TISSUE ENGINEERING: Part B
Volume 28, Number 1, 2022

DOI: 10.1089/ten.teb.2020.0246

termis
Tissue Engineering
& Regenerative Medicine
International Society

REVIEW ARTICLE

Exosomes: A Tool for Bone Tissue Engineering

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Mesenchymal stem cells (MSCs) have been repeatedly shown to be a valuable source for cell-based therapy in regenerative medicine, including bony tissue repair. However, engraftment at the injury site is poor. Recently, it has been suggested that MSCs and other cells act through a paracrine signaling mechanism. Exosomes are nanostructures that have been implicated in this process. They carry DNA, RNA, proteins, and lipids and play an important role in cell-to-cell communication directly modulating their target cell at a transcriptional level. In a bone microenvironment, they have been shown to increase osteogenesis and osteogenic differentiation *in vivo* and *in vitro*. In the following review, we will discuss the most advanced and significant knowledge of biological functions of exosomes in bone regeneration and their clinical applications in osseous diseases.

Keywords: exosomes, mesenchymal stromal cells, bone tissue engineering, bone defect, regenerative medicine

Impact Statement

Mesenchymal stem cells have been shown to be a promising tool in bone tissue engineering. Recently, it has been suggested that they secrete exosomes containing messenger RNA, proteins, and lipids, thus acting through paracrine signaling mechanisms. Considering that exosomes are nonteratogenic and have low immunogenic potential, they could potentially replace stem-cell based therapy and thus eradicate the risk of neoplastic transformation associated with cell transplantations in bone regeneration.

Introduction

Bone defects and their surgical reconstruction represent a major financial burden for worldwide health care systems.[1–4] Tumors, trauma, surgery, or inflammatory diseases can lead to critical-sized bone defects, necessitating extensive surgical reconstruction. Bone has limited self-regenerating capabilities. Similarly, alternative therapeutic options for bone repair, such as autologous or allogenic transplantation or prosthetic material, come with considerable drawbacks. For instance, implantation of prosthetic material is expensive, and postoperative physical rehabilitation is time consuming and difficult for patients. Bone autografts also cause donor site morbidity, are limited in their availability, and have long operating times. Bone allografts are also disfavored as they lead to an increased immunogenic response, are expensive, and often lead to nonunion.[1–4]

Taking into account our rapidly aging population and an increase in obesity-related diseases, bone defects and consecutively the cost for repair and reconstruction are only going to rise in the coming years.[3,5] Thus, alternative approaches to repair bone are highly sought after.

Bone repair using culture-expanded mesenchymal stem cells (MSCs) has been shown to be highly effective in bone regeneration.[6–10] It was initially thought that the MSC's differentiation potential would be crucial in tissue engineering using MSCs. However, MSC engraftment and differentiation at the injury site are poor and do not correlate with the good clinical results.[4,11,12] Thus, it has been proposed that MSC-derived paracrine signaling may be responsible for the tissue regeneration and not the MSCs themselves.[4,11,12] In a bone regeneration environment, Osugi *et al.*[13] showed increased expression of osteogenic markers *in vitro* and enhanced bone formation in calvarial defects *in vivo* using bone marrow stem cell (BMSC)-derived conditioned medium

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(CM), further supporting a paracrine signaling mechanism of MSCs. Recently, exosomes released by MSCs have been implicated to play an important role as key signaling mediators between cells.

Exosomes are biomolecular nanostructures released from a variety of different cells that play an important role in cell-to-cell communication by delivering functional biomolecules, for example, microRNA (miRNA), messenger RNA (mRNA), proteins, and lipids. Exosomes can directly modulate the function of the target cell by triggering gene transcription and therefore protein levels and are thus involved in a vast amount of physiological processes, which makes them an interesting target for clinical application and modulation of diseases.[14–17] As such, applications of exosomes have shown promising results in neurodegenerative diseases,[18–21] in modulating the immune response,[16,18,22,23] as vehicles for antigen delivery,[18,24,25] in cardiac and pulmonary disease,[16,26–29] and acute kidney injury.[30,31] In contrast, tumor-derived exosomes are also implicated in promoting tumor progression and metastasis[16,25,32–35] and the spread of infectious diseases.[14,36,37]

In a bone microenvironment, exosome treatment leads to increased osteogenic potential *in vitro* and *in vivo* and has been successfully applied in a variety of osseous diseases.[4,38–40] This review aims to provide an overview over the current knowledge of exosomes in the context of bone regeneration.

Biogenesis of Exosomes

During biogenesis of exosomes, endosomal vesicles are formed by invagination of the plasma membrane. Early endosomes develop into late endosomes and accumulate intraluminal vesicles (ILVs). These multivesicular bodies fuse with the plasma membrane and release ILVs, now exosomes, or merge with lysosomes for degradation[16,41–43] (Fig. 1). After exocytosis, exosomes communicate with other cells by fusion with the plasma membrane of the target cells and release their cargo to regulate cellular processes.[41] Exosomes can be isolated using a variety of different methods, most commonly differential ultracentrifugation. Characterization of exosomes can be achieved based on detection of surface protein markers, determination of size and morphology, or size distribution. Exosome biogenesis, isolation, and characterization have been described in detail elsewhere.[41,44–46]

Exosomes in Cell-to-Cell Communication

The cargo of exosomes contains DNA, miRNA, mRNA, proteins, and lipids (Fig. 1).[41,42] By delivering their cargo, exosomes are able to modify gene expression and signaling pathways of the recipient cell and are thus involved in regulation of physiological processes of the target cells, such as activation, migration, differentiation, apoptosis, or necrosis.[14,16,42,47]

