membrane contents (such as phosphatidylserine), nuclear material, and cellular organelles. Unlike apoptotic bodies, microcycles are shedding directly from the healthy cell membrane. Like the apoptotic body, these EVs have a heterogeneous morphology, and usually, their sizes range from 100 nm to 1000 nm [130]. Microvesicles can affect the expression of genes by transmitting mi-RNA to neighbour cells. Besides, because the MVs releasing from the cell is not dependent on endocytosis, they lack endocytosis-related proteins [129]. Exosome sizes range from 30 nm to 120 nm. These vesicles are produced during late endosome membrane inward invagination, which leads to multiple vesicular bodies (MVBs) formation. The MVBs are composed of many exosomes released by the MVBs membrane attaching to the cell's plasma membrane [131].

As mentioned above, exosomes are nanoscale vesicles that carry the mother cell's contents and properties and deliver them to the target cell through their desirable properties [29]. These vesicles have a bilayer lipid membrane composed of cholesterol, sphingomyelin, and ceramide [132]. Exosomes carry a variety of components, including lipids, proteins, ssDNA, dsDNA, RNA, and miRNA [133]. After producing and releasing the exosome into the intracellular environment, these vesicles can affect the target cell in various ways, such as fusion, receptor-mediated endocytosis, juxtacrine signaling, micropinocytosis, and soluble signaling, and alter their actions [134,135]. Table 4 lists some of the markers that are commonly seen in exosomes (Table 4). Studies hypothesise that exosomes can transfer small amounts of cytokines to target cells and are a more efficient mechanism for stimulating surrounding cells than cytokines dumped into the intracellular

Table 4
Exosomes commonly expressed markers.

	Example	function
Tetraspanins	CD63, CD9, CD81, CD82	Cell adhesion, regulation of cell Motility and morphology, fusion, signaling, and other functions [201,202]
Fusion proteins	Flotillins, annexin	Exosome release from host cell [203] Induce cell proliferation, migration, differentiation, promote the cytoskeletal organization [204,205]
Adhesion molecules	Various integrin	Guiding exosomes to specific tissues [206]
Channel-building proteins	Connexins-43	Transmembrane transport of ions and small molecules between the cytoplasm of two attaching cells [207]
Heat shock proteins	HSC70 and HSC90	Inhibit caspase activation and apoptosis [208]
Major histocompatibility complex	MHC-1, MHC-2	Presentation of peptides to T cells [209]
Rab GTPases family proteins	Rab-27a, Rab11, and Rab35	Regulate exosome release, regulate membrane components recycling from the endosomal compartment to the plasma membrane [210]
Lysosomal proteins	Lamp2b	Role in chaperone-mediated autophagy [211]
Multivesicular body biogenesis	Alix and TSG101	Endolysosomal and multivesicular body trafficking and biogenesis [212]

Lamp2b (lysosome-associated membrane glycoprotein 2), TSG101 (tumor susceptibility gene 101) protein, Alix (ALG-2-interacting protein X), HSC (heat shock cognate).

environment [136].

Many studies and clinical trials have been used from MSCs derived exosomes due to their suitable characteristics such as small size, biocompatibility, ability to migrate to the site of injury, and anti-inflammatory and regenerative properties [137,138].

Nowadays, MSCs derived exosomes have been used to treat diseases such as liver disease [139], cardiovascular diseases [140], allergies [140], bronchopulmonary dysplasia [141], neurodegenerative disease [142], wound healing [143], atopic dermatitis [144], rheumatoid arthritis [145], systemic lupus erythematosus [146], bone fractures [147], cartilage repair [148], and eventually osteoporosis [149]. The development of various studies on the use of exosomes in diagnosing and treating diseases provides an important basis for their subsequent medicine applications. MSCs derived exosomes play a role in treating osteoporosis by affecting the target cell in various ways. Fig. 3 summarizes the MSC-derived exosome-related mechanisms in the osteoporosis treatment.

