Biomaterials Functionalized with MSC Secreted Extracellular Vesicles and Soluble Factors for Tissue Regeneration

Meadhbh Á. Brennan, Pierre Layrolle, and David J. Mooney*

The therapeutic benefits of mesenchymal stromal cell (MSC) transplantation are attributed to their secreted factors, including extracellular vesicles (EVs) and soluble factors. The potential of employing the MSC secretome as an alternative acellular approach to cell therapy is being investigated in various tissue injury indications, but EVs administered via bolus injections are rapidly sequestered and cleared. However, biomaterials offer delivery platforms to enhance EV retention rates and healing efficacy. This review highlights the mechanisms underpinning the therapeutic effects of MSC-EVs and soluble factors as effectors of immunomodulation and tissue regeneration, conferred primarily via their nucleic acid and protein contents. Discussed is how manipulating the cell culture microenvironment or genetic modification of MSCs can further augment the potency of their secretions. The most recent advances in the development of EV-functionalized biomaterials that mediate enhanced angiogenesis and cell survival, while attenuating inflammation and fibrosis, are presented. Finally, some technical challenges to be considered for the clinical translation of biomaterials carrying MSC-secreted bioactive cargo are discussed.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) are major cell candidates in regenerative medicine. MSCs can be isolated from diverse tissues in the body including the bone marrow,[41] adipose tissue,[2] umbilical cord,[14] placenta,[5] and dental pulp[6] (Figure 1). Some evidence indicates that MSCs in these tissues have a perivascular origin.[7] MSCs are identified by their plastic adherence, expression of surface markers such as CD44, CD73, CD90, CD105, and tri-lineage differentiation potential[1,8,9] and have demonstrated efficacy in preclinical[10,11] and clinical trials[12–14] in a number of tissue repair settings. In spite of the ability of MSCs to successfully mediate healing, the engraftment of transplanted MSCs is rather low,[15,16] and it is now generally considered that MSCs impart their therapeutic benefit primarily through paracrine mechanisms.[17,18] The MSC secretome can be collected in vitro as MSC-conditioned media (MSC-CM) and comprises both soluble factors, including cytokines, chemokines, and growth factors,[19] as well as vesicular secretions termed extracellular vesicles (EVs).[20] MSC-CM enhances in vitro cell proliferation,[21–23] migration,[22,24] angiogenesis,[25] and imparts anti-apoptotic[22,25] and anti-inflammatory[26] effects. In vivo, MSC-CM has demonstrated healing in diverse preclinical indications including cutaneous wounds,[22] Parkinson’s disease,[27] liver failure,[28] and myocardial infarction (MI).[29,30] Indeed, significant data indicate that MSC-CM can achieve similar results as direct MSC delivery in various preclinical injury models,[31–33] while others report inadequate healing by MSC-CM compared with MSC transplantation.[34,35] Recently, a number of clinical cases suggest the healing potential of MSC-CM.[36–38]

In 2009, the therapeutic effects of MSCs in a kidney injury model were attributed to MSC-secreted EVs.[39] Shortly after, heart regeneration was enhanced by delivery of MSC-EVs in a mouse model of myocardial ischemia injury.[40] Since then, EVs isolated from MSC-CM have been shown to effectively promote repair in many tissues and organs, including cardiovascular,[41] musculoskeletal,[23,42] neurological, renal,[43] and pulmonary indications[45] in preclinical studies. The mechanisms of tissue repair in vivo mediated by MSC-derived EVs include immune modulation,[46] enhanced angiogenesis[47] inhibition of apoptosis,[48] and reduction of fibrosis.[49] MSC secretome-based therapeutics have a number of potential advantages over MSC therapy, whose clinical translation is hampered by invasive harvesting procedures, limited sources, and high production costs. First, soluble factors and EVs can be filter sterilized. Moreover, EVs may have extended stability, with reduced demands on
frozen storage conditions, and can be utilized directly upon thawing.\textsuperscript{[50]} Furthermore, they can potentially be lyophilized for an off-the-shelf product, while retaining functionality.\textsuperscript{[21,51]} Together, these may reduce the manufacturing costs compared to cell therapy.

