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Harnessing extracellular vesicles to direct endochondral repair of large bone defects

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Large bone defects remain a tremendous clinical challenge. There is growing evidence in support of treatment strategies that direct defect repair through an endochondral route, involving a cartilage intermediate. While culture-expanded stem/progenitor cells are being evaluated for this purpose, these cells would compete with endogenous repair cells for limited oxygen and nutrients within ischaemic defects. Alternatively, it may be possible to employ extracellular vesicles (EVs) secreted by culture-expanded cells for overcoming key bottlenecks to endochondral repair, such as defect vascularization, chondrogenesis, and osseous remodelling. While mesenchymal stromal/stem cells are a promising source of therapeutic EVs, other donor cells should also be considered. The efficacy of an EV-based therapeutic will likely depend on the design of companion scaffolds for controlled delivery to specific target cells. Ultimately, the knowledge gained from studies of EVs could one day inform the long-term development of synthetic, engineered nanovesicles. In the meantime, EVs harnessed from *in vitro* cell culture have near-term promise for use in bone regenerative medicine. This narrative review presents a rationale for using EVs to improve the repair of large bone defects, highlights promising cell sources and likely therapeutic targets for directing repair through an endochondral pathway, and discusses current barriers to clinical translation.

Cite this article: *Bone Joint Res* 2018;7:263–273.

Keywords: Extracellular vesicles, Critical-sized bone defects, Endochondral ossification, Mesenchymal stromal cells

Introduction

Unlike many fractures, larger bone defects resulting from high-energy trauma or osseous tumour removal do not heal without intervention. Current treatment options include the use of autologous or allogeneic bone grafts,[1,2] sometimes preceded by an induced membrane procedure to improve defect vascularization.[3] In the United States, for example, it was estimated in 2009 that half a million bone grafts were performed per year at a cost of $2.5 billion; this number was expected to double by 2020.[4] For problematic defects, such as those in the lower limbs, bone grafts cannot ensure healing.[5] Failed treatment necessitates challenging revision surgeries, with associated pain and risk of infection. If these also fail, amputation is a likely outcome. The lifetime healthcare costs of lower limb amputation were estimated to exceed $500 000,[6] but lost quality of life for these patients cannot be fully quantified. These realities underscore the need for better strategies to stimulate bone healing.

Among the wide range of biologicals being considered to improve bone repair, there is recent interest in the therapeutic application of extracellular vesicles (EVs). The term EV has been adopted by the research community to describe multiple types of secreted, membrane-enclosed vesicles.[7] Although there has been past inconsistency with nomenclature,[8] two distinct classes of EVs are known as exosomes (30 nm to 150 nm), which are released from multivesicular bodies when docking with the plasma membrane, and microvesicles (50 nm to 1000 nm), which result from plasma membrane budding at the cell surface. Additional EV subclasses include apoptotic bodies, ectosomes, and oncosomes (all >1 µm). EVs were once thought to represent cellular waste, although in recent years, their importance to widespread physiological

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doi: 10.1302/2046-3758.74.BJR-2018-0006

Bone Joint Res 2018;7:263–273.

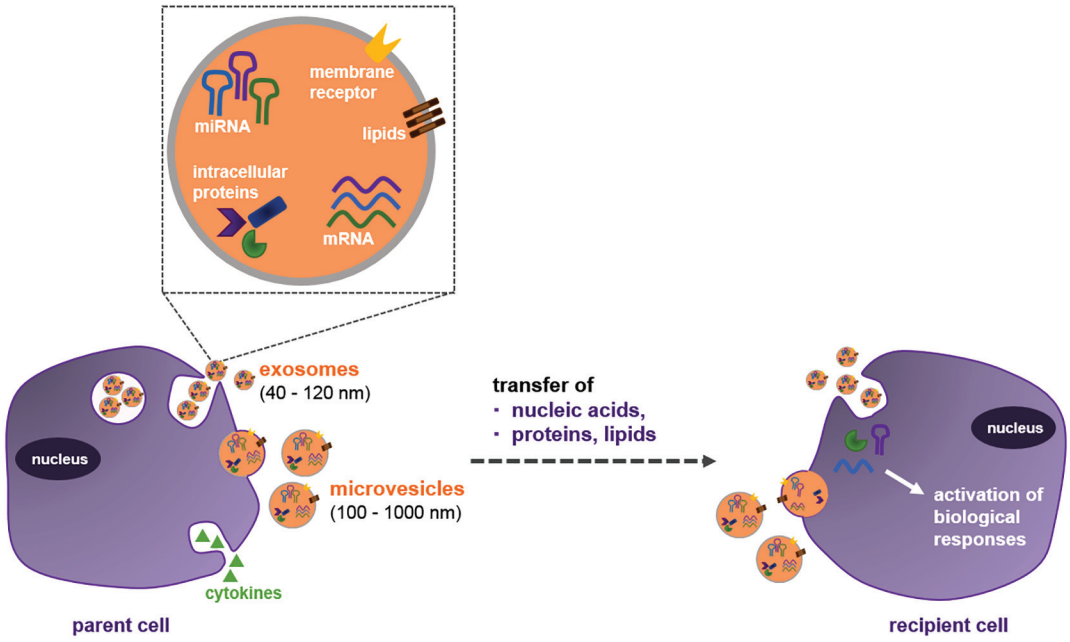

Fig. 1

Diagram showing intercellular communication by extracellular vesicles (EVs). Two principle EV fractions are understood to play roles in intercellular communication: exosomes, which are released from multivesicular bodies after fusing with the parent cell plasma membrane; and larger microvesicles, which bud directly from the parent cell membrane. Each fraction contains unique profiles of intravesicular RNAs and protein as well as membrane-bound receptors and lipids. These fractions stimulate responses within recipient cells by direct activation of recipient cell surface receptors, by transfer of vesicle contents to the recipient cell cytosol after fusion with the plasma membrane, and by intracellular trafficking of vesicle contents following endocytosis.