Although therapeutic effect differences resulting from differences in tissue source used to isolate MSCs, cell culture conditions, and different exosome separation methods between different Word groups have not yet been systematically studied, they are likely to significantly affect the therapeutic potential of exosomes [150]. The production of EVs, including exosomes, for clinical applications, should be done with a Good Manufacturing Process (GMP) and by Quality Control (QC) to be reliable and used for repeatable studies in academic settings [151,152]. The necessary criteria for QC of extracellular vesicles have been set by the International Association of Extracellular Vesicles (ISEV) called MISEV2018 and have been available to researchers [153]. In addition, the Korean Ministry of Health, Food and Drug Administration (MFDS) has published the world's first guide to EV therapy products as a guide to the quality, non-clinical and clinical evaluation of extracellular vesicle therapy products [137]. In the following, we will discuss about MSCs derived exosomes related treatment mechanisms in treating osteoporosis.

7.1. Stimulation of MSC osteoblastic differentiation

MSCs derived from human pluripotent stem cells (hiPSC-MSC) produce exosomes (hiPSC-MSC-Exos) that reflect the benefits of both MSCs and iPSCs. CCK-8 assays show that hiPSC-MSC-Exos increase osteogenic proliferation and differentiation in ovariectomized rat BMMSCs in vitro. Also, RT-PCR and Western blot analysis showed that the expression of RUNX-2, COL1, ALP, and OPN was significantly upregulated compared to the control group in hiPSC-MSC-Exos treated cells. Therefore, using these exosomes can lead to improved ossification in osteoporotic ovariectomized rats [154].

In the study by Jieyuan Zhang et al., a scaffold consisting of tricalcium phosphate (β-TCP) and MSCs derived exosomes labeled by DIO was added to the hBMSC cell culture plate. After release from the scaffold, These exosomes are internalized and captured by hBMSC And stimulate osteogenic differentiation. Microarray analysis shows that the expression of many genes in treated cells changes and increases. The results of the bioinformatic analysis showed that many of upregulated genes play a role in the PI3K/Akt signaling pathway, ECM-receptor interaction, and focal adhesion [155]. So these exosomes play a role in osteogenic differentiation and proliferation of MSCs and lead to the recruitment of residents and circulated MSCs to the osteoporotic site. Because resident MSCs play a significant role in tissue damage repairing, increasing their migration ability is particularly important in improving osteoporosis [155].

Adipose tissue MSCs derived exosomes contain large amounts of miR-375. This miR acts as a positive regulator of MSCs osteogenic differentiation, increases alkaline phosphatase activity, and also increases calcium deposition in MSCs [156]. miR-375 stimulates osteogenic differentiation in MSCs by targeting Insulin-like Growth Factor binding protein 3 (IGFBP3) [157]. Insulin-like Growth Factor (IGF) signaling is