Exosomal proteins and lipids are mainly involved in biogenesis and trafficking of exosomes and fusion with target cells but have also been shown to directly activate signaling processes in target cells.[48–50]

However, exosomal miRNA may be the most important regulator of cell–cell communication. MiRNAs are small noncoding RNAs that act as negative regulators of post-transcriptional gene expression. They bind to complementary sequences located in the 3′ untranslated and the 5′ end ''seed'' region of mRNAs.[51,52] The ''seed'' region spans from nucleotide position 2 to 7 and plays an important role in target recognition.[52] This leads to destabilization and consequent degradation of mRNA and thus suppression of expression of their target genes.[51,53] It has been suggested that a majority of protein-coding genes may be controlled by miRNAs.[52,54] Packaged in exosomes, miRNAs are protected from degradation by serum ribonucleases.[55]

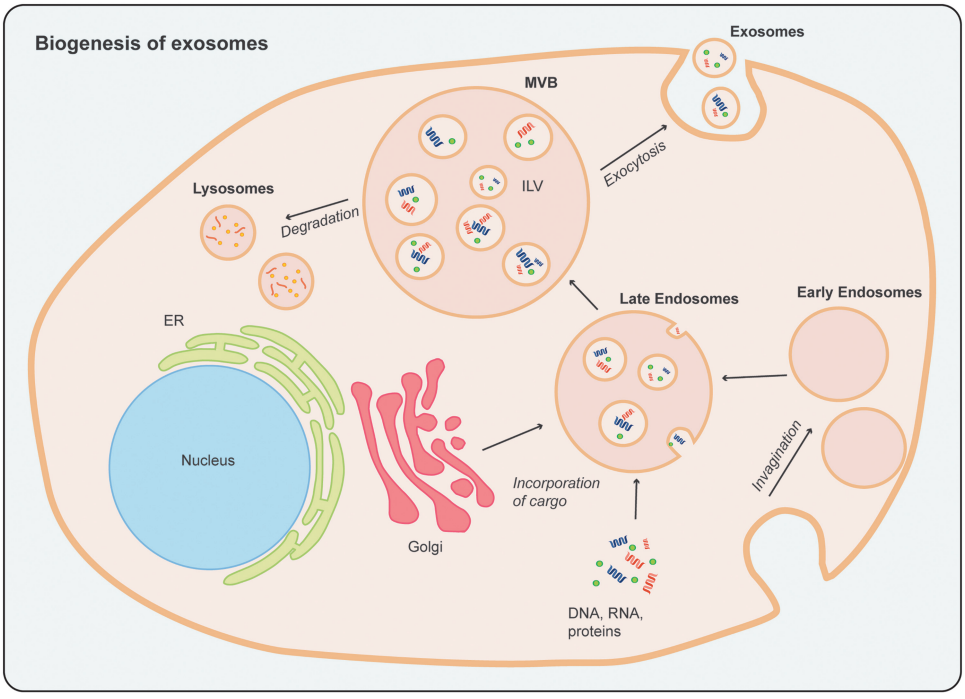

FIG. 1. Schematic of the biogenesis of exosomes. Invagination of the plasma membrane to form early endosomes. Involving the Golgi apparatus, DNA, RNA, proteins, and lipids are incorporated by invagination of the endosomal membrane to form late endosomes. A multivesicular body is formed containing multiple intraluminal vesicles. The multivesicular body is either processed by lysosomes for degradation or fuses with the cell plasma membrane to release the intraluminal vesicles, now called exosomes, into the extracellular space. This process is called exocytosis. ER, endoplasmatic reticulum; ILV, intraluminal vesicle; MVB, multivesicular body.

Exosomes in Bone Tissue Engineering

Exosomes can be isolated from a variety of different cell sources, and exosome cargo has been shown to be reflective of function of their parent cells.[16,38,40,47,55–58] Below, we will provide an overview over MSC-derived exosomes and exosomes from other parent cell sources (Tables 1 and 2).

MSC-derived exosomes

BMSC-derived exosomes. In bone regeneration, the most widely used source of exosome parent cells is BMSCs,[58–74] including human BMSCs (hBMSC),[58–64,72,74] mouse BMSCs,[65] rat BMSCs,[66–71,75] and rabbit BMSCs.[73] Target cells for *in vitro* experiments include hBMSCs,[58,59,62,64,72] mouse BMSCs (mBMSC),[65] rat BMSCs,[61,66,67,69] human[60,75] and mouse[70] osteoblasts, and human umbilical vein endothelial cells (HUVECs).[63,70,74]

Treatment with BMSC-derived exosomes led to increased expression levels of osteogenic growth factors and bone-related proteins, such as Osteopontin (OPN), Runt-related transcription factor 2 (RUNX2), collagen type 1 (COL1), transcription factors, for example, RUNX2, bone morphogenetic protein (BMP) 9, transforming growth factor (TGF)-β1, alkaline phosphatase (ALP), collagen type 1 alpha 1 (COL1A1), and extracellular matrix molecules,[58–60,62,64–69,72] as well as increased calcium deposition and matrix mineralization[60,64,66–70,72] *in vitro*. Moreover, three studies indicated increased proliferation[68,70,75] and migration[70] after exosome treatment *in vitro*. BMSC-derived exosomes have also been applied in several *in vivo* experiments. Increased bone formation and enhanced expression levels of osteogenic markers have been shown after implantation of BMSC-derived exosomes on the back of immunocompromised mice,[58,62] in rat[60,63,64] and mice[72] calvarial defects, in a mouse[61] and rat[69,70] femur fracture, in a model of osteonecrosis of the femoral head (ONFH) in rats[67,74] and rabbits,[73] in a tibial distraction model in rats,[68] in a rat model of radiation-induced bone loss,[71] and after intravenous injection in a mouse model of systemic lupus erythematosus (SLE).[65]