EVs can be administered via a number of routes, including intravenous, intraperitoneal, or subcutaneous injection, and the delivery route drastically alters the in vivo bio-distribution of administered EVs.\textsuperscript{[52]} The most common mode of EV delivery for tissue repair is via local injection of EVs directly into sites of injury. However, this may lead to rapid clearance of EVs from the site. In some cases, repeated injections over the healing period are required.\textsuperscript{[53]} Much in the same manner as biomaterials can aid in the retention and therapeutic potential of MSCs delivered by either local or intravenous means,\textsuperscript{[54,55]} the use of biomaterials to deliver EVs has emerged as a promising strategy in regenerative medicine to overcome the low retention rates of bolus injections of EVs. Binding to, or encasing EVs in a biomaterial matrix has been shown to extend their bioavailability following delivery, permit sustained and controlled release, maintain stability of EV cargo, and potentially augment therapeutic potency.\textsuperscript{[56–59]} It must be noted that the various bioactive factors in EVs and the soluble fraction secreted by MSCs have multiple biological activities, and as such may cause unanticipated side effects. While no such observations have been reported to date, the local delivery of the MSC secretome, as opposed to systemic delivery, may help to ensure any effects are not wide spread.

In this review, we outline the components of the MSC soluble secretions and EVs and review their potential as an acellular regenerative medicine strategy. Known mechanisms of tissue healing mediated by the MSC secretome are highlighted, and the impact of culture stimuli on the secretome is reviewed. We then focus on recent advances in the administration of MSC-derived EVs and soluble secretions via biomaterial delivery platforms. Finally, we discuss future challenges in the field of EV-functionalized biomaterials, including standardization, scalability, and clinical translation.

2. MSC Soluble Secretions

Soluble components of MSC-CM include a wide variety of secreted growth factors, chemokines, and hormones with immunomodulatory, angiogenic, and anti-apoptotic functions. Soluble factors contained in MSC-CM at the highest concentrations include vascular endothelial growth factor VEGF,\textsuperscript{[23–27,62,63]} basic fibroblast growth factor (FGF2),\textsuperscript{[62]} Angiopoietin-1 (AGPT1),\textsuperscript{[22]} insulin-like growth factor (IGF-1),\textsuperscript{[22,63,64]} placental growth factor (PIGF),\textsuperscript{[62]} platelet-derived growth factor (PDGF),\textsuperscript{[65]} sphingosine 1-phosphate (S1P),\textsuperscript{[22]} transforming growth factor-beta (TGF- β1),\textsuperscript{[22,64,66]} interleukin (IL)-6,\textsuperscript{[25,27]} IL-8,\textsuperscript{[67]} monocyte chemoattractant protein (MCP-1),\textsuperscript{[25,62]} hepatocyte growth factor (HGF),\textsuperscript{[22,63,64]} macrophage inflammatory protein-1α (MIP-1α),\textsuperscript{[22,66]} MIP-1β,\textsuperscript{[68]} monokine induced by IFN-γ (MIG),\textsuperscript{[69]} brain-derived neurotrophic growth factor (BDNF),\textsuperscript{[27]} glial cell-derived neurotrophic factor (GDNF),\textsuperscript{[27]} prostaglandin E2 (PGE2),\textsuperscript{[69]} stromal cell-derived factor-1α (STF-1α),\textsuperscript{[70]} and tumor necrosis factor alpha (TNF-α) stimulated gene/protein 6 (TSG-6).\textsuperscript{[71]}

Meadhbh Á. Brennan is a postdoctoral fellow in Professor Mooney’s laboratory at the School of Engineering and Applied Sciences (SEAS) at Harvard University. She earned her Ph.D. in Biomedical Engineering from the University of Southampton and B.Eng. degree in Biomedical Engineering from the National University of Ireland, Galway. As part of her current Marie Skłodowska Curie fellowship, she is researching the therapeutic potential of mesenchymal stromal cell-secreted factors, particularly extracellular vesicles and mechanobiology cues that can regulate their immunomodulatory roles in tissue repair.

