MUSCLE AND BONE (L BONEWALD AND M HAMRICK, SECTION EDITORS)

Exosomes and Extracellular RNA in Muscle and Bone Aging and Crosstalk

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Abstract

Purpose of Review Extracellular vesicles (EV), which include exosomes and microvesicles, are membrane-bound particles shed by most cell types and are important mediators of cell-cell communication by delivering their cargo of proteins, miRNA, and mRNA to target cells and altering their function. Here, we provide an overview of what is currently known about EV composition and function in bone and muscle cells and discuss their role in mediating crosstalk between these two tissues as well as their role in musculoskeletal aging.

Recent Findings Recent studies have shown that muscle and bone cells produce EV, whose protein, mRNA, and miRNA cargo reflects the differentiated state of the parental cells. These EV have functional effects within their respective tissues, but evidence is accumulating that they are also shed into the circulation and can have effects on distant tissues. Bone- and muscle-derived EV can alter the differentiation and function of bone and muscle cells. Many of these effects are mediated via small microRNAs that regulate target genes in recipient cells.

Summary EV-mediated signaling in muscle and bone is an exciting and emerging field. While considerable progress has been made, much is still to be discovered about the mechanisms regulating EV composition, release, uptake, and function in muscle and bone. A key challenge is to understand more precisely how exosomes function in truly physiological settings.

Keywords Bone · Muscle · Exosomes · miRNA · Crosstalk · Aging

Introduction

Extracellular vesicles (EV) are increasingly being recognized as new players in cell-to-cell communication, leading to changing paradigms in our understanding of how cells communicate with their neighbors and how they crosstalk with

cells in distant tissues (reviewed in [1–7]). EV are membrane-bound particles shed from cells which carry their cargo of proteins, mRNAs, and microRNAs (miRNAs). These EV dock with a target cell, delivering their cargo to the cell and thereby altering its differentiation and/or function. Several types of EV have been described, with the most widely studied being exosomes (20–140 nm), which are released from multivesicular bodies, and microvesicles (100 nm–1 μm), which bud from the plasma membrane (Fig. 1) [8, 9]. Although initially studied in the cancer field, EV are now known to be shed by most normal cell types, with their cargo reflecting the differentiated state of the parent cells. Accumulating evidence suggests that exosomes are essential for cell-to-cell, organ-to-organ, and whole-body communication and have pleiotropic effects on the functions of neighboring or distant cells, tissues, and organs [9–13]. For example, EVs released by cells of one tissue or organ can travel in the circulation and may bind to cells of another tissue or organ through receptor-ligand interactions [14]. They can also spread through the body and anchor and/or fuse with the membrane to deliver cargos to distant recipient cells [15, 16].

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This article is part of the Topical Collection on Muscle and Bone

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EV Biogenesis
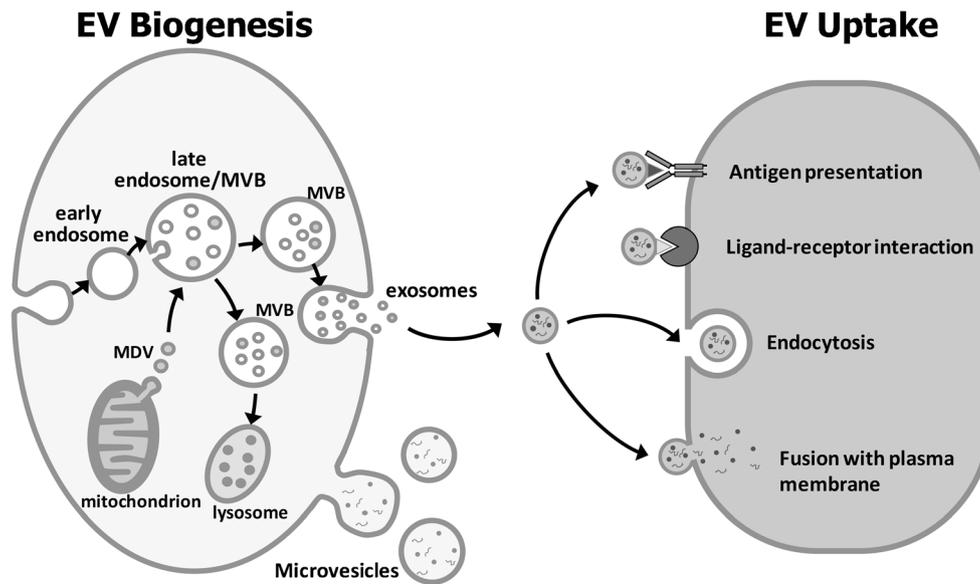
EV Uptake

Fig. 1 Biogenesis and uptake of extracellular vesicles. Exosomes form by inward budding of the late endosomal membrane to generate smaller internal vesicles within a multi-vesicular body (MVB). The MVBs can fuse with the lysosome for degradation of their cargo or fuse with the plasma membrane, releasing the internal vesicles, termed exosomes (20–140 nm), extracellularly. Mitochondrial-derived vesicles (MDV) (70–150 nm) can enter the endosome and its derivative MVBs and can also contribute to the exosomal population. Microvesicles (100 nm–1 µm) form by outward budding of the plasma membrane. EV can interact with recipient cells by signaling via antigen-antibody or ligand-receptor interactions. The most common mechanism of EV uptake involves endocytosis or the EV can fuse with the plasma membrane. Upon fusion or uptake, the EV cargo is released into the target cell (diagram modified from [19, 69])