Adipose tissue-derived exosomes (ASC). Li *et al.*[76] harvested exosomes from osteogenically induced hASCs and showed increased ALP staining and activity and cell matrix mineralization and enhanced expression of osteoblastogenesis-related genes (*RUNX2*, *ALP*, *COL1A1*) in osteogenically induced hBMSCs. Respectively, they showed increased bone formation and enhanced expression of key osteogenic markers in a mouse calvarial defect treated with a PGLA scaffold with hASC-derived exosomes. After lentiviral transfection of hASCs, Chen *et al.*[77] isolated miR-375-overexpressing exosomes and were also able to show increased osteogenic potential in hBMSC-target cells *in vitro* and enhanced bone formation in rat calvarial defects *in vivo* compared to an untreated control group.[77]

Other MSC-derived exosomes. Imitating steroid-induced bone loss, Guo *et al.*[78] could show inhibition of decreased proliferation and reduced apoptotic effects in hBMSCs after treatment with exosomes from synovial-derived MSCs. In a rat model of steroid induced ONFH, treatment with exosomes led to reduced ONFH.[78]

Liu *et al.*[79] confirmed these findings in a similar animal model using human induced pluripotent stem cell (hiPSC)–MSC-derived exosomes and also showed increased tube formation in HUVECs *in vitro* and increased vascularization *in vivo.* hiPSCs are reprogrammed cells that can be used to create case-specific embryonic stem cells (ESCs) and can differentiate into every cell type. Moreover, they display lower immunogenic potential and do not raise ethical concerns as the use of ESCs does,[80] which makes them an ideal parent cell for isolation of exosomes. Treatment with hiPSC-MSC-derived exosomes led to increased proliferation, ALP activity, and matrix mineralization, as well as enhanced expression of osteogenic genes and proteins in BMSCs from ovariectomized rats[80] and hBMSCs[81] *in vitro* and increased bone formation and angiogenesis in rat calvarial defects *in vivo*.

Exosomes can also be isolated from human umbilical cord-derived MSCs (hucMSCs). Kuang *et al.*[82] isolated exosomes from Wharton's jelly of hucMSCs and were able to attenuate decreased proliferation and apoptotic effects in steroid-treated murine osteocyte like cells. In a rat model of steroid-induced ONFH, treatment with exosomes led to reversed bone loss and prevented osteonecrosis.[82] In HUVECs, treatment with hucMSC-derived exosomes led to increased expression of angiogenic markers, increased migration, and tube formation, but did not lead to changes in expression of osteogenic marker genes in osteoblasts.[83,84] In a steroid-induced model of ONFH in rats[85] and a femoral fracture model in mice[83] and rats,[84,86] hucMSC-exosome treatment led to increased neovascularization and enhanced bone regeneration *in vivo*. Yang *et al.*[87] created a rat model of disuse osteoporosis by hind limb unloading. In hence isolated rat BMSCs, they demonstrated a decrease of apoptosis-related proteins and a rescue of decreased proliferation *in vitro* and an increase in bone volume *in vivo* after treatment with hucMSC-derived exosomes.[87]

Wang *et al.*[51] isolated MSC-derived exosomes in different stages of osteogenic induction and demonstrated increased ALP activity, increased calcium and phosphate deposition, and enhanced matrix mineralization in hMSCs after exosome treatment *in vitro*; however, they did not further specify the parent cell source.

Exosomes from other parent cell sources

The osteoblast–osteoclast interaction is very important in maintenance of bone homeostasis. Receptor activator of nuclear factor kappa-B ligand (RANKL) is a transmembrane protein, expressed by osteoblasts, that binds to the receptor RANK on monocytes to initiate their differentiation into osteoclasts.[38,88,89] Mature osteoclasts continuously require RANKL stimulation to resorb bone.[89] Deng *et al.*[88] demonstrated that osteoblasts release microvesicles containing RANKL protein, thus stimulating osteoclast differentiation *in vitro*. Cui *et al.*[90] showed an upregulation of osteogenic marker genes and enhanced matrix mineralization in mBMSCs after treatment with exosomes derived from mineralizing osteoblasts. Deng *et al.*[91] decreased generation of osteoblast-derived exosomes and thus serum levels of RANKL through imipramine and thus were able to inhibit osteoclast differentiation and activation *in vitro* and attenuate decreased bone mineral density and show increased bone volume in ovariectomized mice *in vivo.*