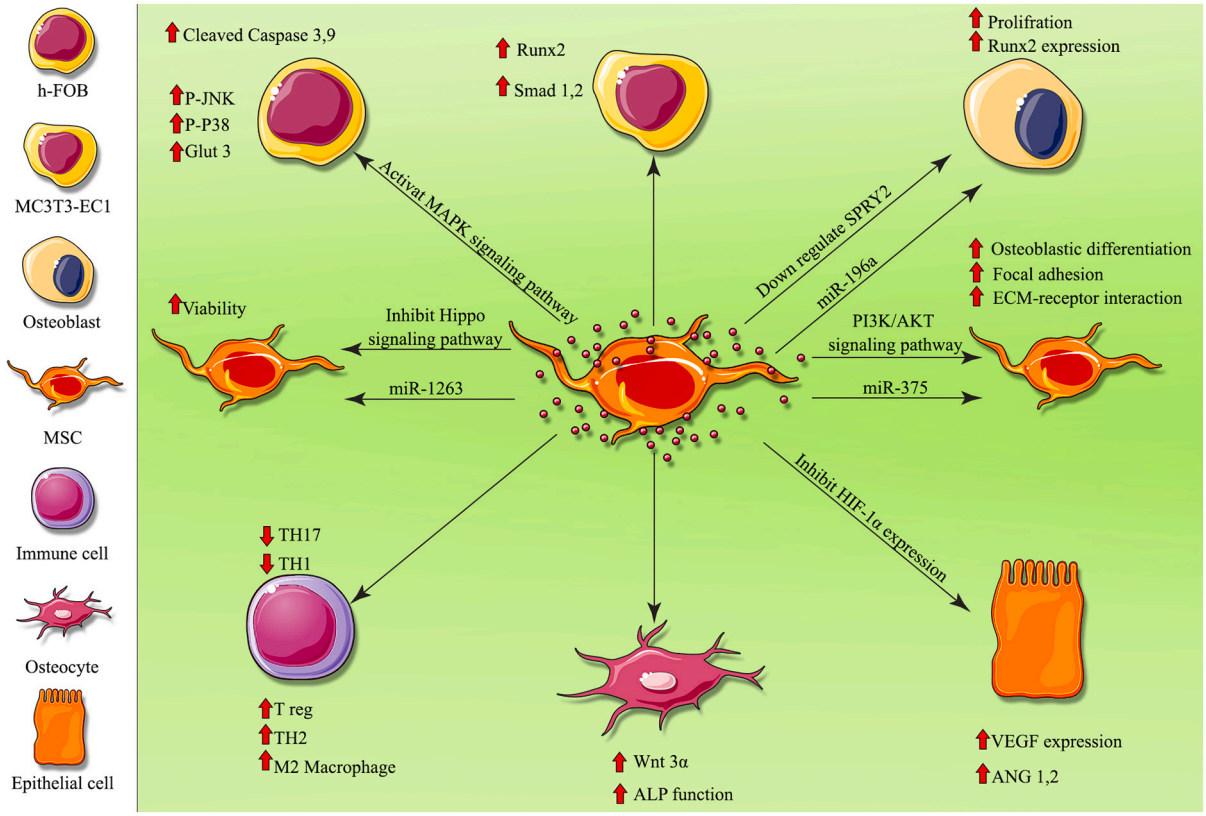

Fig. 3. Mechanisms involved in the improvement of osteoporosis by mesenchymal stem cell exosomes.

one of the pathways involved in bone growth, and its activation regulates by six ligands called IGFBP1–6 [158]. Studies have shown that IGFBP3, as a negative regulator of IGF signaling, inhibits osteogenesis in MSCs. Thus, miR-375 stimulates osteoblastic differentiation in MSCs by inhibiting IGFBP3 gene expression [157].

miRNA array analysis shows that the amount of miR-128-3p in mesenchymal stromal/stem cells exosomes in aged rats is much higher than in young rats. Evidence from experimental studies suggests that the transfer of miR-128-3p to young MSCs downregulates the expression of osteogenic-related proteins such as Runx2, collagen I, and ALP and suppresses their osteogenic differentiation. Therefore, young rats MSCs-derived exosomes have a high potential compared to aged rats MSCs-derived exosomes to induce osteogenic differentiation in mesenchymal stem cells [159].

7.2. Increased survival in cells involved in osteoporosis treatment

Many in vivo and in vitro studies have shown that MSCs derived exosomes stimulate osteoblasts, osteocytes, MSCs, and endothelial cells proliferation, survival, and migration [3]. In a study by Bao-cheng et al., CCK-8-related analysis and annexin V-FITC/PI assays showed that HUCMSC-derived exosomes help osteoporosis treatment by preventing BMSCs number reduction [160]. RNA-seq analysis to identify involved exosomal mi-RNAs in the treatment of osteoporosis indicates the presence of large amounts of miR-1263 in them [161]. miR-1263 inhibits Hippo signaling pathway and apoptosis by binding to 3′ UTR Mob1 (regulates YAP expression, which is the main regulator of the Hippo signaling pathway) and increases survival in BMSC [160].

In a study performed by Y.XIE et al., Western blot results show that after treating human fetal osteoblastic 1.19 (hFOB) cell line with MSCs-Exo, the expression level of cleaved Caspase-3 and 9 decreased. Therefore, it is suggested that MSCs derived exosomes play an important role in improving osteoporosis by inhibiting apoptosis in the osteoblast cell line [162].

7.3. Regulation of osteoblasts proliferation and differentiation

Umbilical cord mesenchymal stromal/stem cell-derived exosomes increase osteoblasts proliferation in a dose-dependent manner [163]. Experimental studies show that treatment of the hFOB 1.19 cell line with MSCs-Exo increases the expression levels of p-p38 and p-JNK and activates the MAPK signaling pathway. CCK-8 assay analysis in this study showed that the proliferation of hFOB 1.19 treated with MSCs-Exo increases through the activation of the MAPK pathway [164]. Besides, it was found that the mRNA levels and GLUT3 protein (osteoblast differentiation and maturation marker) increased in the treated cells [162].

