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Regenerative medicine 2.0: extracellular vesicle–based therapeutics for musculoskeletal tissue regeneration

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In recent years, extracellular vesicles (EVs) have emerged as prominent mediators of the homeostasis, repair, and regeneration of musculoskeletal tissues including bone, skeletal muscle, and cartilage. Accordingly, the therapeutic potential of EVs for regenerative medicine applications has not gone unnoticed. The use of EVs for the treatment of musculoskeletal injury and disease in veterinary species is a nascent but rapidly expanding area of research. Recent studies in this area have demonstrated the safety and feasibility of EV products in dogs and horses. While early clinical responses to EV-based therapeutics in companion animals have been favorable, more rigorously designed, sufficiently powered, and placebo-controlled clinical trials are required to fully elucidate the clinical benefits and best-use scenarios for EV therapeutics in veterinary medicine. Additionally, clinical translation of EV-based therapeutics will require Good Manufacturing Practice–compliant methods to scale up and purify EV products. Despite these challenges, EVs hold great promise in the regenerative medicine landscape, particularly in the treatment of musculoskeletal injury and disease in companion animals.

The Confusing Landscape of Mesenchymal Stromal Cell Therapies

Mesenchymal stromal cells (MSCs) have been demonstrated in numerous studies to aid in bone repair,[1] skeletal muscle regeneration,[2–4] and cartilage regeneration.[5] While MSCs and stem cell (SC) therapies have generated widespread enthusiasm as a means to repair, regenerate, or minimize loss of musculoskeletal tissue, their clinical efficacy for specific disease indications such as osteoarthritis (OA) and other musculoskeletal conditions in veterinary and human patients is still somewhat ambiguous. Several issues have contributed to conflicting opinions about the clinical efficacy of SCs. For instance, there is in fact no standard clinical definition for what constitutes SC therapy. The term "stem cell therapy" is used to describe a multitude of cell-based therapeutics referring to concentrates enriched for SCs but containing other cell populations (eg, bone marrow aspirate concentrate and adipose stromal vascular fraction), purified stromal cells that are culture expanded and isolated as pure multipotent cell populations, or even platelet-rich plasma concentrates. Cell-based therapeutics may be sourced from the patient's own tissue (autologous) or from a donor (allogenic) and can be isolated from adult or juvenile individuals. Finally, the specific mechanisms of action and relative efficacies of SC treatments are highly dependent on the donor species, tissue source, method of isolation and preparation, and delivery method.[6] Thus, there is considerable variation in what may be delivered to the patient and, even within a defined category of SC treatment, inconsistent reproducibility from one treatment to another. Further, SC therapies are clinically used for a variety of medical conditions with varying severity of disease. Taken together, these variables have made it extremely difficult to define the clinical efficacy of SC treatments and develop best practices for their use.[7]

Extracellular Vesicles and Exosomes as Alternatives to MSCs

The capacity for MSCs to differentiate into multiple cellular lineages was originally believed to be the primary mechanism for MSC-mediated tissue healing and regeneration.[8] It is now widely accepted that the MSC secretome (ie, biological factors that are secreted by MSCs such as cytokines, growth factors, and extracellular vesicles [EVs]) is largely responsible for the proregenerative properties of MSCs.[9,10] EVs are a key component of the MSC secretome and are considered to be prime mediators of MSC function. EVs are nanoscale, membrane-bound vesicles released by MSCs (and many other cell types), which contain a variety of bioactive cargo that function in cell signaling.[11] EVs are broadly characterized as either (1) ectosomes, which originate from the cell membrane, or (2) exosomes, which originate from within the cell and contain important cell signaling molecules such as mRNAs, microRNAs (miRNAs), cytokines, and

other proteins. Exosomes, which form intracellularly within multivesicular bodies, are typically 50 to 200 nM in size and are released upon multivesicular body fusion with the plasma membrane.[11,12]