and pathological processes has been recognized.[9-11] For example, EVs can either stimulate or suppress immune responses to pathogens and cancer cells. Circulating EVs are thought to play a role in immune tolerance, yet they can also contribute to the progression of autoimmune diseases, including rheumatoid arthritis and diabetes.[12] EVs exert their effects on target cells through multiple mechanisms, directly activating recipient cell surface receptors, transferring their membrane contents to the recipient cell plasma membrane, and delivering packaged cargo into the recipient cytosol.[13] This cargo includes proteins, mRNAs, and microRNAs that, importantly, reflect the state of the parent cell (Fig. 1).

It may be possible to exploit this natural mechanism for intercellular signalling to overcome some important limitations of cell therapies for bone defect repair. It is known that successful repair, which occurs during fracture healing, involves multiple contributing cell populations, including osteochondral progenitors and macrophages. For each of these populations, multiple signalling pathways regulate essential repair activities.[14,15] Consequently, delivery of any single pathway regulator may have limited impact on improving repair. Because EVs stably package protein and nucleic acid signals for transfer between cells, they have the potential to activate complementary, pro-regenerative signalling pathways in the same target cells, and to stimulate multiple target populations. This property could make them an efficient therapeutic vehicle for bone regenerative medicine.

EVs are already being evaluated in clinical trials, mainly for the diagnosis and treatment of specific cancers.[16] Their potential uses in regenerative medicine are still largely speculative.[11,13,17-20] This review focuses on the possibility for harnessing EVs to enhance the endochondral repair of large bone defects. The following sections present the general hypothesis that parent cells can be manipulated *in vitro* to produce EVs with complementary, pro-regenerative signals that direct endogenous cells to complete one or more limiting steps to bone repair. A rationale for the use of EVs is presented, along with promising cell sources and likely therapeutic targets for directing repair through an endochondral pathway. Finally, barriers to clinical translation are discussed.

The papers included in this narrative review were identified using PubMED and Web of Science prior to 15 December 2017. With the exception of studies describing matrix vesicles, most of the research discussed below has been published since 2014, demonstrating the relatively nascent state of this research area.

Rationale for directing endochondral repair of large bone defects

The long bones are formed through a developmental programme known as endochondral ossification, which essentially involves the generation of a cartilage template that is remodelled into vascularized bone.[21] Bone fractures are repaired through a similar endochondral process: the fracture gap is bridged by a cartilaginous callus

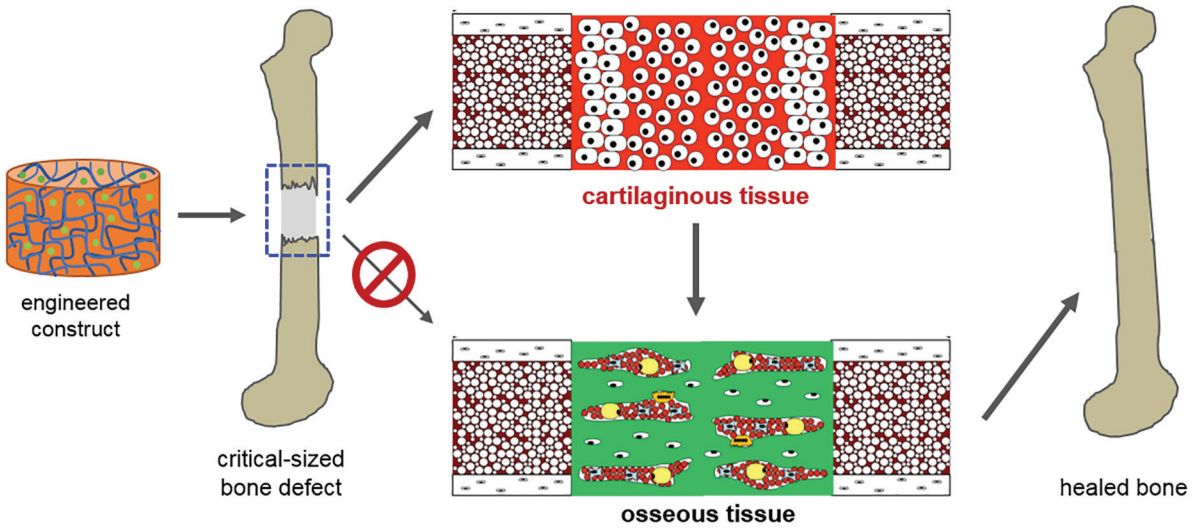

Fig. 2

Directing endochondral repair of large bone defects. One paradigm for bone regenerative medicine is modelled on the processes of long bone development and successful (fracture) repair. Instead of designing scaffold/biological constructs for the direct stimulation of osteogenesis, constructs can be engineered to undergo an initial chondrogenesis phase, which serves as an efficient template for ordered osteogenic remodelling by successive waves of repair cells. It is noteworthy that cartilage, an avascular tissue, is more resilient to the vascular deficiency within larger bone defects.