Due to the presence of MiR-122-5p in the BMSCs derived exosomes, these vesicles can increase proliferation and differentiation in osteoblasts by negatively regulating Sprouty 2 (SPRY2) and activating the RTK/Ras/MAPK signaling pathway [165]. SPRY2 (Antagonist of fibroblast growth factor (FGF) pathways via inhibition of FGF-mediated phosphorylation of ERK1/2) contributes to osteoporosis development by inhibiting the activation of the MAPK/ERK signaling pathway [166]. IHC and Western blot analyses show that the use of BMMSC-Exos can significantly increase the expression of osteogenesis-related genes such as OCN, OPN, and OGN. Besides, the expression of osteogenesis-associated transcription factors such as BMP-2, Smad1, and RUNX2 was also increased in BMMSC-Exos treated MC3T3-E1C cell line (mouse muscle-derived osteoblast precursor cell line) [166].

Given the presence of osteogenic miRNAs such as miR-196a, miR-27a, and miR-206 in MSCs derived exosomes, treatment of osteoblasts with these exosomes leads to an increase in the expression of osteogenic genes such as RUNX-2, ALP, OCN, and OPN in them. Also, alizarin red staining and qRT-PCR show that miR-196a further enhances this action than other miRNAs [167].

A study by J. ZHOU et al. Showed that using hUCB-MSCs derived exosomes increases the expression of proteins involved in the Wnt signaling pathway in osteocytes, such as β-catenin and Wnt3a. As mentioned, activation of the Wnt/β-catenin signaling pathway stimulates osteoblastic proliferation and differentiation in resident cells at the osteoporotic bones and helps to improve osteoporosis [168]. Wnt3a induces bone formation in vivo by increasing ALP activity, increasing the deposition of minerals such as calcium, and increasing the expression of OC and OPN [169].

7.4. Induction of angiogenesis

MSCs derived exosomes can stimulate angiogenesis by increasing proliferation, migration, and tube formation in endothelial cells and help improve osteoporosis. Using a specific RNA inhibitor (siRNA) to inhibit hypoxia-inducible factor-1α (HIF-1α) indicates that the inhibition of this protein expression suppresses vascular endothelial growth factor (VEGF) expression and angiogenic index in endothelial cells will be reduced. Therefore, it is suggested that these exosomes regulate VEGF expression in endothelial cells by overexpression of HIF-1α [170].

A study conducted by Ryoko Takeuch et al. shows that using MSCs exosomes in rats containing bone defects leads to increased angiogenesis at the injury site. qRT-PCR analysis showed that the expression of angiogenic genes such as VEGF, ANG-1, and ANG-2 in the endothelial cells at the site of osteoporosis increased in these rats. Also, Micro-CT analysis showed an increase in new bone formation in rats treated with MSCs derived exosomes compared to the control group [171].

7.5. Immunomodulatory effect

Since uncontrolled inflammation leads to increased bone resorption and suppression of bone formation, the regulation of inflammatory responses at the damaged tissue site is particularly important to tissue regeneration. The MSCs derived exosomes' anti-inflammatory functions and immunomodulation has been widely reported in studies [172]. The miR-223 in these exosomes increases the differentiation of macrophages into the M2 phenotype. When PBMCs are co-cultured with these exosomes, PBX/knotted homeobox 1 (PKNOX1) expression, an important regulator of macrophage polarization are decreases [173]. Considering the role of T cells as one of the main regulators of osteoclast and osteoblast formation and based on these cells' role in various diseases, their activity regulating can play an important role in osteoporosis treatment [174]. Also, BMMSC derived exosomes increase Th1 to Th2 plasticity and decrease Th17 differentiation in PBMCs [175]. These exosomes increase the differentiation, proliferation, and suppressive potential of Treg cells by inhibiting inflammatory cytokines and increasing anti-inflammatory cytokines [137,175]. Therefore, the use of mesenchymal stromal/stem cells-derived exosomes can provide a suitable regenerative environment for the activity of cells involved in bone regeneration and the treatment of osteoporosis.