Among the types of EVs studied, exosomes have garnered the most interest for clinical use in regenerative medicine as they are known to play a foundational role in MSC-associated proregenerative activities. Indeed, exosomes derived from MSCs recapitulate many of the biological activities of MSCs themselves and can even "home" to sites of tissue injury, thereby affecting tissue healing and regeneration.[10,13–15] In addition, exosome biogenesis is highly regulated, with directed and selective packaging of content for release into the extracellular space in response to specific signals or conditions.[16] This last feature is of particular importance and represents a clear potential advantage over MSC-based therapies: exosomes can be more readily manipulated to display specific surface proteins for enhanced tissue targeting, and their cargo can be enriched for bioactive components such as proregenerative and immunomodulating mRNAs, miRNAs, and cytokines. The ability to modulate exosome cargo and tissue-homing properties to achieve a predictable and specific biological effect can be harnessed for the development of precise and customizable therapeutics.[17] Furthermore, unlike MSCs, exosomes cannot self-replicate, thereby alleviating some of the safety concerns with live cell therapies. Early data further suggest that exosomes retain their biological activity at room temperature following lyophilization, which holds promise for the long-term storage and shipment of exosome therapeutics at room temperature.[18] Exosomes therefore hold great promise as an alternative to cell-based therapies in regenerative medicine.

EV-Based Therapy in Musculoskeletal Tissue Repair and Regeneration

Because of their unique properties, EV-based therapies have potential utility in a wide variety of musculoskeletal tissue regeneration settings. While not yet available for clinical use, research exploring the mechanisms and proregenerative effects of EVs for musculoskeletal tissue repair, particularly those derived from MSCs and progenitor cells, is a rapidly expanding area of clinical research. While the majority of studies reviewed here use the term "exosome" to describe small EVs, it is important to note that there is likely a mixture of EV subtypes that fall within a similar size range, and therefore many investigators accept that EV isolates frequently contain a heterogenous mixture of both exosomes and small EVs not of endosomal origin. What follows is a summary of recent published research surrounding the use of EV therapeutics in musculoskeletal tissue regeneration.

Bone regeneration

EVs from osteogenic precursors have been shown to promote osteoblastic differentiation of naïve adipose-derived SCs in vitro.[19,20] Likewise, EVs secreted by mechanically stimulated osteocytes will promote in vitro recruitment and osseous differentiation of MSCs.[20] MSC-derived EVs have also been shown to enhance bone regeneration in rodent models of fracture healing,[21–24] osteoporosis,[25] and radiation-induced bone loss.[26] Intravenous administration of bone marrow–derived MSC (BM-MSC) EVs to rats that received radiation to the distal femur resulted in reduced bone loss compared to irradiated femurs in rats that did not receive EV treatment.[26] At a cellular level, in vitro incubation of irradiated BM-MSCs with nonirradiated BM-MSC–derived EVs led to the functional recovery of the irradiated BM-MSCS by alleviating senescence-associated protein expression and restoring proliferative capacity.[26] Furthermore, MSC-derived EVs can accelerate fracture healing as demonstrated in a mouse model of delayed fracture healing in which delayed union was attenuated by delivery of MSC-derived EVs into the fracture site.[21] Zhang et al[22] reported similar findings in a rat femur fracture model, with BM-MSC EVs accelerating repair and angiogenesis in femur fractures. In another study,[25] EVs secreted by induced pluripotent SCs (iPSCs) enhanced proliferation and osteogenic gene signaling in BM-MSCs from ovariectomized, osteopenic rats. Following implantation of scaffolds containing iPSC-derived EVs into calvarial defects in osteopenic rats, bone regeneration and angiogenesis scores improved compared to rats that received only the scaffold without EVs.[25] Liao et al[27] investigated the use of MSC-EVs to treat osteonecrosis of the femoral head in rabbit models. Treatment of rabbits with EVs engineered to overexpress miR-122-5p, a miRNA that promotes the differentiation of osteoblasts, resulted in increased bone mineral density, trabecular bone volume, and mean trabecular plate thickness in the femoral head.[27] Collectively, these studies describe a role for EVs in osteocyte signaling and suggest possible clinical applications for bone repair and regeneration.