formed by progenitor cells migrating from the nearby periosteum; upon chondrocyte hypertrophy and calcification, the callus is remodelled into bone.[15] This repair process is often compromised in defects beyond a critical size (critical-sized bone defects, or CSBDs), leading to nonunion. While comorbidities and biomechanical factors (e.g. stability of bone fixation) can influence the success of healing, deficiencies in repair cell numbers and inductive growth factor levels are limiting to the initiation of an endochondral repair pathway.[22]

There has been increasing focus in the orthopaedic research community on 'developmental engineering' strategies for large defect treatment that aim to mimic the endochondral ossification programme of development and fracture repair (Fig. 2).[23-25] Common components of these strategies are mesenchymal stromal/stem cells (MSCs), which have the potential to self-renew and differentiate into the constitutive cells of bone, including osteoblasts and hypertrophic chondrocytes (growth plate).[26,27] For example, Scotti et al[28,29] demonstrated that hypertrophic cartilage engineered *in vitro* from human MSCs could form a functional bone organ when implanted ectopically in nude mice. Bahney et al[30] subsequently demonstrated that cartilaginous grafts, derived either from fracture callus or from MSCs pre-differentiated *in vitro*, can stimulate bone healing following implantation into murine CSBDs. Other tissue-engineered cartilage constructs have since been tested in various bone formation models *in vivo*.[31-36] While these results are promising, their clinical translation relies on production of cartilage templates outside the body under carefully controlled conditions to ensure safety and efficacy. Alternatively, if the template could be formed within the defect by either exogenous (i.e. culture-expanded) and/or endogenous progenitor cells, this should reduce the complexity – and related cost – of treatment.[37]

Rationale for using EVs *versus* their parent cells

Relative to fractures, larger bone defects are characterized by an ischaemic microenvironment, with extreme deficiencies in oxygen and nutrients near their core.[5,38,39] This harsh microenvironment presents a major challenge for the use of cell-based therapies, because the implanted cells compete with endogenous progenitor cells (i.e. migrating into the defect) for limited oxygen and nutrients (Fig. 3). This should be especially true for repair cells expanded under high serum and normoxia prior to implantation.[40,41] It has been demonstrated that most culture-expanded MSCs, for example, die or undergo phagocytosis by macrophages in the first couple weeks after implantation within CSBDs.[42,43] Recent studies have demonstrated that these cells cannot adapt to the ischaemic environment, particularly upon the depletion of glucose stores.[44] In contrast to culture-expanded cells, their EVs should neither tax the defect for oxygen and nutrients nor actively produce cellular waste.

If EVs can deliver pro-regenerative paracrine signals produced by their parent cells, they would present a treatment alternative with potential safety advantages. One key advantage would be the inability to undergo malignant transformation. However, because tumour cell EVs have been shown to transfer oncogenic molecules to recipient cells,[45] EVs from culture-expanded cells cannot be assumed to be safe in terms of tumourigenicity. Regarding their storage in the absence of cryoprotectants, EVs retain their activity after freezing and thawing

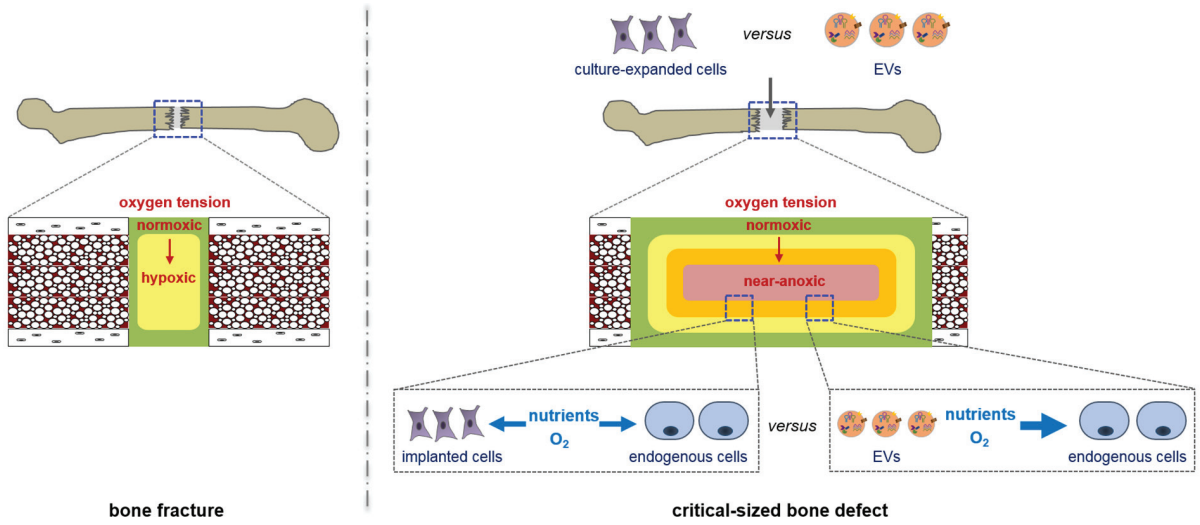

Fig. 3

Diagram showing the potential advantage of extracellular vesicles (EVs) within a large bone defect microenvironment. As opposed to simple fractures, bone defects beyond a critical size are characterized by severe nutrient deficiency and near-anoxia within their core. While exogenous cells implanted into these defects may secrete pro-regenerative factors, they also compete with endogenous repair cells migrating into the defect for scarce oxygen and nutrients. In contrast, the same pro-regenerative signals packaged within EVs would not necessarily tax the defect for nutrients and oxygen, potentially permitting enhanced repair by endogenous cells.

better than cells do, although changes to their properties and function have been reported and are dependent on the precise storage conditions.[46] While these theoretical advantages are promising, more pre-clinical studies are needed that provide head-to-head comparisons of EVs with their candidate parent cells, in order to demonstrate a clear therapeutic advantage in the context of bone repair.