The EV protein cargo can theoretically have direct effects in target cells, and the mRNAs can be expressed in target cells, thereby altering their function. EVs also contain miRNA cargo, which are single-stranded non-protein-coding RNAs of about 22–26 nucleotides in length. These function mainly as gene repressors at the transcriptional or post-transcriptional level by binding to the 3′-untranslated regions of mRNA transcripts and suppressing mRNA translation and/or stimulating mRNA degradation [17]. If delivered to cells via EV, miRNAs could regulate gene expression in the target cell. It is thought that the packaging of protein, mRNA, and miRNA cargos into EV protects them from degradation by extracellular proteases and nucleases. By expressing specific cell surface proteins, ligands, and receptors that may interact with proteins and/or receptors on target cells, the EV may also have increased specificity (i.e., a "molecular address") to deliver their cargo to a defined target cell. In theory, this provides a more efficient mechanism for cell-cell communication compared to signaling via diffusible ligands/soluble mediators. Not only are EV involved in cell-cell communication, but increasing evidence suggests they play an important role in pathological and disease processes and that analysis of EV as circulating biomarkers may be a useful tool for diagnosis and monitoring of disease. Furthermore, numerous studies have examined potential applications of EVs for treatment of disease and as a drug delivery mechanism. In this review, we provide a brief overview of EV biogenesis and uptake and then focus on recent studies that have revealed important functions for exosomes in bone and muscle cells and their role in mediating crosstalk between these two tissues. We also review their role in musculoskeletal aging and discuss current limitations in the field.

EV Biogenesis and Uptake

Exosomes are derived from the endosomal cell sorting machinery (reviewed in [1, 18, 19]). An early endosome forms via inward budding of the cell membrane. After this, the endosomal membrane undergoes a second inward (intraluminal) budding to generate smaller vesicles within the late endosome lumen to form a multivesicular body (MVB) (Fig. 1). The MVB then either fuses with a lysosome for degradation or fuses with the plasma membrane, thereby releasing the intraluminal vesicles, termed exosomes, into the extracellular space. Mitochondrial-derived vesicles may also enter the MVBs and can contribute to the final exosome population. Formation of intraluminal vesicles is thought to be initiated by enrichment of microdomains on the late endosomal membrane with tetraspanins (e.g., CD9, CD63). Proteins of the Endosomal Sorting Complex Required for Transport (ESCRT) family also play a key role. ESCRT I and II are recruited to the site and may initiate the intraluminal budding process. Other ESCRT proteins (e.g., ESCRT III) are then recruited through accessory proteins, ALIX, TSG101, and others to complete the budding process. Proteins involved

in exosome biogenesis are therefore commonly identified in exosome proteomic profiles. Alternative pathways for exosome biogenesis have also been proposed and are described in detail in other excellent review articles [1, 18, 19]. Release of exosomes from the cell surface is thought to be a cytoskeleton-dependent p53-controlled process involving ESCRT III and GTPases of the Rab family (e.g., Rab 5, Rab 27, Rab35).

The mechanisms for microvesicle biogenesis are less well defined, but this type of vesicle forms via outward budding and fission of the plasma membrane of the cell (Fig. 1). Outward membrane budding may be induced by translocation of phosphatidylserine from the inner to the outer membrane leaflet in localized domains. The budding process appears to be finalized by contraction of cytoskeletal structures through actin-myosin interactions, mediated via a signaling cascade involving ARF6, phospholipase D, and ERK (reviewed in [1, 18, 19]).

Three main mechanisms for EV interaction with target cells have been proposed (Fig. 1) (reviewed in [1, 19]). The EV may bind to the cell surface via antigen-antibody interaction or ligand-receptor interactions and may potentially trigger signaling via surface receptors, even without EV entry into the cell. The most common mode of EV uptake into target cells involves internalization via endocytotic processes, such as clathrin, caveolin, or lipid raft-mediated endocytosis, micropinocytosis, or phagocytosis. For EVs entering by endocytosis, the mechanism for transfer out of the endosomal compartment remains unclear, but they may be discharged via lysosomes or released into the cytoplasm. A third proposed mechanism for EV uptake is through direct fusion of EV with the plasma membrane of the cell and release of cargo directly into the cytoplasm. Whether an exosome will bind to a particular cell depends on the EV content and characteristics of its parent cell and the EV uptake mechanism(s) may also be dependent on the cell types of the donor and recipient cells.

EV Function in Bone Cells

Although EV were initially studied in the cancer field, they are now known to be released by virtually all cell types and bone cells are no exception. EV are released by the major bone cell types, osteocytes, osteoblasts, and osteoclasts and also by bone marrow stromal (stem) progenitor cells (BMSCs). Accumulating evidence suggests that EV play important roles in communication between these cell types to regulate bone formation and resorption, thereby regulating bone modeling and remodeling. Although we have used terminologies as reported in the various cited articles, readers should keep in mind that many studies describing "exosomes" or "microvesicles" are likely looking at mixed populations and

it may be more appropriate to consider them under the generic term "extracellular vesicles" (EV).