Muscle regeneration

EVs released by muscle progenitor cells have been found to contain signals that promote muscle regeneration following injury or stress.[28] Several in vitro studies have demonstrated that EVs mediate various steps in the process of myogenesis. For instance, myotube-derived EVs facilitate the differentiation of myoblasts into mature myotubes.[29] In another study,[28] treatment of human adipose-derived SCs with EVs derived from differentiating myoblasts led to the development of a myotube-like phenotype and promoted the expression of several myogenic genes, demonstrating that skeletal muscle–derived EVs can instruct adipose-derived SCs to adopt a myogenic lineage. Muscle precursor cells have also been found to secrete EVs that communicate with fibroblasts to regulate collagen synthesis during extracellular matrix remodeling, facilitating muscle repair in response to hypertrophic stimuli.[30] Nakamura et al[14] demonstrated that local injection of EVs secreted by MSCs into the injured tibialis anterior muscle of mice accelerates skeletal muscle regeneration, as demon-

strated by an increased diameter of myofibers, reduced fibrotic area, and increased capillary density. EVs derived from myogenic precursors were also shown to improve muscle regeneration following local injection into the injured muscle of mice, with the injured muscle showing reduced collagen deposition and a greater number of myofibers 1 week after EV injection.[28] In a more recent study,[15] systemic administration of EVs isolated from adipose-derived MSCs resulted in an increase in the cross-sectional area of regenerating myofibers and a reduction in the number of infiltrating macrophages in the injured tibialis anterior muscle of mice. In all of these studies, the investigated EVs were found to contain proregenerative cargo including proteins and miRNAs.[14,15,28] More recently, Leng et al[31] compared the benefits of systemic administration of MSC-derived EVs, serum-derived EVs, or myotube-derived EVs on muscle function in mice with the progressive muscle wasting disease, Duchenne muscular dystrophy (DMD). EVs from all 3 sources resulted in improved skeletal muscle function and myoarchitecture.

Cardiac progenitor cells (CPs) represent another source of EVs that have been found to enhance skeletal muscle regeneration. EVs produced by CPs have been shown to improve the repair and functionality of both cardiac and skeletal muscle.[32–34] In a mouse model of DMD, injection of CP-EVs into the soleus muscle of mice resulted in a greater number of myofibers, increased tissue levels of protein markers for myofiber differentiation, and decreased inflammation and fibrosis compared to vehicle-injected mice.[32] Importantly, CP-EVs also improved the functionality of skeletal muscle in these DMD mice by restoring soleus isometric contractile properties to levels seen in wild-type mice by 3 weeks following EV delivery. Another study[33] demonstrated that CP-EVs normalized gene expression in the skeletal muscle of DMD mice toward that of wild-type mice, and CP-EV delivery improved the isometric force produced by the soleus muscle.

Cartilage regeneration

A number of preclinical studies suggest that EV-based therapies can be used to enhance cartilage healing or prevent cartilage degradation. EVs secreted by chondrogenic progenitor cells (CPCs) have been shown to enhance cartilage repair in mice.[35] Weekly intra-articular (IA) injection of EVs derived from CPCs into a surgically induced mouse model of OA was found to reduce OA severity by decreasing collagen type I expression, increasing aggrecan and collagen type II expression, and reducing cartilage matrix loss.[35] CPC-derived EVs contained miRNAs involved in processes that are important for chondrogenesis and modulation of inflammation. In another study,[36] weekly IA injections of MSC-derived EVs led to the early and more complete regeneration of cartilage and subchondral bone in an osteochondral defect in rats. In a rodent chronic OA model, IA injection of EVs derived from embryonic MSCs prevented cartilage and matrix degradation.[37] Similarly, EVs derived from infrapatellar fat pad MSCs ameliorated OA severity in a surgically induced mouse model of OA.[38] These EVs reduced articular cartilage damage, improved mouse mobility, inhibited apoptosis, and enhanced matrix synthesis in chondrocytes.[38] In a rabbit osteochondral defect model, a combination of MSC-derived EVs and hyaluronic acid administered via IA injection resulted in improved cartilage regeneration and mechanical function compared to rabbits treated with hyaluronic acid alone.[39]

Extracellular Vesicles in Veterinary Medicine

While still an emerging area of research in veterinary medicine, several studies have recently characterized the cargo and proregenerative effects of EVs from companion species, most notably dogs[40–42] and horses.[43,44] These early studies have shown promising results, both in vitro and in vivo.