Cell sources of EVs for bone regenerative medicine

To date, only a handful of studies have evaluated EVs harvested from culture-expanded cells within pre-clinical models of bone injury. These studies are summarized in Table I, along with studies using models of osteochondral defect repair, which may offer insights into the endochondral repair of bone defects. To date, little consideration has been made for strategies to alter EV composition in order to better direct tissue repair.

Mesenchymal stromal/stem cells. MSCs from human adults have been shown to generate bone tissue in a variety of experimental models.[47] These laboratory observations have encouraged the development of bone graft substitutes that incorporate MSCs into osteoconductive scaffolds, with the idea that the MSCs will form new bone upon implantation. There are several completed and ongoing clinical trials applying autogenous or allogeneic MSCs for treatment of complex fractures or nonunions. While the first clinical studies using culture-expanded MSCs were promising,[48] the cumulative results have been mixed.[22] Pre-clinical studies of MSC fate following implantation or systemic delivery have shown that the large majority of culture-expanded cells contribute to repair indirectly, through their paracrine effects on endogenous cells at the site of injury. For example, MSCs enhance recruitment of endogenous repair cells through secretion of angiogenic and chemotactic factors.[49] But if MSCs do not directly participate in new bone formation, their implantation might be inefficient for stimulating repair, since they compete with endogenous repair cells for limited oxygen and nutrients (Fig. 3). In place of MSCs, the mediators of their paracrine effects might be delivered to bone defects.

Pro-regenerative effects by MSC-derived EVs have been extensively reported in pre-clinical models of acute kidney injury, liver and lung injury, myocardial infarction, and hindlimb ischaemia.[11,13] In some studies, the effects of MSC-EVs were comparable to those of direct MSC administration, suggesting that EVs relay essential paracrine effects of their parent MSCs. Specific effects attributed to MSC-EVs include increased angiogenesis, inhibition of apoptosis, and reduction of oxidative stress. MSC-EVs have been reported to contribute adenosine triphosphate (ATP) production through their surface kinases, which is thought to improve endogenous cell survival at sites of injury.[50,51] MSC-EVs also have established immunomodulatory effects that could impact bone repair.[13] For example, EVs from adipose-derived MSCs cultured under hypoxia caused an M1-to-M2 shift in bone marrow macrophage phenotype, which was associated with pro-regenerative EV effects in a muscle injury model.[52]

Many of the studies to date that have evaluated exogenous EVs for stimulating bone and cartilage repair have used primary MSCs as the donor cells (Table I). In one of

Table I. Studies evaluating the use of exogenous extracellular vesicles (EVs) to alter bone or cartilage repair *in vivo*

First author	EV fractions studied	Cell source of EVs	Experiment model	Delivery method	Outcome measures	Key findings
Furuta et al (2016)[53]	Multiple*	Human BM-MSCs	Murine femoral fracture	Injection (×2)	X-ray, μCT, histology/IHC	Injections of MSC-EVs rescued delayed fracture healing in CD9-/- mice and enhanced normal healing in wild type mice
Li et al (2017)[77]	Exosomes	Rabbit BM-MSCs (+/- HIF-1α overexpression)	Rabbit steroid-induced avascular necrosis of femoral head	Injection	MRI, histology/IF	Exosomes from HIF-1α-overexpressing MSCs promoted increased trabecular bone generation and neo-vascularization in femoral heads compared with unmodified MSC-EVs and saline-treated groups
Qi et al (2016)[63]	Exosomes	Human iPSC-derived MSCs	Rat critical-sized cranial defects (×2)	TCP scaffold	μCT, histology/ histomorphometry/ IHC	EVs from iPSC-derived MSCs dose-dependently enhanced bone formation and vasculogenesis compared with TCP controls
Qin et al (2016)[54]	Multiple	Human BM-MSCs	Rat critical-sized cranial defects (×2)	Hydrogel scaffold	μCT, histology	MSC-EVs stimulate bone formation compared with hydrogel controls
Xie et al (2017)[79]	Multiple	Rat BM-MSCs	Subcutaneous implantation in nude mice	Bovine DBM scaffold	μCT, histology/IHC	EVs from rat MSCs enhanced vessel formation within DBMs implanted subcutaneously, although they did not independently enhance bone formation compared with scaffold-only controls
J. Zhang et al (2016)[64†]	Exosomes	Human iPSC-derived MSCs	Rat critical-sized cranial defects (×2)	TCP scaffold	μCT, histology/IHC	EVs from iPSC-derived MSCs dose-dependently enhanced bone formation compared with TCP controls
S. Zhang et al (2016)[57]	Exosomes	Human ESC-derived MSCs	Rat osteochondral defects	Weekly injections	Histology/IHC	EVs from ESC cell-line-derived MSCs enhanced cartilage repair score (O'Driscoll) and cartilage marker deposition by six weeks
S. Zhang et al (2018)[58‡]	Exosomes	Human ESC-derived MSCs	Rat osteochondral defects	Weekly injections	Histology/IHC	EVs from ESC cell-line-derived MSCs enhanced cartilage repair score (Wakitani) as early as two weeks