Table 1. Mesenchymal Stem Cell-Derived Exosomes in Bone Regeneration

Parent cells	*Target cells*	*Isolation*	*Characterization*	*Preconditioning of parent cells*	*In vitro*	*Animal models*	*In vivo*
hBMSCs[58–64] mBMSCs[65] rBMSCs[66–71,75] hBMSCs from the jaw[72] Rabbit BMSCs[73] hBMSCs from traumatic ONFH patients[74]	hBMSCs[58,59,62,64,72] mBMSCs (MRL/lpr)[65] Human osteoblasts[60,75] mouse Osteoblasts[70] BMSCs from SFHN rat[66] rBMSCs[67,69] irradiated rBMSCs[71] hUVECs[63,70,74]	ExoQuick-TC[58,59,62,66] UC[60,61,63–65,68–73,75] Total Exosome Isolation Kit[67,74] UF[69,70]	TEM[58,60,62–64,66–74] DLS[59,69] Atomic force microscopy[59] Laser Doppler micro-electrophoresis[59] Flow cytometry[59,60,69] WB[61–67,70–74] FTE[61] NTA[62–64,67,69,70,72–74] TRPS[68]	Osteogenic induction[58,59,62] HIF-1α-adenovirus transfection[67] Dimethyloxaloylglycine-stimulation[63] miR-122-5p transfection[73]	Increased expression of osteogenic proteins/genes[58–60,62,64–69,72] Increased mineralization[60,64,66–70,72] Decreased adipogenesis[66] Increased angiogenesis[63,64,70,74] Increased proliferation[68,70,75] Increased migration[70] Reduced oxidative stress, accelerated DNA repair, attenuated inhibition of proliferation, and osteogenic differentiation potential in irradiated BMSCs[71]	Implantation in the back of immunocompromised mice[58,62] IV injection in a mouse model of SLE[65] Calvarial bone defect in rats[60,63,64] and mice[72] Femur fracture in mice [61] and rats[69,70] ONFH model in rats[67,74] and rabbits[73] Tibial distraction model in rats[68] Radiation-induced bone loss in rats[71]	Increased vascularization[58,63,64,70,73,74] Increased bone formation[58,60,61,63–74] Increased expression of osteogenic proteins/genes[58,62,65,69,70] Increased expression of angiogenic proteins/genes[58,70] Reduced oxidative stress[71] Prevention of bone mineral loss[67,73,74]
hASCs[76,77]	hBMSC[76,77]	UF[76] UC[76,77]	TEM[76,77] NTA[76,77] WB[76,77]	Osteogenic differentiation[76] miR-375 lentiviral transfection[77]	Increased proliferation[76] Increased migration[76] Increased mineralization[76] Increased expression of osteogenic proteins/genes[76]	Calvarial bone defects in mice[76] and rats[77]	Increased bone formation[76,77] Increased expression of osteogenic proteins/genes[76]
Synovial-derived MSCs[78]	hBMSCs Treated and untreated with steroids[78]	UF[78] UC[78]	DLS[78] TEM[78] WB[78]		Increased proliferation in untreated BMSCs[78] Inhibition of decreased proliferation of steroid treated BMSCs[78] Inhibition of apoptotic effects[78]	ONFH model in rats[78]	Prevention of bone mineral loss and cystic degeneration[78] Increased bone formation[78]
hiPSC-MSCs[79–81]	rBMSC-OVX[80] hBMSCs[81] hUVECs[79]	UF[80,81] UC[79–81]	TRPS[80,81] WB[79–81] TEM[79,81] NTA[79]		Increased proliferation[79–81] Increased mineralization[80,81] Increased expression of osteogenic proteins/genes[80,81] Increased migration[79] Increased tube formation[79]	Calvarial bone defects in OVX rats[80] and rats[81] ONFH model in rats[79]	Increased bone formation[80,81] Increased expression of osteogenic proteins/genes[80,81] Increased vascularization[79,80] Increased expression of angiogenic proteins/genes[80] Prevention of bone mineral loss[79]
human Wharton's jelly of umbilical cord MSCs[82]	Mouse osteocyte-like cells treated with steroids[82]	exoEasy Maxi Kit[82]	TEM[82] DLS[82] WB[82]		Inhibition of decreased proliferation of steroid-treated cells[82] Inhibition of apoptotic effects[82]	ONFH model in rats[82]	Prevention of bone mineral loss[82]
hucMSCs[83–87]	hUVECs[83,84] human Osteoblasts[83] mouse Osteoblasts[84] rBMSCs from DOP model[87]	UC[83–87] UF[83,84,86]	TEM[83–87] WB[83–87] NTA[83,87] DLS[84]	Hypoxic conditions[83]	Increased expression of angiogenic proteins/genes[83,84] Increased proliferation[83,84] Increased migration[83,84] Increased tube formation[83,84] Inhibition of decreased proliferation in DOP[87] Inhibition of apoptotic effects in DOP[87]	ONFH model in rats[85] Femur fracture in mice[83] and rats[84,86] DOP model in rats[87]	Increased bone formation[83–87] Inhibition of apoptotic effects[85,87] Increased vascularization[84,85] Increased expression of osteogenic proteins/genes[86]
hMSCs[51]	hMSCs[51]	UC[51] UF[51]	TEM[51] NTA[51]	Osteogenic induction[51]	Increased mineralization[51]		

ASC, adipose tissue-derived MSC; BMSC, bone marrow stem cell; DLS, dynamic light scattering; DOP, disuse osteoporosis; FTE, high-resolution frequency transmission electric-field imaging; h, human; HIF-1α, hypoxia-inducible factor-1 alpha; hiPSC, human induced pluripotent stem cell; hucMSC, human umbilical cord-derived MSC; hUVEC, human umbilical vein endothelial cell; IV, intravenous; m mouse; MRL, Murphy Roths Large; MSC, mesenchymal stem cell; NTA, nanoparticle tracking analysis; ONFH osteonecrosis of the femoral head; OVX, ovariectomy; r, rat; SLE, systemic lupus erythematosus; TEM, transmission electron microscopy; TRPS, tunable resistive pulse sensing; UC, ultracentrifugation; UF, ultrafiltration; WB, western blot.