EVs were recently found to improve canine tendon cell survival and proliferation in vitro[40] and to modulate inflammatory responses in canine T cells.[41] Equine MSC-derived EVs are enriched for proregenerative miRNAs known to modulate immune and inflammatory processes[43] and are capable of stimulating the proliferation of equine chondrocytes while inhibiting chondrocyte cell death in vitro.[44] Other investigators have developed methods to optimize the content of MSC-derived EVs for equine disease–specific uses. Weiss et al recently demonstrated that pretreatment of adipose-derived MSCs with 5-azacytydine and resveratrol, which has previously been shown to reverse equine metabolic syndrome–related cellular dysfunction in equine adipose-derived MSCs,[45] resulted in production of EVs with enhanced cellular rejuvenation properties in vitro in MSCs isolated from horses with equine metabolic syndrome.[46]

Although the majority of in vivo EV studies to date have been performed in rodent and rabbit models, a small number of publications describing EV use in companion species with naturally occurring musculoskeletal disease have been published in recent years. These studies, while not well controlled or sufficiently powered, have reported no significant adverse events and suggest potential clinical benefits.[42,47–49] For example, the effect of MSC-EVs on cartilage repair was investigated in a canine chondral defect model.[47] Intra-articular injection of MSC-EVs resulted in improved cartilage regeneration, demonstrating potential benefits for cartilage repair in dogs. A lyophilized MSC-derived EV product was also recently evaluated in a small number of dogs with naturally occurring OA.[42] In this study, MSC-derived EVs were injected into the knee or elbow joints of 3 dogs with radiographic and clinical signs of OA. No systemic adverse reactions were reported, and no progression of lameness was observed at least 80 days after treatment.[42] Similarly, when conditioned media containing EVs from equine MSCs was injected locally into the injured tendons or ligaments of 13 horses, no adverse reactions were reported and conditioned media–treated horses demonstrated

enhanced healing-related neovascularization and a lower rate of reinjury as compared to placebo-injected horses.[49] While these results support safety, more rigorously designed, sufficiently powered, and placebo-controlled clinical trials are required to understand the potential benefit and best-use scenarios for EV therapeutics in veterinary patients.

Optimizing EV-Based Therapeutics for Clinical Use

Drug delivery

Because EVs are nanoparticles with lipid bilayers, they are quite stable in circulation and can cross various tissue barriers, such as the blood-brain barrier,[50] with relative ease as compared with MSCs. As a result, EV cargo that would normally be destroyed or rendered biologically inactive (such as mRNA and miRNA) in circulation is also protected from degradation. Further, the lipid bilayer of EVs boasts surface markers that permit specific tissue targeting and allow EVs to "home" to sites of injury and inflammation, which can be engineered to target specific tissues. These features make EVs an attractive vehicle for drug delivery. The earliest example of the use of EVs as a drug delivery vehicle involved the use of curcumin, a potent anti-inflammatory agent, complexed with EVs and then delivered to myeloid cells in vivo.[51] Other work has demonstrated successful EV-based chemotherapy delivery to tumors.[52–55]

EVs have been utilized as drug delivery vehicles to treat various musculoskeletal conditions in rodent models. In one such study, Gao et al[56] demonstrated that a phosphorodiamidate morpholino oligomer (PMO), which is used to treat DMD, can be conjugated to the surface of EVs. Systemic injection of PMO-conjugated EVs into a mouse model of muscular dystrophy resulted in increased dystrophin expression in skeletal muscles, significantly improving the therapeutic efficacy of PMO.[56] Furthermore, the conjugation of a skeletal muscle–targeting peptide to these EVs further enhanced the delivery of PMO-conjugated EVs to skeletal muscle. A more recent study[57] utilized EVs as a delivery vehicle for myostatin propeptide for the treatment of DMD. Myostatin propeptide has low serum stability when given IV as a free drug; however, by anchoring myostatin propeptide to the surface of EVs, the serum stability, delivery efficiency, and therapeutic efficacy was enhanced, ultimately resulting in increased muscle mass and function and improved bone regeneration in DMD mice.[57]