*Based on the described isolation method, the specification of exosomes does not seem consistent with criteria established by a position paper from the International Society of Extracellular Vesicles[7]
†Follow-up study to Qi et al (2016)[63]
‡Follow-up study to S. Zhang et al (2016)[57]
BM-MSC, bone-marrow-derived mesenchymal stem cells; μCT, micro-computed tomography; IHC, immunohistochemistry; MSC-EVs, mesenchymal stem cell extracellular vesicles; HIF-1α, hypoxia-inducible factor 1-alpha; MRI, magnetic resonance imaging; IF, immunofluorescence; iPSC, induced pluripotent stem cell; TCP, tricalcium phosphate; DBM, demineralized bone matrix; ESC, embryonic stem cell

the most promising studies, Furuta et al[53] first demonstrate delayed endochondral ossification during fracture repair in CD9 global knockout mice, which display reduced exosome secretion, suggesting a role for exosomes in normal fracture healing. When the authors injected exogenous EVs from human bone marrow MSCs into the fracture site of CD9-/- mice, they observed a rescue in delayed healing; moreover, MSC-EVs enhanced fracture repair in wild type mice. Another study has shown that human bone marrow MSC-derived EVs stimulate bone formation within critical-sized calvarial defects made in immunocompetent rats.[54]

Pluripotent stem cells. While the extensive literature on MSCs supports their evaluation as a potential source of therapeutic EVs, alternative cell sources should also be considered. There has been longstanding interest in the use of embryonic stem cell (ESCs) for bone regenerative medicine.[55] ESC lines have been used to generate cells with MSC properties.[56] Using human ESC-derived MSCs as a source of exosomes, Zhang et al[57] demonstrated that weekly injection of these vesicles enhanced cartilage repair in a rat osteochondral defect model. A follow-up to this original report demonstrated that enhanced cartilage repair was associated with enhanced proliferation, reduced apoptosis, and increased M2 macrophage polarization within the defects.[58] It is noteworthy that there is significant osseous injury within these 1 mm deep osteochondral defects (thickness of rat trochlear cartilage is ~ 200 microns). The early histology shown by the authors suggests that enhanced repair of subchondral bone occurred through an endochondral pathway.[58]

Recent work in bone regenerative medicine has focused on the application of induced pluripotent stem cells (iPSCs), which avoid sourcing barriers associated with using ESCs and autologous MSCs.[59] As with ESCs, protocols have been established for generating cartilage- or

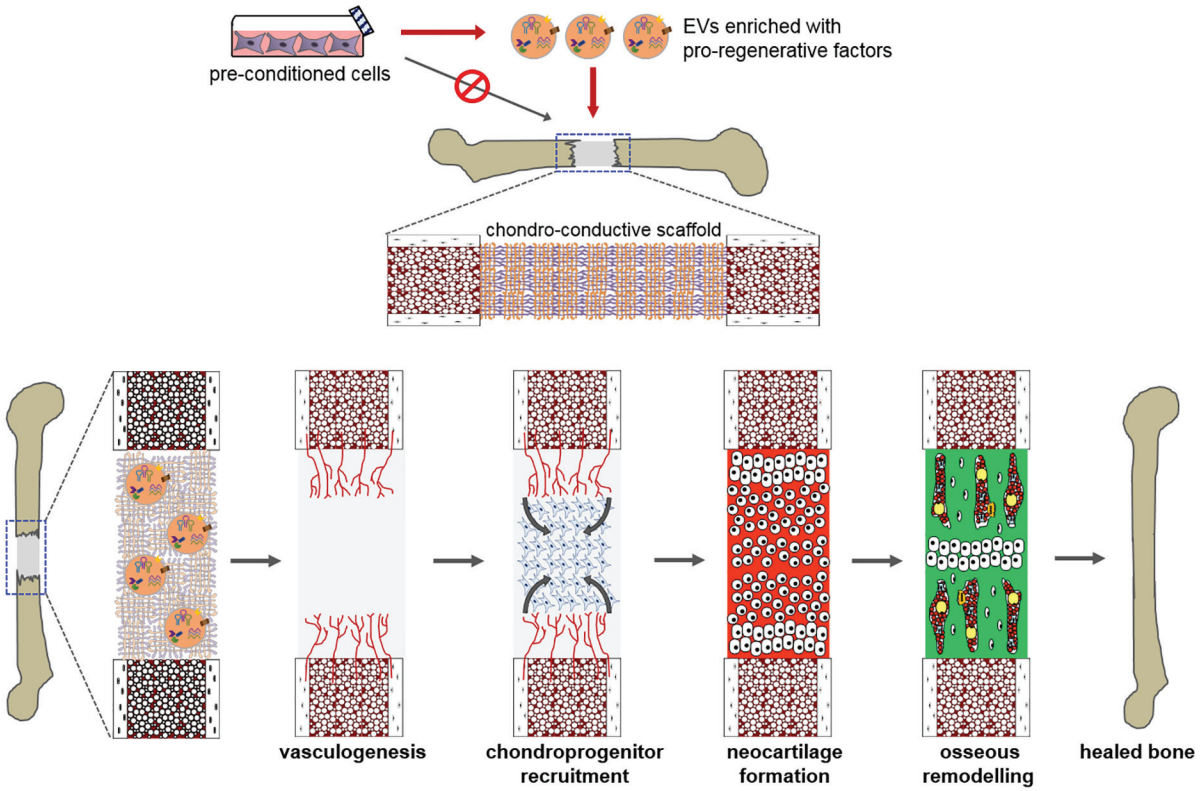

Fig. 4

Diagram showing therapeutic targets for endochondral repair. Bottlenecks to endochondral bone repair include progressive vascularization, chondroprogenitor recruitment, neocartilage formation, and osseous remodelling. It may be possible to deliver extracellular vesicles (EVs) harvested *in vitro* from promising parent cell cultures that stimulate endogenous cells to overcome one or more of these bottlenecks. An ideal scaffold for this approach would not only be chondroconductive but would also control the release of therapeutic EVs, in order to match the migration timeframe of target repair cells (endothelial, chondroprogenitor) into the defect.