Table 2. Exosomes Derived from Other Parent Cell Sources in Bone Regeneration

Parent cells	*Target cells*	*Isolation*	*Characterization*	*Preconditioning of parent cells*	*In vitro*	*Animal models*	*In vivo*
hMSC-derived adipocytes[96]	hMSC-derived osteocytes[96]	UC[96]	TEM[96]		Transfer of adipogenic mRNAs[96] Increased expression of adipogenic genes[96]		
Mouse dendritic cells		UC[96] UF[96]	WB[96] NTA[96] TEM[96]	Treatment with TGF-β1 and IL10[96]			Inhibition of inflammatory alveolar bone loss[97]
Osteoclasts from miR-214 knock-in mouse[92,93] Osteoclasts from osteoporotic patients and mice[92] Mouse osteoclasts[89,92,93] Mouse osteoclast precursors[89]	Mouse osteoblasts[92,93] Osteoblasts from OVX-mice[92] 1,25(OH)2D3-stimulated mouse marrow[89]	UC[92,93] ExoQuick-TC[89]	WB[89,93] NTA[93] FACS[92] DLS[92] TEM[89]	miR214-mimic and antagomir transfection[92,93]	Osteoclast-derived miR-214 inhibits osteoblast activity[92,93] Exosomes from osteoclast precursors stimulate osteoclastogenesis, Exosomes from osteoclasts inhibit osteoclastogenesis[89]	IV-injection in WT mouse[93] OVX-induced osteoporotic mouse model[92,93]	Osteoclast-derived miR-214-3p inhibits bone formation and expression of osteogenic markers in WT mice[93] Inhibition of osteoclast-derived miR-214-3p promotes bone formation in OVX mice[92,93] Inhibition of exosome release in osteoclasts from OVX mice leads to increased bone formation and promotes osteoblast activity[92]
Mouse osteoblasts[88,90,91]	Mouse osteoclasts[88] mBMSCs[90] Mouse macrophages[91]	UC[88] Exoquick-TC[90]	WB[88] TEM[88] SEM[90] NTA[91]	PTH[88] Imipramine[91]	Enhanced RANK-mediated osteoclast precursor differentiation[88] Increased expression of osteogenic proteins/genes[90] Increased mineralization[90] Inhibition of osteoclast differentiation and activation[91]	OVX-induced osteoporotic mouse model[91]	Inhibition of decrease of bone mineral density[91] Increased bone volume[91]
Mouse osteocytes[94]	Mouse osteoblasts[94]	UC[94]	WB[94] TEM[94]	Myostatin[94]	Decreased expression of osteogenic proteins/genes[94] Inhibition of osteoblastic differentiation and activity[94]		
Human monocytes[95]	hBMSCs[95]	UC[95]	Flow cytometry[95] WB[95]		Increased expression of osteogenic proteins/genes[95]		

FACS, fluorescence-activated cell sorting; IL10, interleukin 10; mRNA, messenger RNA; PTH, parathormon; SEM, scanning electron microscopy; TGF, transforming growth factor; WT, wild type.

In contrast, exosomes derived from osteoclasts containing miR-214 have been shown to inhibit osteoblast activity *in vitro*[92,93] and decreased bone formation and expression of osteogenic marker genes *in vivo*.[93] Respectively, inhibition of exosome release from osteoclasts led to increased bone mineral density and upregulated osteoblast activity in ovariectomized mice.[92] Huynh *et al.*[89] demonstrated that exosomes derived from osteoclast precursors stimulated osteoclastogenesis, whereas exosomes derived from mature osteoclasts inhibited osteoclastogenesis.

Similarly, exosomes derived from osteocytes led to inhibition of osteoblast differentiation and activity in osteoblasts *in vitro*,[94] whereas exosomes derived from human monocytes led to increased expression of osteogenic marker genes in hBMSCs *in vitro*.[95]

Martin *et al.*[96] investigated the interaction between adipocytes and osteoblasts and the paracrine mechanisms influencing MSC differentiation. They showed that hMSC-derived adipocytes produce extracellular vesicles containing adipogenic miRNAs decreasing osteoblast marker expression in osteocytes.[96] This is important in the context of bone loss in osteoporosis and the accompanying accumulation of adipocytes in the bone marrow, leading to an increased fracture risk.[96]

Other promising cell sources for isolation of exosomes and application in a bone microenvironment include dendritic cells. Dendritic cells are major regulators of the immune response, including direct modulation of T cells.[97] Elashiry *et al.*[97] loaded dendritic cell-derived exosomes with TGF-β1 and interleukin 10 (IL10), which were taken up by dendritic cells and T cells *in vivo*. Exosome treatment led to inhibition of maturation of dendritic cells and suppression of Th17 effector cells and thus stimulation of regulatory T cells, ultimately leading to inhibition of bone loss in inflammatory alveolar bone loss.[97]

The impact of tissue origin of exosomes

Interestingly, exosomes derived from naive MSCs can influence osteogenesis in target cells in different ways depending on their parent cell and can induce lineage-specific changes in naive MSCs *in vitro*, as well as *in vivo*.[58,60,72,98] Narayanan *et al.*[58] investigated whether exosomes derived from osteogenic MSCs were able to induce osteogenic differentiation of naive BMSCs. Thus, they compared the gene expression profiles in BMSCs treated with exosomes from noninduced BMSCs and with exosomes derived from BMSCs upon osteogenic differentiation. They were able to show that both regular and osteogenically induced exosomes led to a significant upregulation in expression levels of BMP9 and TGF-β1 *in vitro* and increased calcium phosphate nucleation *in vivo*, which was however more pronounced in the induced exosomes.[58] Qin *et al.*[60] have also shown upregulation of three important miRNAs in BMSC-derived extracellular vesicles and have identified miR-196a as a key player in osteoblastic differentiation and expression of osteogenic genes. Baglio *et al.*[98] characterized the small RNA content of hASC- and hBMSC-derived exosomes, investigating whether they differ respective to their parent cell. Interestingly, they found that the exosomal RNA content is not reflective of RNA content of parent cells, while miRNAs and transfer RNAs indicative of the parent cell origin were selectively packaged into exosomes.[98] Interestingly, in a recent study, Li *et al.*[72] demonstrated that exosomes isolated from neural crest-derived BMSCs from the jaw enhanced osteogenic differentiation of mesoderm-derived BMSCs from the ilium in a coculture *in vitro*. In a calvarial defect *in vivo*, they were able to show increased bone formation upon treatment with iliac BMSCs cultured with jaw BMSC exosomes compared to BMSCs cultured with iliac BMSC exosomes, thus supporting packaging of cell-specific signaling molecules into exosomes.[72]