Pierre Layrolle is Director of Research at Inserm, leading a research team entitled “Inflammation and cell communications in bone pathologies” at the University of Nantes, France. He holds a Ph.D. in Biomaterials from the University of Toulouse. After a 2 year post-doctorate in Tsukuba, Japan, he joined the tissue engineering company IsoTis in The Netherlands where he was a senior researcher. In 2003, he was recruited by Inserm in Nantes to conduct translational research on bone tissue engineering using stem cells and biomaterials. He is the coordinator of several European projects (Reborne, Orthounion, Paragen, Maxibone) that support the completion of clinical trials in bone regeneration.

David J. Mooney is the Robert P. Pinkas Family Professor of Bioengineering at Wyss Institute for Biologically Inspired Engineering at Harvard University. He earned his Ph.D. degree in chemical engineering from the Massachusetts Institute of Technology and B.S. degree in chemical engineering from the University of Wisconsin, Madison. His laboratory aims to make cellular and molecular therapies more effective and practical to treat disease using novel biomaterials. Biomaterials developed in his laboratory are used in a variety of drug delivery, immunotherapy, and regenerative medicine projects to promote regeneration or targeted destruction of tissues and organs in the body.
The bioactivity of MSC-secreted soluble factors has been tested in vitro and in preclinical models by depletion, inhibition, or by delivery of recombinant proteins. Many trophic factors each perform diverse functional activities as outlined in Figure 2. For example, HGF and MCP-1 promote angiogenesis,[66,72,73] while also imparting an anti-apoptotic effect.[68,74] Similarly, VEGF serves as one of the primary MSC mediators of angiogenesis,[66,75] while also attenuating apoptosis in vitro and in vivo,[74,76,77] and promoting cell proliferation and differentiation.[64,78–80] Many other soluble secretions contribute to angiogenesis, such as FGF2,[81–83] IGF-1,[84] and Ang-1,[85] while cell migration and the recruitment of endogenous MSCs is promoted by PlGF,[86] PDGF,[65] MIP-1α,[68] and SDF-1α.[87] IL-6 demonstrates angiogenic,[66,88] and anti-apoptotic ability,[25] and depletion of IL-6 and IL-8 from MSC-CM led to a marked attenuation of its regenerative capability in models of cutaneous wound healing.[67] MSCs are a potent source of immunomodulatory soluble factors such as PGE2,[90–92] while TGF-β1 is a vital mediator of the anti-inflammatory and therapeutic potential of MSC and MSC-CM, as demonstrated in a rat model of ischemic brain injury.[93] The anti-inflammatory and tissue-protective protein TSG-6 mimicked the beneficial effects of MSC transplantation in rats with global cerebral ischemia[94] and reproduced the healing induced by MSC-CM after peritoneal injury.[95] TSG-6 also demonstrated healing ability by reducing the influx of neutrophils to the site of tissue injury.[96] Interestingly, evidence shows that some soluble secretions act in concert to enhance tissue healing. For example, MCP-1 participates in mediating angiogenesis induced by TGF-β[85] and VEGF.[97] Furthermore, a synergistic effect of VEGF and FGF2[82] and also VEGF with Ang-1 in promoting vascularization in vivo has been demonstrated.[75]

3. MSC EVs

EVs are nanoparticles secreted by all cells that typically contain lipids, proteins, and nucleic acids, thereby acting as cell–cell communicators. EVs were early recognized as being functionally active as intercellular messengers, particularly in immunomodulation and cancer.[98–100] EV research has grown exponentially since it was recognized in seminal studies that EVs containing mRNA, miRNA, and protein, exchanged genetic information between cells and acted as sophisticated effectors of recipient cell behavior.[101–103] EVs are currently categorized based on their biogenesis and size, with EVs of endosomal origin released when multivesicular bodies fuse with the plasma membrane termed exosomes (50–150 nm), while microvesicles (MVs, 100–1000 nm), which are generally larger, are released directly by outward budding of the plasma membrane.[104] The recently identified nonmembranous nanoparticles termed exomeres are ≈35 nm in size.[105] Other vesicles, called apoptotic bodies (800–5000 nm), are membrane-bound vesicles released as a consequence of membrane blebbing during programmed cell death.[106] EV isolation methods include ultracentrifugation (UC) with or without density gradient separation, size exclusion chromatography, polymer-based precipitation, and immunoaffinity.[107] Uptake of EVs by
recipient cells is mediated by various routes of endocytosis and micropinocytosis \[108,109\] and membrane fusion. \[110\] The precise mechanisms of EV secretion and uptake were comprehensively reviewed recently by Mathieu et al. \[104\]. CD81, CD63, CD9, synaptenin-1, Alix, TSG101, and flotillin-1 are proteins associated with EVs from MSCs \[111,112\] and from other cell types \[113,114\] and as such these are generally used as EV-identifying markers. MSC-EVs have been reported to contain about 850 different proteins, \[111\] over 200 mRNA, \[39\] and \(\approx 60\) miRNAs. \[115\] When EVs are separated according to size, small and large EVs were found to contain distinct proteins, mRNA, and miRNA, and interestingly only small EVs showed therapeutic effects in a model of acute kidney injury. \[50\] When EVs are separated based on their membrane lipids, distinct protein and RNA cargos are also observed. \[116\] In this review, we use the term EVs to refer to exosomes and MVs collectively, as endorsed by the International Society of Extracellular Vesicles (ISEV), \[117\] since there is an overlap in size between both subsets and there is still no consensus on distinct surface markers of each type.