Osteoblastic EVs

Osteoblast-derived exosomes were characterized by Ge et al., who examined the proteomic profile of exosomes from culture supernatants of mouse MC3T3 osteoblast-like cells [20]. They identified a proteome of 1069 proteins, consisting predominantly of plasma membrane and cytoplasmic proteins. Pathway analysis showed that the exosome cargo included proteins involved in exosome biogenesis, formation, and uptake and several proteins involved in osteogenesis. Proteins associated with the eukaryotic initiation factor-2 pathway were enriched in MC3T3-exosomes. This could potentially play a role in osteogenesis, since EIF2α plays a role in BMP2-induced osteoblast differentiation [21], although functional assays were not carried out in this study.

Cui and colleagues performed functional studies with exosomes from mineralizing pre-osteoblastic MC3T3-E1 cells, which they termed mineralizing osteoblasts (MOBs), and showed that they promoted differentiation of ST2 bone marrow stromal cells into osteoblast-like cells [22]. Microarray analysis showed that MOB-exosomes contained 457 miRNAs, of which 43 were highly expressed, including several "osteo-miRNAs" known to be expressed in osteoblasts (miR-1192, miR-680, and miR-302a). Treatment of ST2 cells with MOB-exosomes upregulated 91 and downregulated 182 miRNAs. Several of the upregulated miRNAs were also detected in MOB-exosomes, including miR-3084-3p, miR-680, miR-677-3p, and miR-5100, which were highly expressed. These miRNAs target gene pathway networks converging on the β-catenin gene (Ctnnb1), a key transcription factor for osteoblast differentiation. Other miRNAs upregulated in ST2 cells (miR-667-3p, miR-6769b-5p, miR-7044-5p, miR-7668-3p, and miR-874-3p) are predicted to inhibit Axin 1, an inhibitor of Wnt/β-catenin signaling. Their work confirmed that Axin 1 was repressed and β-catenin was enhanced in ST2 cells treated with MOB-exosomes, thereby promoting osteogenesis, and raised the possibility that osteoblast-EV could play a role in recruitment of marrow stromal cells into the osteogenic pathway. The findings also highlight the possibility of using exosomes as a pro-osteogenic therapy in bone diseases and bone tissue engineering.

In addition to their osteogenic properties, osteoblast-EVs can contain RANKL protein, suggesting they may regulate osteoclast formation. Deng and colleagues showed that RANKL was present in EV from the UAMS-32P stromal/osteoblastic cell line [23]. EV from these cells promoted formation of TRAP-positive multinucleated cells in RAW264.7 osteoclast precursor cell cultures, presumably through RANKL on the surface of the EVs interacting with its receptor, RANK, on osteoclast precursors. In support of this, these

authors showed that the EV bound to the surface of cells that expressed RANK. However, they were internalized in cells that did not express RANK or when RANK-RANKL interactions were inhibited. These studies suggest that release of RANKL-containing EV from osteoblasts may represent a novel mechanism for osteoblast-osteoclast cell-cell communication and regulation of bone resorption.

In addition to their role in bone, osteoblasts play important roles in other biologic processes, such as providing stem cell niches for hematopoiesis and modulating bone metastasis of various types of cancer. A recent study using the human osteoblast cell line, SV-HFO, showed that EV from both non-mineralizing human osteoblasts (NMOBs) and MOB contained an extensive proteome and were taken up by the human prostate cancer cell line, PC3 [24]. Bioinformatic analysis of the proteomes of the osteoblast-EV and the RNA expression profiles of PC3 cells treated with osteoblast-EV showed that they converged on pathways related to cell growth and survival. Functional studies confirmed that osteoblast-EV stimulated PC3 prostate cancer cell growth. This highlights the importance of EV-mediated signaling in crosstalk between normal and cancer cells in the metastatic site, and particularly in the bone microenvironment.

Osteoclastic EVs

Osteoclast-enriched cultures generated from M-CSF-dependent mouse marrow precursors produce exosome-like EVs [25]. Interestingly, EVs from osteoclast precursors enhanced 1,25-dihydroxyvitamin D_3-dependent formation of osteoclasts in mouse marrow cultures. In contrast, EVs from differentiated osteoclasts inhibited osteoclast formation in the same assay. These data suggest that osteoclast-EVs can locally regulate osteoclastogenesis, either positively or negatively, depending on the differentiation state of the producer cell. Mechanistically, this study showed that EVs from mature osteoclasts contained the RANKL receptor, RANK, which may compete with RANK on osteoclast surfaces for binding to RANKL. Thus, the EV may function like a decoy RANKL receptor, similar to the naturally occurring RANKL inhibitor, osteoprotegerin [25].