In addition to undifferentiated MSC-derived exosomes displaying certain characteristics of their parent cells, multiple studies have shown increased osteogenic effects *in vitro* and *in vivo* after treatment with exosomes derived from osteogenically differentiating hBMSCs[53,58,59,62] and hASCs.[76]

Besides the osteogenic lineage commitment, previous studies have also shown specific alterations in exosomal cargo upon induction of adipogenic and chondrogenic differentiation in hBMSCs.[62,96]

Thus, exosomal cargo is highly dependent on tissue origin of parent cells and can induce lineage-specific changes in target cells.

The mechanisms of MSC-derived exosomes in bone regeneration

The pro-osteogenic effect of MSC-derived exosomes on bone regeneration may be due to (1) direct modulation of the osteogenic differentiation process of the neighboring target cells[53,60,61,99,100] and (2) stimulation of angiogenesis and thus optimizing the microenvironment to create ideal conditions for bone regeneration.[13,64,101–103]

Exosomes modulate the osteogenic differentiation process of target cells. Exosomes have been shown to carry specific osteogenic proteins, extracellular matrix proteins, and bone-related miRNAs.[53,61,67] Parent cells can be modified to express specific proteins and miRNAs, which allow targeted delivery of stimulating factors to neighboring cells.[63,67,73,77,83,88,92–94,97]

Xu *et al.*[53] demonstrated significant upregulation of seven miRNAs and downregulation of five miRNAs in BMSC-derived exosomes upon osteogenic induction that play important roles in modulating osteogenesis in target MSCs. For example, miR-218 enhances Wnt signaling by downregulating Wnt antagonists DKK2 and SFRP2 in hASCs thus leading to increased bone formation.[99] Furuta *et al.*[61] have demonstrated reduced fracture healing and delayed callus formation due to impaired endochondral ossification in $CD9^{-/-}$ mice that are known for decreased exosome production compared to wild-type mice. They were able to rescue this effect by injection of BMSC-derived exosomes and also found increased bone union rates in exosome-treated fractures in wild-type mice compared to untreated fractures.[61] Interestingly, levels of cytokines monocyte chemoattractant protein (MCP)-1, MCP-3, and stromal cell derived factor (SDF)-1 promoting osteogenesis and angiogenic factors were lower in MSC-derived exosomes than in CM. However they could show enhanced levels of miR-21 in both CM, as well as exosomes.[61] miR-21 promotes osteogenic differentiation and increases matrix mineralization by directly repressing expression of Smad7 by inhibition of translation.[61,104]

Parent cells can also be genetically modified or medically or environmentally stimulated to manipulate cargo load of exosomes: hypoxia-inducible factor-1α (HIF-1α) is an important factor regulating osteogenesis and angiogenesis under hypoxic conditions by promoting expression of osteogenic and angiogenic marker genes.[67] Treatment with exosomes secreted by BMSCs overexpressing HIF-1α after adenovirus transfection led to increased expression of osteogenic markers, such as osteocalcin (OCN) and ALP, *in vitro* and enhanced trabecular density in a steroid-induced ONFH model *in vivo*.[67] Similarly, Liu *et al.*[83] demonstrated increased bone fracture healing in mice after treatment with exosomes secreted in hypoxic conditions through HIF-1α activation and subsequent packaging of miR-126 into exosomes.

Further studies have shown increased osteogenic effects *in vitro* and *in vivo* in gain-of-function experiments with parent cells overexpressing miR-122-5p[73] or miR-375.[77] On the contrary, overexpression of miR-214 in osteoclasts led to downregulation of osteoblast activity *in vitro* and decreased bone formation *in vivo*.[92,93]

Treatment of dendritic cells with immunomodulatory TGF-β1 and IL10 led to selective packaging into exosomes and resulted in an inhibition of inflammatory alveolar bone loss.[97] Treatment of osteoblasts with parathormon (PTH) resulted into an exosome-mediated enhancement of osteoclast precursor differentiation,[88] whereas treatment of osteocytes with Myostatin led to decreased osteoblastic differentiation potential and activity through downregulation of miR-218 in exosomes.[94]

In conclusion, exosomal miRNAs regulate osteogenic differentiation by activating or deactivating different cellular signaling pathways and thus modulating gene transcription and expression of their target proteins. They also function as vehicles for delivery of important signaling molecules by protecting them from degradation.