bone-forming cells from iPSCs,[60,61] sometimes through the induction of an intermediate MSC phenotype.[62] Exosomes harvested from human iPSC-derived MSCs have been reported to dose-dependently enhance neovascularization and/or bone formation within rat cranial defects.[63,64]

Monocytes/macrophages. Another possible source of therapeutic EVs are cells of the monocyte/macrophage lineage, which play prominent roles in bone repair, including endochondral fracture repair.[65] Macrophage-derived EVs have been shown to improve intestinal regeneration in a mouse model of radiation injury through the delivery of multiple wingless-related integration site (WNT) proteins to intestinal stem cells.[66] An important question related to the application of macrophage-derived EVs concerns what source phenotype is best suited to enhance the repair of larger bone defects. While anti-inflammatory (M2) macrophages are known to mediate regenerative effects in wound healing, pro-inflammatory (M1) macrophages can also contribute to bone repair. For example, Zhan et al[67] reported that EVs from M2 macrophages increased the proliferation and migration of Schwann cells *in vitro* compared with EVs from M1 macrophages; this was associated with enhanced Schwann cell infiltration and axon formation by M2 macrophage EVs in a rat sciatic nerve injury model. However, Seebach et al[42] have demonstrated that CSBD repair enhanced by exogenous MSC delivery is associated with the recruitment of M1, but not M2, macrophages. The most suitable macrophage phenotype for therapeutic EV production may depend on the specific regenerative activities to be stimulated.

Engineering EVs through their parent cells. The therapeutic efficacy of EVs harvested *in vitro* will not only be determined by the choice of parent cells, but also by how those cells are conditioned prior to EV harvest. Multiple studies have reported that pre-conditioning various stem cell populations under low oxygen tension improves the pro-angiogenic activity of their EVs both *in vitro* and *in vivo*.[52,68] EVs collected from MSCs while they were stimulated with pro-inflammatory cytokines or with lipopolysaccharide displayed anti-inflammatory effects on recipient lymphocytes and macrophages, respectively.[69,70]

In addition to pre-conditioning, parent cells can also be genetically modified in order to enrich their EVs with pro-regenerative signals.[71] Horizontal transfer of transgene

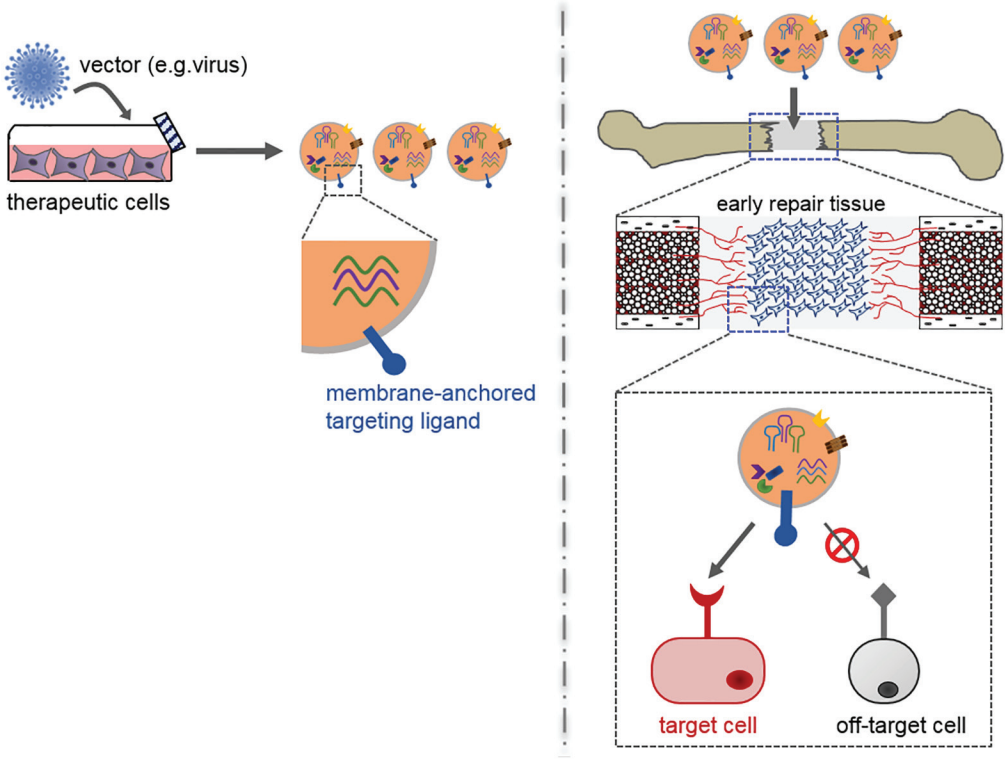

Fig. 5

Diagram showing the genetic engineering of extracellular vesicles (EVs) for targeting endogenous repair cells. The efficacy of exogenous EVs may be improved by introducing targeting ligands onto their surface that recognize cell surface receptors specific for key repair cells. One way in which to introduce these ligands would be to genetically engineer parent cells to express them within the extracellular domain of a membrane-anchored fusion protein. The fusion protein would then be expressed on the surface of EVs secreted by the parent cells. In addition to potentially improving the efficiency of target cell uptake of EV contents, this strategy could also limit nonspecific or adverse effects in off-target cells.

products have been demonstrated following parent cell modification using plasmid, adenoviral, and lentiviral vectors.[71,72] In addition to transgene mRNA or cDNA, microRNAs can also be introduced to EVs using this approach.[73,74] Which signals to introduce or enrich within EVs would depend on the specific target activities to be stimulated in the recipient population(s).