Li and co-workers showed that osteoclast-derived EV can transfer miR-214-3p to osteoblasts and inhibit osteogenesis in vitro and in vivo [26••]. Their work showed that increased expression of miR-214-3p in human bone biopsies correlated with increased serum exosomal miR-214-3p and that serum miR-21-3p levels were higher in elderly patients with fractures compared to age-matched controls without fracture. They also found that miR-214-3p serum levels increased with aging in females, regardless of fracture status. Osteoclasts rather than osteoblasts provided the major source of serum exosomal miR-214-3p. Increased osteoclastic and serum miR-214-3p were also associated with decreased bone formation rates in an aged mouse model of ovariectomy-induced bone loss. Targeting of miR-214-3p overexpression to osteoclasts inhibited bone formation in mice, and inhibition of miR-214-3p using an osteoclast-targeted antagomir promoted bone formation.

In a related study, Sun and colleagues showed that osteoclasts release exosomes enriched in miR-214 [27]. These were taken up into osteoblasts via interaction of ephrin A2 on the exosomes with its receptor EPHA2 on osteoblasts. They also showed that bone formation was reduced in transgenic mice overexpressing miR-214 in osteoclasts and confirmed that osteoclast-derived exosomes are present in the circulation and can regulate osteoblast function. The serum exosome miR-214 levels were increased in osteoporotic patients and ovariectomized mice. They further showed that osteoblasts incubated with exosomes from osteoporotic compared to non-osteoporotic patients had higher levels of miR-214, leading to reduced expression of osteogenesis-related genes. Inhibition of exosome release in ovariectomized mice using in vivo delivery of *Rab27a* siRNA improved the bone mass. Together, these studies provide compelling in vivo evidence for an important role of osteoclast-EV in regulating bone formation. A proposed mechanism by which miR-214-3p regulates osteogenesis is through targeting of ATF4, an osteogenic transcription factor, to inhibit bone formation [28]. Additionally, miR-214-3p enhances osteoclast formation through activation of the PI3K/AKT pathway, mediated via inhibition of phosphatase and tensin homologue (PTEN) [29].

Osteocytic EVs

Signaling molecules produced by osteocytes are widely believed to serve as soluble factors that contribute to bone remodeling, as well as signaling to distant organs (reviewed in [30,31]). However, whether osteocytes communicate via EV release is less clear. Osteocyte-derived vesicles have been observed in the bone extracellular matrix in transgenic mice expressing a membrane-bound GFP in osteocytes [32]. Furthermore, release of osteocyte-derived microvesicles was observed by Veno et al. using live-cell imaging in osteogenic calvarial cell cultures from the same mouse model [33].

Recently, Qin et al. characterized the morphology and biological features of exosomes from Ocy454 osteocyte-like cells [13]. These vesicles have a diameter of 50–100 nm, a cup-shaped appearance, and express CD63 as shown by immunogold-labeling (Fig. 2(A)), consistent with previous descriptions of exosomes [11, 13]. The microRNA, miR-218, stimulates Wnt/β-catenin signaling and promotes osteogenic differentiation of bone marrow stromal cells by inactivation of Wnt inhibitors, SOST, DKK2, and SFRP2 [34]. Interestingly, Qin et al. showed that in response to the muscle-released myokine, myostatin, osteocytes produced greater amounts of SOST, RANKL, and DKK1, which was
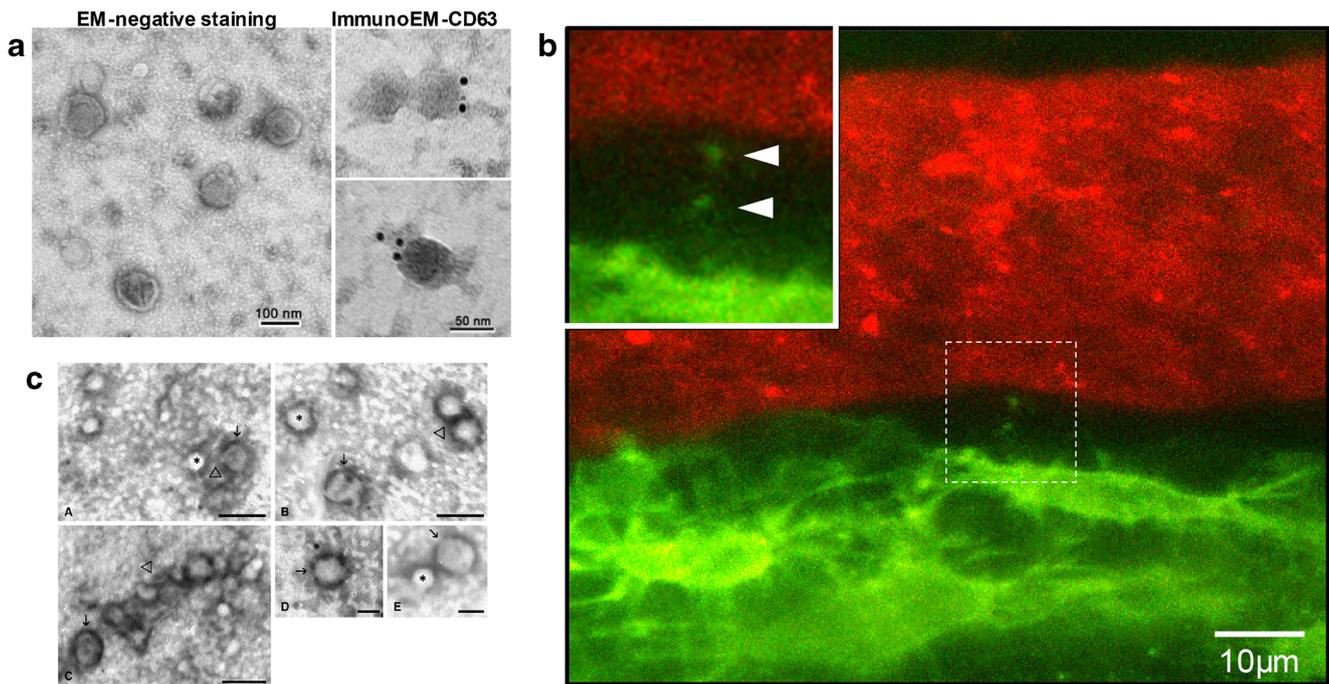
Fig. 2 (**A**) Morphology and structure of exosomes obtained from Ocy454 osteocyte-like cells by electron microscopy analysis. The exosomes were stained with uranyl acetate (EM-negative staining) or labeled with 10-nm immunogold using antibody against the exosomal membrane marker CD63 and stained with uranyl acetate (ImmunoEM-CD63). Exosomes were visualized using a Hitachi H7000 electron microscope (reproduced from Qin et al. [13], with permission from the American Society for Biochemistry and Molecular Biology). (**B**) Still frame from intravital confocal imaging in calvarial bone of a Dmp1-mGFP transgenic mouse. Osteocytes are GFP-positive (green), and the vascular lumen is labeled with Texas Red-conjugated high MW dextran. Note an osteocyte that is releasing small vesicle-like particles adjacent to the lumen of the vascular channel (also see enlarged inset, arrowheads). (**C**) TEM images of microvesicles released from C2C12 cells. The microvesicles were isolated from culture supernatants by 110,000×g centrifugation, then fixed, negatively stained, and observed by TEM. The images show small vesicles of 50–80 nm in diameter. Some of these microvesicles apparently contain a diffuse electron-dense material ($\rightarrow$) while others appear completely electron transparent (*). A tendency to aggregate (Δ) occasionally appears. Within panel C, bar = 100nm (images A, B and C) and bar = 50nm (images D and E) (reproduced from Guescini et al. [45] with permission from Elsevier)