Stimulation of angiogenesis by exosomes in bone formation. Bone regeneration is highly dependent on angiogenesis. A sufficient blood supply is essential for bone growth, but also vascular endothelial growth factor (VEGF) acts directly on vascular endothelial cells, which in turn stimulates osteoblast activity and maturation.[64] MSC-derived exosomes and conditioned media contain angiogenesis-enhancing factors, thus creating an optimal niche for bone regeneration.[40,63,64,67,69,70,73,74,79,80,83,101–103]

It is well known that hypoxic conditions lead to stimulation of angiogenesis through activation of the HIF-1α complex and subsequent translocation in the nucleus and transcription of angiogenic marker genes.[63] Thus, multiple studies have shown that treatment with exosomes derived from cells in hypoxic conditions or hypoxia-simulated conditions led to increased tube formation, increased proliferation and migration in HUVECs, and increased neovascularization in animal models *in vivo*.[63,67,83] Moreover, several authors showed increased expression of osteogenic and angiogenic factors in exosome-treated BMSCs,[64] increased proliferation and migration in exosome-treated HUVECs[70,74,79] *in vitro*, and increased bone formation and neovascularization[64,69,70,79,80] *in vivo*. Takeuchi *et al.*[64] further supported their findings by reversing the pro-angiogenic effect of BMSC-derived exosomes by adding a VEGF inhibitor. Xu *et al.*[69] identified miR-224-3p as a key regulator in angiogenesis in bone formation and were able to demonstrate increased angiogenesis by downregulation of miR-224-3p through inhibition of downregulation of target gene FIP200 (focal adhesion kinase family interacting protein of 200 kDa).

Exosomes in Osseous Diseases

Exosomes have been shown to be effective in the treatment of various skeletal diseases involving bone tissue, such as osteomyelitis,[105] SLE,[65] ONFH,[66,67,73,74,78,79,82,85] bone fractures,[61,69,70,83,84,86] osteoporosis,[80,87,91,92] and radiation-induced bone loss.[71]

Osteomyelitis is an inflammatory process that ultimately leads to bone destruction.[106] The most common microorganism causing this disease is *Staphylococcus aureus*.[106] Besides its extracellular localization, *S. aureus* can also be found intracellularly colocalizing with lysosomes. Due to this intracellular localization, therapeutic efficacy of antibiotic treatment has been shown to be limited.[105] Yang *et al.* showed that by incorporating linezolid into macrophage-derived exosomes, they could enhance the efficacy of the antibiotic in an intracellular environment *in vitro* and *in vivo*.[105]

SLE is an autoimmune disease that, among others, affects bone tissue and can cause osteopenia. Liu *et al.*[65] demonstrated rescue of impaired osteogenic differentiation in BMSCs derived from a mouse model of SLE after coculture with wild-type BMSC, through alteration of miR-29b and Notch1 levels. They were able to show a reversal of this rescue effect after knockdown rab27a, thus blocking exosome secretion, *in vitro* and *in vivo*.[65] Respectively, they confirmed their results after direct treatment of SLE-BMSCs *in vitro* and *in vivo* with BMSC-derived exosomes, showing reduced levels of miR-29b, downregulation of Notch, increased osteogenic differentiation, and bone formation.[65] Thus, they identified exosomes as an important target for pharmaceutical approach to improve osteopenia in SLE.[65]

Long-term steroid use, osteoporosis, or trauma can ultimately lead to reduced blood supply to the femoral head and thus cause death of osteocytes resulting in collapse of the femoral head and hip joint pain and dysfunction.[66,73,74] Resulting ONFH represents a major burden for the health care system resulting in total hip arthroplasty in 65% of patients.[66,73] Multiple authors were able to demonstrate decreased fatty degeneration of the bone marrow, reduced apoptotic effects, increased osteogenic differentiation, and increased tube formation, proliferation, and migration *in vitro*[66,67,73,74,78,79,82] and increased bone regeneration and neovascularization *in vivo*[67,73,74,78,79,82,85] after exosomal treatment.

Impaired fracture healing due to reduced blood supply, insufficient immobilization, infection, and other causes leads to nonunion or delayed union in about 10% of cases with numbers increasing with an aging population.[61,69,107,108] Thus, new pharmacological treatment approaches are much needed. Furuta *et al.*[61] were able to identify MSC-derived exosomes as important regulators in tissue repair process by showing reduced fracture healing in mice secreting reduced levels of exosomes. Xu *et al.*[69] demonstrated significantly reduced fracture healing in exosomes derived from MSCs from aged rats and were able to attenuate this effect by treatment with an miR-128-3p inhibitor. Multiple authors were able to show increased fracture healing treated with exosomes derived from naive MSCs[70,84,86] and hypoxic MSCs.[83,108]

Osteoporosis, one of the most problematic challenges in orthopedic surgery, is common in our aging society, but can also be caused by trauma, infection, skeletal defects, or tumors.[80,107] Ovariectomy (OVX) in mice or rats represents a very commonly used model for osteoporosis in research. Qi *et al.*[80] were able to show enhanced cell proliferation and increased expression of osteogenic markers after treatment of OVX-BMSCs with hiPSC-MSC-derived exosomes. *In vivo*, they demonstrated increased bone regeneration and angiogenesis in a calvarial defect model.[80] Sun *et al.*[92] identified exosomes as vehicles for transfer of miR-214 and were able to attenuate impaired osteoblast activity in ovariectomized rats by blocking the secretion of osteoclast-derived exosomes through rab27, thus blocking the transfer of miR-214. Yang *et al.*[87] demonstrated rescue of decreased proliferation and decreased apoptosis-related proteins in rat BMSCs from a disuse osteoporosis model *in vitro* and attenuated decreased trabecular thickness and increased bone volume *in vivo* after treatment with hucMSC-derived exosomes. Deng *et al.*[91] demonstrated that treatment with imipramine led to reduced production of osteoblast-derived exosomes and thus decreased serum levels of RANKL, which ultimately protected against bone loss in OVX mice.