Significant recent work has identified the protein and nucleic acid effectors of EV-mediated proangiogenic, anti-apoptotic, and anti-inflammatory actions (Figure 2), which are integral in facilitating the tissue healing cascade. Many studies attribute the EV-mediated therapeutic effects observed in preclinical studies to their nucleic acid contents. Interestingly, RNase treatment of EVs abrogated their effects on kidney recovery in a murine model of acute kidney injury, highlighting RNA content as the major therapeutic component. \[39\] Indeed, reduced fibrosis and enhanced tissue regeneration conferred by EVs in an acute kidney injury model was mediated by EV-shuttled HGF mRNA, \[118\] while miRNA cargo of EVs, such as miR-21 and miR-494, was suggested to promote myogenesis and tissue healing after skeletal muscle injury. \[119\] Apoptosis plays a role in tissue damage following injury such as ischemia \[120\] and MSC-EVs have demonstrated the ability to promote tissue regeneration by attenuating apoptosis in preclinical injury models. \[128,129\] In terms of identifying the EV components responsible, studies have shown that protein cargo transferred by EVs, such as 14-3-3ζ, has prosurvival

Figure 2. Functional MSC-EV cargo and MSC secreted soluble factors potentially mediating tissue repair. MSCs secrete soluble factors and EVs that promote tissue regeneration through a coordinated mobilization of various cell types and triggering of numerous cellular processes. Nucleic acid cargo, and luminal and membrane protein contents of EVs, as well as secreted soluble proteins, are categorized for clarity into four panels according to their identified roles as anti-apoptosis agents, promoters of cell migration and proliferation, proangiogenic molecules, and as anti-inflammatory agents (each type of biological activity is also depicted in the relevant panel). Together these activities induced by the MSC secretome act in concert to effect tissue healing. Created with BioRender.com.
effects,\cite{123} while IL-10 mRNA shuttled in EVs also rescued cell viability.\cite{124} The miRNA cargo of EVs in particular exhibits pro-survival effects, as exemplified by knockout of Argonaute-2, a key miRNA effector molecule, which abrogated EV-mediated nerve repair in a rat optic nerve crush model.\cite{125} miR-21 has been identified as an important EV component that promotes cell survival in vitro\cite{126} and in a rat MI model\cite{127} through activation of the PI3K/Akt pathway. miR-let7-5-p shuttled in EVs also contributes to promoting cell survival.\cite{128} Endogenous cell migration and proliferation at the site of injury are integral steps in the tissue healing cascade. EVs promoted ischemic wound healing and the long noncoding (lnc) RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was responsible for EV-mediated increases in cell migration,\cite{24} while EV-shuttled Wnt5a and Wnt5b also mediated increased migration and proliferation.\cite{129} Several other MSC-EV shuttled proteins such as fibronectin,\cite{130} and CD73\cite{46} have been shown to promote cell proliferation by activating the AKT and ERK signal transduction pathways. The mechanisms by which cells package proteins into EVs is still being elucidated but it appears to be a regulated process since EVs are enriched for proteins compared to their parental cells.\cite{130} Interestingly, EV-shuttled insulin-like growth factor receptor (IGF-1R) mRNA is translated to the corresponding protein in recipient cells, thereby potentiating the cells to IGF-1 and enhancing proliferation.\cite{44}