Osteoblast-derived EVs have been loaded with antiosteoclast drugs (dasatinib and zoledronate) as a potential treatment for osteoporosis.[58] After encapsulation in EVs, both drugs maintained their ability to target osteoclasts in a mouse model of osteoclast overactivation.[58] In another study, Luo et al[59] conjugated a BM-MSC–specific aptamer to the surface of BM-MSC–derived EVs to achieve targeted delivery to BM-MSCs following systemic injection. Intravenous injection of these aptamer-functionalized EVs resulted in greater accumulation within the limbs of mice 6 hours postinjection compared to injection of EVs without the aptamer.[59] Furthermore, after intravenously injecting aptamer-functionalized EVs into an ovariectomized mouse model of osteoporosis, mice demonstrated significantly higher trabecular volume, number, and thickness in the distal femur compared to mice that received either the vehicle or EVs without the aptamer.[59] Thus, conjugation of a bone marrow targeting aptamer to EVs can enhance their accumulation in bone to better promote osteogenesis and bone repair.[59]

One of the challenges of engineering EVs is doing so without altering their biodistribution and impairing their ability to transfer cargo to recipient cells. Gao et al[56] demonstrated that this limitation can be overcome by identifying a peptide, CP05, which specifically binds a conserved exosomal surface protein to allow for direct anchoring of peptides to exosomes regardless of their origin and without altering their biodistribution. This study thus demonstrated that CP05 can be used as an anchor peptide to enable direct modification, cargo loading, and capture of exosomes.[56]

Enriching EVs with beneficial miRNAs

In addition to loading EVs with drugs, it is also possible to enrich EVs with beneficial miRNAs to enhance reparative and regenerative processes in recipient cells. In one such study, synovial MSCs (sMSCs) were transfected with miR-140-5p,[60] a miRNA that is known to modulate cartilage homeostasis. Unaltered sMSC-EVs enhanced the proliferation and migration of articular chondrocytes in vitro but decreased chondrocyte extracellular matrix secretion. EVs enriched with miR-140-5p similarly enhanced proliferation and migration of articular chondrocytes but reversed the decreased extracellular matrix secretion that was observed with unaltered EVs. In an OA rat model, IA injection of these miR-140-enriched EVs minimized cartilage loss as compared to untreated rats.[60] Other investigators have utilized plasma-derived EVs enriched with miR-140 to induce differentiation of BM-MSCs into chondrocytes.[61] Together, these studies suggest that EVs enriched for miR-140 may have therapeutic applications in cartilage repair and OA treatment. EVs have also been enriched with other specific miRNAs to affect specific conditions. As one example, EVs were enriched with miR-26a, a skeletal muscle–associated miRNA, and conjugated to a skeletal muscle–targeting peptide.[62] Following local IM injection into the anterior tibial muscle of mice with renal disease–associated muscle wasting, the muscle cross-sectional area significantly increased and renal disease–induced muscle atrophy of the injected muscle was attenuated.

Considerations for Clinical Translation

Isolation and purification

As scientists continue to explore the myriad of ways in which EV-based therapeutics can be applied to clinical conditions, it is paramount that parent

cell culture conditions and EV isolation and purification methods are optimized and standardized. Good Manufacturing Practice–compliant isolation and purification processes that can produce consistent concentrations of EVs with the appropriate surface markers and cargo are still in development. Current methods to isolate and purify EVs include ultracentrifugation, size-based isolation, immunoaffinity capture, EV precipitation, and microfluidics-based isolation.[63] Ultracentrifugation is currently considered the gold standard technique for isolating EVs.[63] However, EVs are lost during sample processing with ultracentrifugation, resulting in a relatively lower EV yield. Another challenge with many current EV isolation techniques is that other proteins can contaminate the EV pellet and may not result in highly pure populations of EVs. It is likely that microfluidic sorting methods will be the means by which some of these challenges can be overcome. To maximize EV recovery while reducing contamination, it may be necessary to combine isolation techniques.