associated with reduced miR-218 in the parent Ocy454 cells and their exosomes [13]. They further showed that the myostatin-modified osteocyte-EV were internalized by MC3T3 cells, inhibiting osteoblastic differentiation via inactivation of Wnt signaling [13]. These studies show that osteocytes and their exosome cargo can be influenced by muscle-related factors, suggesting that exosomes can provide a mechanism for crosstalk between muscle and bone. This topic is further discussed in later sections of this review.

It is currently unclear whether osteocyte-EV can signal to distant organs, but recent work from Sato et al. provides suggestive evidence that osteocyte-EV are present in the circulation [35]. They showed that ablation of osteocytes in mice modifies miRNA levels in circulating exosomes and that many of the miRNAs downregulated are ones that are expressed at high levels in MLO-Y4 osteocyte-like cells. Exosomes are too large to travel down the canalicular fluid space, and therefore, it seems logical that osteocyte-EV in the circulation must arise from osteocytes located adjacent to a blood vessel and/or bordering on the marrow space. Support for this comes from the work of Wang et al., using mice expressing a membrane-targeted GFP in osteocytes [36]. They showed using intravital imaging that osteocytes adjacent to blood vessels shed vesicle-like particles from their dendrite tips (see Fig. 2(B)). They further showed that GFP-positive EV were present in the blood and in the bone marrow.

It is currently unclear whether there are specific cell surface markers that can be used to identify the cell-specific origin of bone cell-derived EV (e.g., osteocyte, osteoblast, osteoclast) and/or to enrich for EV of specific bone cell types. Potential markers expressed on the osteocyte cell surface include E11/gp38 and PHEX. However, expression of these marker genes is not uniquely restricted to osteocytes and may be limited to early rather than mature osteocytes. Dentin matrix protein-1 and Sclerostin are expressed in early and mature osteocytes, respectively, but it is not clear whether they are cell surface markers. Fluorescence-activated cell sorting of osteoblasts has been performed using antibodies to alkaline phosphatase, which could potentially be applied to circulating EV [37]. Surface markers that could potentially be used to identify EV of osteoclast origin include DC-STAMP, OSCAR, and calcitonin receptor. Future studies are therefore needed to define which markers or sets of markers are optimal for distinguishing subpopulations of circulating EV derived from different bone cells.

EVs from Bone Marrow Stromal Cells

Human bone marrow stromal cells (BMSCs) are an attractive cell source for stem cell therapy treatments, including potential applications in bone regeneration. Recent studies have shown that exosomes from BMSC may play a role in osteoblastic differentiation, bone healing, and regeneration. One such study demonstrated that EVs from human BMSC upregulated expression of osteogenic marker genes and promoted osteoblastic differentiation in a human osteoblast-like cell line, mediated by exosomal miR-196a [38]. These BMSC-derived EVs were used to treat critical-sized calvarial defects in rats and promoted bone formation and bone healing. Similarly, Martins et al. showed that hBMSCs grown under osteogenic conditions shed EVs that have osteoinductive potential by promoting expression of *BMP2, SP7, SPP1, BGLAP/ IBSP*, and alkaline phosphatase in uncommitted BMSC cells [39]. They proposed that these BMSC-derived EV may have potential application in bone regenerative therapies. In further support of this, exosomes from MSC from human induced pluripotent stem cells were combined with scaffolds of tricalcium phosphate and found to promote bone regeneration [40].