The resolution of inflammation is vital in order for tissue healing to occur and MSC-EVs have demonstrated anti-inflammatory ability in various injury models including skeletal muscle injury,\cite{119} cutaneous wound healing,\cite{133,134} and MI.\cite{121} A comprehensive characterization of the protein cargo of MSC-EVs found that angiogenic signaling proteins feature prominently,\cite{117} while miR-30b\cite{138} and miR-125a\cite{139} are also important EV cargo responsible for EV-mediated angiogenesis in vitro and in vivo.

4. Enhancing the Therapeutic Efficacy of the MSC Secretome

While MSCs secrete many therapeutic entities, several strategies have been explored to increase the potency of the secretome by altering culture conditions or by genetically modifying MSCs to overexpress a desirable gene (Figure 3).

4.1. Impact of Culture Conditions

Cell culture is usually performed under atmospheric oxygen levels (normoxia 21% O₂); however, this does not mimic...
the low oxygen conditions of the MSC niche or most tissues in the body. Indeed, oxygen tension varies from 1% to 6% in the bone marrow, and 4% to 14% in the heart and peripheral blood (reviewed in Ref. [140]). Hypoxia is one of the most prevalent elements of tissue injury and is a common environmental feature of transplanted MSCs.[141] Hypoxia preconditioning has been found to enhance the healing effects of transplanted MSCs,[142] and hypoxia culture of MSCs induces enhanced secretion of therapeutic soluble factors such as VEGF, FGF2, IGF, HGF, TGF-β, IL-1, -6, -8, Angiopoietin, Oncostatin M, thrombopoietin, PDGF-BB, Leptin, and Neurotrophin-3, -4, as compared to normoxia.[22,23,25,32,142–146] Hypoxia-derived CM promotes proliferation, migration, and tube formation of endothelial cells in vitro.[143,144] and enhanced skin wound healing.[144] restoration of neurological function,[146] and liver regeneration[142,147] compared to normoxia-derived CM. The oxygen level of the cultivation environment also affects MSC-EV cargo and therapeutic potential. MSCs secrete greater quantities of EVs under hypoxia conditions,[148] enhancing angiogenic gene expression, migration, and vascular network formation in vitro.[149,150] Several studies have investigated the underlying mechanisms by which hypoxia-generated EVs confer enhanced regenerative potential. Increased angiogenic EV protein cargo, including FGF2, PDGF, and epidermal growth factor (EGF) was found in EVs secreted from MSCs exposed to ischemic mimicking conditions, with nuclear factor-kappaB (NFκB) signaling identified as the main mediator of EV-induced angiogenesis.[137] miR-210 was also highly implicated in hypoxia EV-mediated proangiogenic effects.[148] EVs generated in hypoxia culture promote repair after MI in preclinical models by transporting miR-26a that targets the anti-angiogenic factor GSK3β,[131] as well as miR-125b-5p[152] and miR-223[153] which act by inhibiting cardiomyocyte apoptosis.

The physical environment of MSCs can also modulate their secretory profile. MSC-CM derived from 3D cell spheroids comprises elevated quantities of anti-inflammatory, anti-apoptotic, and proangiogenic factors such as TSG-6 and stanniocalcin-1 (STC-1).[154] VEGF, FGF2, HGF, and Angiogenin (ANG).[155–157] This MSC spheroid-derived CM leads to enhanced endothelial cell proliferation, migration, and tube formation[155,157] and improved functional recovery in a mouse ischemic model compared to CM derived from monolayers.[156] 3D spheroid-derived CM also confers an anti-inflammatory effect to macrophages, with PGE2 identified as the effector CM molecule.[158] Biomaterial constructs also permit 3D MSC culture, which also regulates their paracrine signaling in terms of soluble secretions.[159,160] and the generation of EVs with enhanced therapeutic potency in a rat model of traumatic brain injury, with enhanced angiogenesis, neurogenesis, and immunomodulation mediating functional recovery.[161] The stiffness of the culture adhesion substrate can also influence the secretion of paracrine factors by MSCs[162–164] via a reactive oxygen species-dependent mechanism.[165] Interestingly, MSC-CM generated from culture on soft substrates demonstrates enhanced wound healing capacity.[163] The surface topography of substrates has also been observed to significantly impact the secretion of soluble factors from MSCs.[70] Another physical stimulus, mechanical stretch, has been observed to affect the secretion of soluble factors by MSCs, whereby secretion of cytokines such as VEGF,[165] IL-6, and IL-8[166] were significantly promoted when MSCs were subjected to cyclic tensile strain. However, others observed no difference in cytokine secretion by MSCs undergoing mechanical stretch.[167] Finally, shear stress can induce secretion of immunomodulatory factors such as PGE2, with the NFκB-COX2 signaling axis mediating these effects.[168] Furthermore, dynamically loaded 3D MSC spheroids secrete more EVs compared to static culture or those in monoculture.[169]