Scale up

To translate EV-based therapeutics for clinical use, scaled-up production methods will be required. Currently, the process of culturing cells and isolating EVs from conditioned media is labor intensive with relatively low yields, making production and purification of clinical-grade EVs inefficient. The limited proliferative capacity of cultured MSCs adds an additional complication to this issue, making it difficult to generate therapeutic concentrations of EVs from autologous MSCs. One way researchers are attempting to address this limitation is with the use of induced pluripotent SCs (iPSCs) rather than MSCs.[64] iPSCs, which are generated by reprogramming somatic cells in vitro, are capable of indefinite propagation and can be derived from adult tissues. Recent work comparing the characteristics of EVs derived from iPSCs to EVs derived from MSCs found that iPSCs produce more than 16 times as many EVs as MSCs under defined culture conditions while maintaining similar physiologic functions.[64] iPSCs can be differentiated into a desired cell type (muscle progenitor, osteoclast, chondrocyte, etc) and therefore may serve as a renewable source for parent cell lines to produce clinical-grade EVs. An additional benefit of iPSC-EV production is that, theoretically, iPSC cell lines can be generated from each individual patient, thus providing an autologous source of iPSCs and EVs for a given patient. However, research to show feasibility of this approach is still evolving.

Other strategies to enhance EV yield include genetic engineering of specific immortalized cell lines designed to produce high yields of cargo-optimized EVs. Ibrahim et al[65] recently demonstrated that engineering skin fibroblasts to overexpress β-catenin and Gata4 conferred immortality to these cells, thus overcoming the limitation of replicative senescence. EVs derived from these immortal cells were shown to be more therapeutically potent, reducing skeletal muscle fibrosis and improving exercise capacity in a mouse model of DMD. Optimization of extraction methods may also serve as a useful strategy to scale up EV production. One such approach was recently described,[66] in which EV isolation was enhanced by use of a modified polymer-based precipitation method that minimized the number of cells needed to extract a therapeutic dose of EVs by 30-fold.

Long-term storage

Evidence now exists that EVs can be cryopreserved at –80 °C for at least 7 months with minimal protein degradation[67] and for at least 2 months with minimal RNA degradation.[68] Longer storage times have yet to be evaluated. EV suspensions are also amenable to lyophilization (freeze-drying) and storage at room temperature while still maintaining their biological activity upon rehydration.[18,40] Rehydrated lyophilized EVs maintained their protein and RNA cargo and biological potency following IV injection into mice.[18] Lyophilized EV products have recently been developed and characterized for possible human,[69,70] canine,[40,42] and equine[71] applications in which EV morphology, surface biomarkers, and biological activity were preserved following rehydration. These studies support the feasibility of developing freeze-dried MSC-derived EV therapeutics that can be stored for long periods of time and shipped without the need for cold chain conditions.

Conclusions and Future Perspectives

Recent progress in EV research has provided evidence that EVs from a variety of cell sources can stimulate the proliferation, differentiation, and rejuvenation of cells from musculoskeletal lineages including osteocytes, myocytes, chondrocytes, tenocytes, and fibroblasts in vitro. Subsequent in vivo studies have demonstrated that EVs enhance the repair and regeneration of bone, skeletal muscle, and cartilage in rodent models. The therapeutic use of EVs for musculoskeletal regeneration in veterinary companion animal species is still an emerging area of research. The limited work in this area to date has provided evidence of safety; however, well-designed prospective studies evaluating larger numbers of animals will be required to fully evaluate the clinical efficacy of EV therapeutics. To translate EV therapeutics to the clinic, strategies to scale up EV production, standardize isolation and purification methods, and store EVs long term are a current area of research requiring more investigation. Finally, the ability to modify EVs for the development of customized and personalized therapeutics makes EVs a promising tool for future veterinary use, especially as precision medicine initiatives become more widespread in veterinary medicine.

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