Interestingly, Furuta et al. showed impairment of bone healing in CD9 null mice, which make reduced amounts of exosomes [41]. Injection with MSC-derived exosomes accelerated fracture healing in this model. In another translational study, it was reported that MSC transplantation rescues impaired bone marrow MSC function and improves osteopenia in a mouse model of lupus [42]. The likely mechanism was through the MSCs releasing exosomes, which transferred Fas to the host bone marrow MSCs, thereby reducing miR-29b levels. This in turn promotes epigenetic regulation of Notch signaling. It is clear from the above studies that BMSC-derived EVs are emerging as novel cell-free-based therapies with potential applications in regenerative medicine, including in bone regeneration.

EV Function in Muscle

Pederson et al. first proposed that muscle may function in an endocrine manner through "myokine" release [43, 44]. Several muscle-secreted peptide hormones and cytokines have been identified that may mediate muscle communication with distant organs, including the liver, pancreas, adipose tissue, brain, and bone, or may regulate myogenesis [43–47]. More recently, several groups have shown that skeletal and cardiac muscle cells secrete EV [48–54]. Accumulating evidence suggests that exosomes released by myogenic cells can transfer their cargo of proteins, mRNA, and miRNA to recipient cells and regulate muscle cell function in an autocrine or paracrine manner. They can also be shed into the circulation [55, 56••] and may have effects on distant tissues. Guescini

et al. showed that muscle-derived EVs (defined as EVs positive for alpha sarcoglycan) represent about 1–5% of the total circulating EVs [55].

Characteristics of Muscle Exosomes

The production of EVs by muscle cells was first reported in 2010 by Guescini et al. [48], who showed that C2C12 myoblasts produced EVs of ~ 50–80 nm in diameter (Fig. 2(C)), consistent with the size range of exosomes [57]. These EV contained known exosome markers, TSG101 and ALIX, demonstrated by immunogold labeling, and also carried mitochondrial DNA in their cargo. Their proteome included many exosome-associated proteins and proteins involved in signal transduction, such as small GTP-binding proteins and 14-3-3 proteins [48]. In a related study, proteomic comparison of EV from C2C12 myoblasts and myotubes showed distinct subsets of proteins that were reflective of the differentiation state of the parent cells [53]. Interestingly, the C2C12-EVs expressed molecules including ITGB1, CD9, CD81, NCAM, CD44, and Myoferlin on their surfaces, which play a role in myoblast fusion by facilitating recognition and subsequent adhesion of competent myoblasts [53].

miRNAs are present in muscle-EV, and over 170 miRNAs have been found in exosomes from C2C12 myoblasts and myotubes [58], including miR-181a, miR-146a, miR-145, miR-378, miR-1, miR-133a, miR-133b, and miR-206, which regulate muscle differentiation [58,59–61]. In particular, miR-133a, miR-133b, miR-1, and miR-206 showed the greatest increase in expression in the cytoplasm and in exosomes during C2C12 differentiation [58] and during development of human skeletal muscle [62]. Fry and colleagues showed that skeletal muscle myogenic progenitor cells also produce exosomes containing miR-206 [63]. These exosomes may regulate extracellular matrix production by fibrogenic cells, preventing excessive extracellular matrix deposition and ensuring optimal early myofiber growth.

Muscle Exosomes in Cell-Cell Communication

As mentioned previously, exosomes are released by many cell types and provide a protected environment for transportation of protein, miRNA, and mRNA to distant target cells [64]. Evidence is accumulating that muscle-EV play a role in crosstalk between myoblasts and myotubes as well as with other tissues. Incubation of C2C12 myoblasts with exosomes from myotubes suppressed myoblast proliferation and promoted differentiation [53]. The cell-to-cell transfer of myotube-derived exosomal cargo was confirmed by labeling the exosomes with GFP and showing transfer of GFP fluorescence into myoblasts. These data support a "feed forward" role for exosomes from differentiated myofibrils by promoting differentiation of more myofibrils. In a follow-up study, the

same authors showed that induction of myotube differentiation may occur via exosomal transfer of miRNAs that silenced expression of SIRT1, a regulator of myogenesis-related gene expression [58].