Priming MSCs with various biologics or chemicals in culture can also alter MSC secretion of therapeutic factors. Priming MSCs with FGF-2 or PDGF upregulates the secretion of HGF and VEGF,[170] and also increases the yield of EVs and EV-mediated angiogenesis in vitro and in vivo.[171] Similarly, MSCs cultured with nitric oxide (NO)-releasing chitosan scaffolds secreted EVs with enhanced angiogenic abilities, and increased VEGF and miRNA-126 levels in EVs were identified as the primary contributing factors.[172] Erythropoietin exposure led to increased secretion of MSC-EVs, EVs enriched in miRNA such as miR-299, miR-499, miR-302, and miRNA-200, and led to a more potent anti-apoptotic ability.[173] Recently, MSCs treated with hydrogen peroxide were found to produce EVs enriched in miR-21[174] known for its anti-apoptotic abilities.[126,127] MSCs production of immunomodulatory factors is highly responsive to inflammatory stimuli. Interferon gamma (IFN-γ) priming increases secretion of soluble factors such as HGF and TBF-β,[69] as well as indoleamine 2,3-dioxygenase which promotes the differentiation of M2 immunosuppressive macrophages,[175] thereby favoring tissue healing. MSC-CM generated from MSCs primed with tumor necrosis factor-α (TNF-α) had elevated levels of IL-6 and IL-8, which led to increased cutaneous wound healing compared to unstimulated MSC-CM.[67] and increased levels of TSG-6, which mediated healing in a murine MI model.[71] EVs secreted by MSCs primed with TNF-α and IFN-γ have enhanced anti-inflammatory capacity[176] and can promote proliferation and osteogenic differentiation of human osteoblasts.[177] Further, priming MSCs with IL-1 generated CM with elevated levels of G-CSF and anti-inflammatory function,[178] and yielded EVs with more potent therapeutic capacity against sepsis compared with EVs from nonprimed MSCs.[179] Engaging Toll-like receptors (TLR) expressed by MSCs provides another approach to alter their secretome. MSCs cultured with a TLR3 agonist secreted elevated levels of factors such as IL-6 and IL-11,[180] while MSCs stimulated with a TLR 4 agonist (LPS) promoted secretion of VEGF, FGF2, IGF-1, and HGF.[145] LPS stimulation of MSCs also led to secretion of EVs with enhanced anti-inflammatory and regenerative capacity via exosome-shuttled miR-let-7b.[181] Preconditioning MSCs with macrophage-activating lipopeptide of 2 kDa (MALP-2), a TLR 2/6 agonist, led to a proangiogenic secretome with increased levels of VEGF, GM-CSF, MCP-3/4, and PECAM-1.[182]

Future investigations into the underlying mechanisms by which MSCs increase secretion of soluble factors and EVs as a consequence of various culture stimuli may yield important insights that might permit further manipulation of the MSC secretome.