Similar to changes in osteoporosis, radiation-induced bone loss is also characterized by fatty marrow and is a common cause for bone fractures in cancer patients.[71] It has been hypothesized that radiation causes damage of the DNA, increases reactive oxygen species, and promotes cell aging of BMSCs, thus reducing the proliferative and differentiative capacity of BMSCs.[71] Zuo *et al.*[71] were able to attenuate radiation-induced bone loss after treatment with BMSC-derived exosomes, thus reducing oxidative stress, accelerating DNA repair, and restoring impaired proliferation differentiation in irradiated BMSCs *in vitro* and reducing bone loss and increasing bone volume *in vivo.*

Exosomes in Cartilage Repair

Osteoarthritis (OA) is a chronic rheumatic joint disease affecting about 250 million people worldwide.[109–111] It mainly occurs in the hip and knee joint and is characterized by cartilage degeneration, bone sclerosis, formation of osteophytes, synovial inflammation, and calcification of menisci and ligaments.[109,110,112] Current treatment is essentially directed at relieving symptoms, such as anti-inflammatory pain medication; however there is no treatment available to repair the damaged cartilage tissue.[109,110,112] Recently, MSC-derived exosomes have shown promise in cartilage repair: In a recent review, Tan *et al.*[111] identified 13 studies describing chondroprotective effects and increased cartilage regeneration after treatment with MSC-derived exosomes in animal models of OA and osteochondral defects.[112–124] In addition, Wong *et al.*[125] further improved the efficacy of exosome treatment in a rabbit osteochondral defect by adding hyaluronic acid. In a model of temporomandibular joint OA, Zhang *et al.*[126] demonstrated an early suppression of inflammation followed by a proliferative phase with increased matrix synthesis after exosome treatment. Toh *et al.*[109] propose that MSC-derived exosomes restore cartilage by modulating the immune response, increasing the anabolic activity and matrix synthesis of cells, and regulating homeostasis in bioenergetics in target cells. Moreover, exosomes carry a unique set of miRNAs that are also known to be potent regulators of chondrogenesis, such as miR-23b, miR-92a, miR-125b, and miR-320.[109,127] A recent review has further described potential applications of exosomes in OA.[107]

Exosomes in Tendon Repair

Like cartilage, tendon also shows limited regeneration abilities and typically exhibits fibrotic healing after injury, thus restricting movement and bearing a high risk of re-tear.[128–131] Tendinopathy or tendon injuries are, however, very common disorders often affecting professional athletes and other people frequently undergoing physical activities.[128,131] Several studies demonstrated increased expression of tenogenic marker genes and increased proliferation and migration *in vitro* and/or increased tendon injury healing *in vivo* after treatment with tendon stem cell-derived exosomes,[128,132] tenocyte derived exosomes,[133] ASC-derived exosomes,[134] and BMSC-derived exosomes.[135,136] The positive effect of exosome treatment in tendon injury has also been ascribed to a reduced pro-inflammatory response in the early stages of tendon repair.[132,136–138] Cui *et al.*[130] recently showed that the fibrotic healing response in tendon injury is mainly mediated by macrophage-derived exosomes containing miR-21-5p. They were able to reduce the peritendinous fibrosis after injury in macrophage-depleted mice, identifying exosomes as an important potential target for pharmacological approaches to improve tendon healing.[130] Treatment with hucMSC-derived exosomes also led to reduced fibrosis after tendon injury, which was ascribed to a decreased expression of miR-21a-3p in the exosomal cargo.[138] A recently published review further analyzes and describes the important mechanism leading to increased tendon healing after exosomal treatment.[131]

Future Perspectives

The adaptability of exosomes in their interaction with target cells makes them a promising tool for translational research purposes. Especially, exosomal mRNA and miRNA display major reprogramming capacity for modulation of the function of recipient cells.[18]

Most importantly, exosomes can be applied in targeted drug delivery, for example, manipulation of exosomes allows enhancement of the bodily response to fight tumor or infection by direct loading of antigenic peptide.[18,25,139,140] Furthermore, exosomes can be loaded with specific miRNAs to modulate the response of target cells on a transcriptional level. This could be applied in a regenerative environment, such as enhancement of proliferation, migration, or differentiation of recipient cells. In bone regeneration, it is hypothesized that many key players regulating osteogenesis are targets of bone-derived exosomes[57,141] and can thus be altered in such a way to promote ossification.

Given their complex biochemical activities, exosomes represent an appealing tool for clinical applications. Considering that they are nonteratogenic and have low immunogenic potential, exosomes could potentially replace stem-cell based therapy and thus eradicate the risk of neoplastic transformation associated with cell transplantations.

In a bone microenvironment, exosomes can be modified to trigger specific changes in the transcriptomic profile of target cells and deliver important growth factors, thus creating an optimized niche to allow bone regeneration.

However, to make the step of standardizing the use of exosomes in a large-scale clinical setting, a uniform method to isolate a highly pure population of exosomes, that is easy and quick to handle, as well as standardized characterization and identification of exosomes, needs to be developed.

Authors' Contributions

J.H. performed literature research and writing of the article. M.F.G., M.T.L., and N.Q. contributed to editing and writing of the article. All authors who participated in the creation of this article are listed.

Disclosure Statement

The authors have no conflict of interest to declare.

Funding Information

This work was supported by the German Research Foundation (HU 2817/1-1) to J.H. and NIH (R01 DE026730; R01 DE027323) to M.T.L.

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Received: August 24, 2020
Accepted: December 8, 2020
Online Publication Date: March 9, 2021