Muscle-EV can be taken up into other organs, where they may have functional effects. For example, fluorescently labeled muscle-derived exosomes injected into the tail vein of C57Bl/6 mice were taken up into most tissues within 24 h, with predominant labeling in the lung, liver, and spleen and uptake also seen in the brain, heart, pancreas, and GI tract (bone was not analyzed) [65]. These authors further showed that mice on a high-fat diet were obese and insulin-resistant and secreted more exosomes compared to mice on standard chow. Muscle-derived exosomes from mice on the high-fat diet induced myoblast proliferation and altered expression of cell cycle-related genes and genes involved in muscle differentiation. These findings suggest that muscle-EV have a paracrine signaling function by altering muscle homeostasis in response to high-fat diet and may have an endocrine function by targeting other tissues in vivo [65]. In a related study, the same group showed that EVs from muscle of mice on a high-fat diet increased the size of isolated islets in vitro and induced proliferation of murine insulin secreting MIN6B1 cells [66]. These effects appeared to be mediated in part through transfer of miR-16, which regulates expression of Ptch1, a regulator of pancreas development. Not only do muscle cells release EV, but they respond to EV from other cells. For example, EV from MSC enhanced muscle regeneration [67] and endothelial-derived EV protected cardiomyocytes from hypertrophy [68].

Exercise-Induced Release of Muscle Exosomes

Exercise has beneficial effects on multiple organ systems, has protective effects on age-related diseases, and attenuates aging effects on mitochondrial function. Release of nucleic acids and peptides from skeletal muscle and other tissues (termed "exerkines") has been proposed to mediate these effects [69]. However, since many growth factors, miRNAs, and nucleic acids would be unstable in the circulation, it has recently been suggested that exosomes and microvesicles are important carriers of "exerkines" that may mediate the systemic effects of exercise [69]. Circulatory EV increase with endurance exercise [70], and the term "exersomes" has been coined to denote these exercise-induced EV [69]. Guescini et al. showed that acute aerobic exercise in young men is associated with increased numbers of circulating exosomes positive for the muscle marker alpha sarcoglycan [55]. Their study also showed upregulation of miR-181a-5p and miR133b in these muscle-derived circulating exosomes following exercise. In a related study, both acute and chronic exercise reduced circulating levels of the muscle-enriched miR-486 in young men [71]. Overall, accumulating evidence supports the idea that EVs

play an important role in muscle crosstalk with other organs and that they may also function to mediate the beneficial effects of exercise on muscle and other tissues.

Muscle Exosomes in Pathological Conditions

Several studies suggest that pathological states, including injury, atrophy, and aging, can alter the cargo of muscle-derived exosomes. One such study showed that muscle denervation by transection of the sciatic nerve resulted in an increase in miR-206 and a decrease in miRs 1, 133a, and 133b in exosomes from cultured myofibers [72]. miR-206 may play a role in the activation of satellite cells that occurs after nerve injury and potentially in regulating muscle mass and new synapse formation during re-innervation. Madison et al. [54] proposed that muscle-EV may play a role in regeneration of motor neurons after nerve injury. Their work showed that exosomes from C2C12 myoblasts promoted cell survival and neurite outgrowth in NSC-34 motor neuron cells. Hudson et al. reported that miR-1 and miR-23a, two miRNAs involved in muscle atrophy, were enriched in exosomes from cultured myotubes in which atrophy was induced by dexamethasone treatment [73]. Their work also showed downregulation of miR-23a in rats under conditions that induced muscle atrophy, suggesting a potential pathogenic role.

Another example of alteration of muscle exosomes under pathological conditions was shown in mice fed on a high-fat diet, as mentioned above [65]. These mice are obese and become insulin-resistant. Muscle-derived exosomes from these mice induced myoblast proliferation and downregulated Myog and Myod1, two markers of terminal muscle cell differentiation [65], suggesting that exosomes can transfer the effects of high-fat diet to other myoblasts. EV may also function in muscle regeneration in response to injury and mechanical stress. This topic has been reviewed by Murphy et al. [7], and the reader is referred to their excellent review for a more in-depth discussion.

Overall, evidence is accumulating that the cargo of muscle-derived exosomes can be changed under pathological conditions and that exosomes contribute to pathogenic processes and/or the propagation of pathogenic responses to neighboring cells as well as distant cells. These findings also suggest that circulating levels of muscle-related miRs could be used as biomarkers of muscle health or disease states and potentially to indicate response to therapies.

Role of Exosomes in Muscle-Bone Crosstalk and Their Potential Role in Musculoskeletal Aging

Mechanical loading through muscle contraction was previously thought to be the main mechanism by which muscle

influences bone mass. Pederson et al. first proposed that muscle functions in an endocrine manner through "myokine" release [43, 44], and evidence is emerging for signaling crosstalk between bone and muscle via circulating and local mediators [74–82] (reviewed in [83–86]). Examples of factors proposed to functionally couple muscle and bone include growth hormone/IGF-1 [87], FGF-2 [78], osteocalcin [81], irisin [88], PGE_2 [74], Wnt3a [82], and β-aminoisobutyric acid (BAIBA) [89]. Myostatin inhibitors also have beneficial effects on bone [90]. An obvious question is whether extracellular vesicles are also mediators of muscle-bone crosstalk.

Exosomes from C2C12 myoblasts were shown to promote differentiation of pre-osteoblastic MC3T3-E1 cells to mature osteoblasts [91]. This was attributed to the function of the exosomes to increase miR-27a-3p levels in the recipient cells, which decreases expression of its target, adenomatous polyposis coli (APC). This in turn activates β-catenin signaling, promoting osteogenesis. Our own recent studies have shown that exosomes from C2C12 myotubes, but not myoblasts, enhance luciferase activity in TOPflash-MLOY4 osteocyte-like cells, which express a luciferase reporter as a readout for Wnt/β-catenin signaling [36]. Previous studies have shown that Wnt/β-catenin signaling enhances osteocyte survival and protects them from apoptotic stimuli and oxidative stress [76, 89, 92].