8. Conclusion and future direction

Overall, MSCs have many desirable properties, and their use in tissue regeneration studies increased over two decades ago. Considering the differentiation of these cells into osteoblasts, reducing the differentiation of osteoclasts and their activity, increasing angiogenesis at the site of injury, and modulating the immune responses at the site of tissue injury, the use of these cells in treating osteoporosis improves the symptoms of the disease and help to treatment of bone density loses. In addition, since MSCs can migrate to damaged tissues, systemic injection of these cells result in their migration to the site of inflammation and helps treat the diseases. Production of chemotactic factors from MSCs at the site of injury leads to the recruitment of tissue residents and circulating MSCs. Thus, mesenchymal stromal/stem cells can accelerate the regeneration of damaged tissues and help treat degenerative diseases such as osteoporosis. However, it has been shown that the use of stem

cells can lead to their accumulation in places such as the lungs and liver and the formation of tumors. MSCs are probably trapped in the lungs due to mechanical and physiological conditions such as small capillary size, large capillary network, and strong adhesion properties of MSCs to arteries. Unwanted entrapment of these cells in the lungs may lead to complications such as embolism in small animal models. In one study, injection of MSCs into the femoral artery in a skeletal muscle injury model showed a considerable proportion of MSCs accumulated in the lungs [176]. This study showed that using sodium nitroprusside vasodilator prior to cell transplantation significantly reduced these cells in the lung [177]. Still, the question of whether the decrease in cell entrapment in the lung is associated with an increase in cells in the target organ and leads to further improvement in patients requires further study. Therefore, the use of therapies that convey the advantages of cell therapy in the absence of its disadvantages is of particular importance. Also, it can be mentioned that many of these studies are preclinical animal studies. As mentioned, MSCs derived exosomes have many characteristics of stem cells and can induce tissue healing in damaged tissues. Due to the nanosize of exosomes, the ability to migrate to the site of injury in the bloodstream and carry various cargos such as enzymes and mi-RNAs, these vesicles have great potential in treating osteoporosis. Therefore, the use of MSCs derived exosomes has increased due to their characteristics.

With all that said, the promising results of EVs using, especially exosomes, for clinical treatment are still challenging, and many questions about them have yet to be answered. Mesenchymal stromal/stem cells derived from various tissues usually have a limited capacity to produce exosomes in a large scale for therapeutic applications. In addition, with all the techniques available for characterizing exosomes, their contents show a lot of heterogeneity in the number of times exosomes are taken from MSCs and can affect clinical functions and outcomes, so the exact content of the exosomes used must be examined. It is also essential to develop an exosome separation protocol that complies with GMP standards to be able to define a single protocol for large-scale clinical applications [136]. However, it seems that the use of exosomes and extracellular vesicles is at the beginning of its path, and many studies are needed to overcome the mentioned defects. Therefore, more experimental research is required to determine the optimal therapeutic dose, time intervals between injections, appropriate injection route for the treatment of various diseases, isolation methods, and optimizing exosome culture conditions to achieve optimal therapeutic results with minimal adverse effects [178]. Future research should also focus on developing a new generation of engineered mesenchymal stromal/stem cells capable of producing large amounts of exosomes. Therefore, it seems that engineered MSCs and manipulated exosomes can have more therapeutic potential than their intact type. Also, it should be considered the internal components of the exosomes produced by them have low heterogeneity at different times of exosome isolation. Also, one of the important factors involved in the repair and regeneration of tissue by MSCs-EVs is their immunosuppressive and supportive function for the dynamic activity of the immune system. It has been shown that in addition to proteins involved in immune suppression in the proteomic profile of MSCs-EVs, some proteins involved in inflammation and complement activation are also abundant in these vesicles [150]. Therefore, the use of these vesicles must be carefully controlled. Finally, it should be noted that the number of studies in this field is low, and most studies are limited to pre-clinical and experimental studies.

Abbreviations

MSC	mesenchymal stromal/stem cells
ES	embryonic stem cells
iPS	induced pluripotent stem cells
EGF	fibroblast growth factor
PDGF	platelet-derived growth factor
Ang-1	angiopoietin-1
KGF	keratinocyte growth factor
SDF-1	stroma-derived factor
BMSCs	bone marrow mesenchymal stromal/stem cells
Runx	Runx-related transcription factor
PPARγ	peroxisome proliferator-activated receptor
TGF-β1	transforming growth factor beta-1
BMP	bone morphogenic protein
Wnt	wingless-type
HH	Hedgehog
NELL-1	Nel-like protein type 1
SFRP1	Secreted frizzled-related protein 1
DKK	Dickkopf protein
WifI	Wnt inhibitor factor I
SOST	sclerostin
TRAP	Tartrate-resistant acid phosphatase
hUCB-MSC	human umbilical cord blood MSCs
TUDCA	tauroursodeoxycholic acid

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval and consent to participate

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Consent for publication

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Further Reading

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