Therapeutic targets for an endochondral repair strategy

For the repair of large bone defects through an endochondral pathway, three interrelated bottlenecks to repair are hypothesized (Fig. 4): 1) the establishment of a vascular network that supports mesenchymal progenitor recruitment deeper within the defect; 2) the chondrogenic differentiation and hypertrophic maturation of these endogenous progenitors; and 3) the osseous remodelling of the hypertrophic cartilage template by subsequent waves of osteoclast and osteoblast progenitors. It may be possible to deliver EV populations that address one or more of these bottlenecks.

Vasculogenesis. The importance of defect vascularization to successful bone repair is well known.[75] Because the MSC secretome includes chemotactic and vasculogenic factors,[49] there has been much interest in using these cells to stimulate vasculogenesis within CSBDs.[76] However, the standard conditions for MSC culture expansion, including atmospheric oxygen and abundant nutrients, are very different from the hypoxic and nutrient-deficient defect microenvironment. Switching MSCs to hypoxia and reduced serum *in vitro* has been shown to increase their vasculogenic and chemotactic factor secretion, potentially enhancing their indirect contribution to bone repair.[40,76,77]

When Anderson et al[78] exposed MSCs to ischaemic conditions, their EVs were enriched with downstream mediators of the platelet derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) signalling pathways. This translated to an EV-dose-dependent increase in tubule formation by endothelial cells *in vitro*. More recently, Gonzalez-King et al[68] genetically modified MSCs to overexpress hypoxia inducible factor (HIF)-1α, a transcription factor that regulates cell responses to hypoxia. HIF-1α-MSC-EVs stimulated increased tubule formation *in vitro* as well as enhanced subcutaneous angiogenesis of EV-laden hydrogels within nude mice. These effects were attributed to increased Jagged1 levels on the HIF-MSC-EVs, as the

pro-vasculogenic effects were blunted with a neutralizing Ab against Jagged1. While pro-vasculogenic effects have been shown for EVs from MSCs grown under standard culture conditions,[63,79] EVs from hypoxia pre-conditioned MSCs have yet to be tested in a CSBD animal model.

Chondrogenesis. Prior studies that tested recombinant vascular endothelial growth factor (VEGF), a potent angiogenic factor important for fracture repair,[15] within rodent CSBDs suggest that vasculogenic stimulation alone may not be sufficient to achieve defect repair.[80,81] During endochondral bone formation, chondrogenesis by recruited mesenchymal progenitors is critical for priming subsequent osteogenesis. Moreover, the formation of hypertrophic cartilage is necessary, as opposed to a fibrocartilage repair tissue that would not support ossification, likely resulting in nonunion.

Martins et al[82] recently reported that EVs harvested from osteogenically differentiated MSCs were able to independently stimulate the osteogenic commitment of naïve, recipient MSCs. A similar observation was made in the context of MSC neurogenic differentiation.[83] These studies suggest that the differentiation of endogenous progenitor cells in a bone defect can be guided by EVs from culture-expanded stem/progenitors directed along a similar pathway *in vitro*. Such lineage guidance using EVs has not yet been described for chondrogenesis. However, it is known that co-culture of MSCs with articular chondrocytes improves chondrogenic differentiation of the MSCs;[84] it is possible that chondrocyte-derived EVs contribute, in part, to the co-culture effect.

Osseous remodelling. During the repair of mechanically stable fractures, hypertrophic cartilage within the fracture callus undergoes mineralization, vascularization, and remodelling into woven bone. Matrix vesicles have long been observed in the hypertrophic cartilage of the callus during fracture repair.[85] These vesicles, which have been proposed to be matrix-anchored exosomes,[86] are secreted by hypertrophic chondrocytes and function to nucleate cartilage calcification.[87] More recent studies have suggested a functional role for the microRNAs enriched within matrix vesicles.[88] Of note, it has been shown that these vesicles contain growth factors that regulate bone formation, such as BMP-2 and VEGF.[89]

It may be possible to produce EVs that mimic matrix vesicles in composition for the purpose of stimulating repair tissue ossification. A possible parent cell population for such EVs would be hypertrophic chondrocytes, which can be derived from MSCs.[28,90] To produce calcification-nucleating, matrix vesicle-mimetic EVs, however, the normal culture conditions for MSC differentiation may require additional optimization.

Challenges for clinical application

A recent position paper from the International Society of Extracellular Vesicles (ISEV) has provided a comprehensive review of the many translational considerations for EV-based therapeutics.[16] Much of the uncertainty about how EVs can be applied clinically is related to persisting gaps in understanding about their biogenesis and function. As with other biological medicinal products, successful translation of EVs to skeletal regenerative medicine will hinge on continued basic science studies that fill in these knowledge gaps.