The microRNA, miR-218, stimulates Wnt/β-catenin signaling and promotes osteogenic differentiation of bone marrow stromal cells by inactivation of Wnt inhibitors, SOST, DKK2, and SFRP2 [34]. Qin et al. showed that in response to the muscle released, myokine, myostatin, Ocy454 osteocyte-like cells produced greater amounts of SOST, RANKL, and DKK1, which was associated with decreased miR-218 in the parent Ocy454 cells and their exosomes [13]. The myostatin-modified osteocyte-EV were internalized by MC3T3 cells, inhibiting osteoblastic differentiation via inactivation of Wnt signaling [13]. These studies show that osteocytes and their exosome cargo can be influenced by muscle-related factors, providing more evidence of EV-mediated crosstalk between muscle and bone.

Osteoporosis, a disease of reduced bone density and quality, is a major clinical problem in the aging population and is associated with a high fracture risk [93, 94]. Sarcopenia, a disease of age-related loss of muscle mass and strength, often accompanies osteoporosis and the muscle weakness contributes to falls that lead to fractures. Since these diseases go hand in hand, common molecular pathogenic mechanism(s) seem likely. This has led to the concept that molecular crosstalk between these two tissues may coordinate age-related degenerative changes. Aging is associated with changes in the cargo of circulating EV, which are likely secondary to cellular changes in the producer cells in response to age-associated stimuli, such as inflammation and oxidative stress [95–98]. Recent evidence suggests that EV may play a role in musculoskeletal aging.

Davis et al. compared miRNA profiles of EVs from the bone marrow interstitial fluid of aged and young mice [99••]. They showed increased expression of the miR-183 cluster (miR-96/-182/-183) in EV from aged mice, which could be mimicked in vitro by experimentally inducing oxidative stress, a hallmark of aging. Treatment of BMSCs from young mice with bone marrow-EV from aged mice inhibited their osteogenic differentiation, and these effects were mimicked by transfection of BMSCs with miR-183-5p, which also induced BMSC senescence. Their data show that the cargo of bone marrow-EVs can be altered by aging and by oxidative stress, and suggest that these EV may play a role in propagating aging effects on stem cell senescence and on impaired osteogenic differentiation.

In a follow-up study, the same group showed that increased oxidative stress in skeletal muscle with aging was associated with increased expression of the senescence-associated microRNA, miR-34a, in skeletal muscle and in muscle-derived circulating EVs [56]. miR-34a was also induced in exosomes from C2C12 myoblasts subjected to oxidative stress. This miRNA was previously shown to be increased with aging in skeletal muscle [100] and in the circulation [101] and has been associated with muscle atrophy [100, 102]. In addition to negative effects on muscle, BMSC treated with these exosomes showed increased senescence. Their data suggest that aged skeletal muscle produces circulating EVs that can induce senescence in stem cell populations in bone and other tissues via their miRNA cargo.

Summary and Conclusions

This review has highlighted exosome-mediated cell-cell communication as an exciting and paradigm-shifting field, with emerging new discoveries providing ever more insight into this previously underappreciated and important mechanism for cell-cell signaling. There is a clear need for future studies to further delineate the role of exosomes in muscle-to-bone crosstalk in aging as well as studies aimed at understanding the mechanisms governing their release and uptake by muscle and bone cells. One key limitation in the field is in showing whether EV-mediated miRNA transfer can alter cell function in a truly physiological setting, which still remains controversial. In a stoichiometric analysis of circulating exosomes, Chevillet et al. estimated that even miRNAs that are fairly abundant are actually present at less than one copy per exosome [103]. It is therefore valid to ask whether miRNAs present in such low amounts per exosome can have effects under completely physiological settings as opposed to controlled experimental studies where large amounts of purified exosomes are added to cells or injected into animals. An argument in favor of a physiological role is that, under in vivo conditions, the producer cells can continually shed EV.

Therefore, over time, target cells could accumulate "hits" from multiple EV, even if each vesicle only delivers a relatively small amount of miRNA to the target cell. Challenges still remain in quantifying and separating different EV subpopulations, as most studies are examining "whole population" effects of EV preparations containing mixtures of exosomes and microvesicles. Future studies using intravital imaging and mouse genetic approaches targeting mechanisms of release and uptake of EV should help to confirm the importance of EV signaling under in vivo physiological and pathological conditions. Such studies will help not only to enhance understanding of EV function, but also to exploit the exciting possibilities for their use in therapeutic applications as well as in diagnosis and monitoring of musculoskeletal disease and aging.

Compliance with Ethical Standards

Conflict of Interest Sarah Dallas and Weiping Qin declare no conflict of interest.

Human and Animal Rights and Informed Consent All reported studies/experiments with animal subjects performed by the authors have complied with all applicable ethical standards (•including the Helsinki Declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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