EV mechanisms of action. The role of EVs in successful endochondral bone repair (e.g. fracture healing) is not completely understood. As early as the 1960s, matrix vesicles were described as playing a role in mineralization of the growth plate.[91] The recent study by Furuta et al[53] described above suggests that EVs have a significant role to play in fracture healing: the authors demonstrated reduced healing in CD9 knockout mice and partial rescue following injection of exogenous, MSC-derived EVs. As the mechanisms governing EV biogenesis and cell uptake become better understood, more conclusive studies into their roles in bone repair may be completed. For example, identifying key protein mediators of biogenesis and uptake should permit genetic engineering tools to be employed for studies of EV knockout and overexpression. Similarly, a better understanding of how pro-regenerative factors are sorted into specific EV subpopulations could be used to block or enhance their loading and demonstrate the importance of their EV-specific delivery *in vivo*. In the absence of this knowledge, however, early therapeutic approaches can be guided by studies of how EVs isolated from candidate parent cells *in vitro* stimulate repair in pre-clinical injury models.

The mechanisms of action for an EV therapeutic will likely depend on the subtype used. While a greater degree of evidence has been provided for exosomes, pro-regenerative actions have also been demonstrated for the microvesicle fraction of parent-cell-conditioned media.[92] While many studies have shown beneficial effects when using a heterogeneous population of EVs,[53,54,79] these studies cannot infer whether the different subpopulations were agonistic or antagonistic to one another. Future studies should consider the relative efficacy of exosomes *versus* microvesicles for each therapeutic application. Their conclusiveness will depend on an improved distinction of these distinct EV subpopulations.[7]

EV delivery. For bone defect repair, medicinal EVs would ideally be delivered directly to the defect site, as opposed to systemic delivery, which would require higher total doses to obtain the same defect concentration, thereby increasing risk of side effects in off-target tissues. Because large defects typically require implantation of a gap-filling scaffold, which supports ingrowth of endogenous repair cells, a promising solution would be to employ the scaffold as a depot for local release of EVs. Such an approach has been used for the delivery of other osteogenic factors, such as recombinant bone morphogenetic

protein (BMP)-2, to bone defects[93,94] However, clinical observations from BMP-2 use demonstrate the need for scaffolds that control the release of an osteogenic therapeutic: burst release of BMP-2 at supraphysiological levels from collagen-based scaffolds has been associated with adverse effects, including perioperative inflammation and pain, resorption of nearby intact bone, and formation of heterotopic bone in adjacent soft tissues.[95-99] It is possible that EVs could have similar adverse effects if delivered by burst release. Additional research into how biomaterials can be used to control the release of different EV populations will complement mechanistic studies of EV action in order to design therapeutic strategies for large bone defects.

In addition to intra-defect release, the specificity of EV action may be improved by targeting them to endogenous repair populations, such as endothelial or chondroprogenitors cells. During normal intercellular communication, EVs deliver their cargo by direct signalling with target cell receptors, through vesicle docking with the target cell plasma membrane, or through endocytosis of the EV and subsequent cargo release. Accordingly, EVs could be engineered to express targeting ligands on their surface, such as membrane-anchored peptides, that specifically recognize cognate receptors on the target cells of interest[100] (Fig. 5). By targeting the vesicles to the repair populations of interest, the per-cell delivery of therapeutic cargo might be enhanced, allowing the total administered EV dose to be lowered. Additional studies are still required to identify the most appropriate target cells and surface receptors for improving bone defect repair. Because these populations may enter the defect over distinct timeframes, information on their migration kinetics will guide the design of scaffolds for controlled release of pre-loaded EVs.

Approval of medicinal EVs. Widespread clinical use of EVs will depend on approval by regional regulatory bodies, such as the United States Food and Drug Administration. These regulatory bodies will require thorough characterization of specific EV therapeutics, including their essential composition and proposed mode of action (MoA), and the qualification of potency assays that reflect the proposed MoA. Demonstration of safety and efficacy in clinically relevant animal models of bone injury will also be important for translation to the clinic. Finally, procedures must be established for EV isolation, storage, and quality control testing that meet Good Manufacturing/Laboratory Practice (GMP/GLP) standards.[16] Due to the complex makeup of EVs, meeting these standards may be more challenging than for biological medicinal products already approved for bone repair, such as BMP-2. Because EVs have a similar degree of complexity as their parent cells, including heterogeneity in composition, EV-based therapeutics may follow a similar regulatory pathway as cell-based therapeutics. For EVs derived from genetically modified cells, the higher degree of manipulation would likely categorize them as Advanced Therapy Medicinal Products,[101] requiring more stringent safety testing.

Long-term prospective. Given the inherent complexity of EVs, information gained from ongoing pre-clinical studies may ultimately be used to develop synthetic vesicles containing only those essential components required for the desired effects in target cells. There have been extensive efforts to develop liposomes as drug delivery vehicles, providing a base of knowledge for the production of artificial, EV-mimetic vesicles.[102] However, development of an effective EV alternative would require a much better understanding of the mechanisms of EV action, in order to determine which components are essential and at what concentrations they should be loaded into artificial vesicles. It is likely that effective, if imperfect, natural EV products may be employed clinically while the research community learns how to reverse engineer their essential functions.

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Funding Statement

- The authors' activity in this area is supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, part of the National Institutes of Health, under award numbers AR069253 and AR071560. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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- E. Ferreira: Reviewing the literature, Preparing and editing the manuscript.
- R. M. Porter: Reviewing the literature, Preparing and editing the manuscript.

Conflicts of Interest Statement

- None declared.