### 4.2. Engineered MSCs

MSCs have been genetically modified in order to stimulate release of soluble or vesicular secretions with potent prosurvival,
proangiogenic, and anti-inflammatory properties. CM from MSCs transduced to overexpress the survival gene Akt1 inhibited apoptosis of cardiomyocytes in vitro and demonstrated an anti-apoptotic effect in vivo leading to reduced infarcted tissue after MI in rats. More recently, EVs derived from Akt-over-expressing MSCs demonstrated enhanced angiogenesis and cardiac regeneration via delivery of PDGF-D. CXCR4-over-expressing MSCs secreted EVs that enhanced proliferation of cardiomyocytes in vitro and increased angiogenesis and prevented apoptosis in a rat MI model, while GATA-4 over-expressing MSCs secreted EVs enriched with miR-19 that mediated cardiomyocyte survival and cardiac repair. miR-126 is considered a chief regulator of angiogenesis and MSCs over-expressing miR-126 improved angiogenesis in the ischemic myocardium and secreted EVs that significantly enhanced angiogenesis in vitro compared to EVs from naïve MSCs. Genetic modification of MSCs can also upregulate therapeutic secretions that promote tissue repair in various other injury models. Skin wound healing and hair follicle regeneration were enhanced by CM from MSCs transduced to overexpress Wnt7a and Nanog, and miR-181c-overexpressing MSC-EVs reduced inflammation in a rat burn model. Additionally, EVs from miR-133b overexpressing MSCs showed an anti-apoptotic and neuroprotective ability in an intracerebral hemorrhage rat model. Hypoxia inducible factor (HIF)-1α overexpression yielded MSC-CM that enhanced endothelial cell migration, while HIF-1α MSC-EVs enhanced angiogenesis in vitro and in vivo, mediated by EV-shuttled Jagged 1 and promoted bone regeneration in a rabbit model of avascular necrosis of the femoral head. Cartilage tissue regeneration in an osteoarthritis rat model was promoted by delivery of EVs secreted from miR-140-5p-overexpressing MSCs that enhanced cell migration and proliferation. miRNAs packaged within EVs by genetic manipulation were observed to inhibit fibrosis in several indications. miR-let7c overexpression in MSCs generates EVs that transfer miR-let7c to damaged kidney cells and inhibit renal fibrosis. EVs from MSCs overexpressing miR-122, an abundant anti-inflammatory liver-specific miRNA, demonstrated an anti-fibrotic effect in hepatic stellate cells in vitro, while EVs from miR-181-5p overexpressing MSCs prevented liver fibrosis via autophagy activation.

5. Biomaterials Functionalized with MSC Secretome

Biomaterials in tissue regeneration execute many roles, including delivery of biologics and providing structural support for infiltration of endogenous cells. They must fulfill many criteria, including biocompatibility, degradability, and favorable mechanical properties and architecture. Biomaterials can be divided into naturally derived, synthetic, or chemically modified naturally derived materials. Natural biomaterials employed in this field include alginate, collagen, hyaluronan, and decellularized extracellular matrix (ECM). While they often provide biomimetic environments and specific cell-binding sites, inherent variability arises according to biological source. Synthetic polymers or ceramics such as polylactide-co-glycolide (PLGA) or beta-tricalcium phosphate (β-TCP) are commonly used biomaterial scaffolds, while gelatin methacrylate (GelMA) represents a natural material with chemical modifications. Advantages of synthetic materials include cost, supply, and batch-to-batch homogeneity. However, they often lack native tissue topography and structure, although they can be chemically modified to have bioactivity (e.g., with tripeptide Arg-Gly-Asp (RGD)). Hybrid hydrogels comprised of both natural and synthetic materials have also been utilized to exploit the biological moieties of natural materials, while benefiting from the advantages of tunable synthetic materials.

Biomaterials may allow one to overcome the low tissue retention of bolus EV and MSC-CM injections and offer a controlled release platform for healing tissues (Figure 4). The stability and retention of EVs following delivery in vivo is recognized as a major hurdle to their clinical translation, as they are cleared rapidly after systemic delivery by the innate immune system in vivo. These challenges echo those observed with delivery of growth factor (GF) therapies, and biomaterial carriers have been used extensively to deliver GFs to overcome their rapid clearance rate and localize their activity. Similar to GF therapies, biomaterial delivery vehicles may also improve retention and optimize therapeutic performance of MSC-CM and EVs. Biomaterials utilized to deliver MSC-CM from various tissue origins have demonstrated healing potential in a myriad of injury indications. For example, in preclinical models, MSC-CM distributed within a hyaluronic acid (HA) hydrogel enhanced tissue regeneration after endometrial injury, while a hydrogel composed of HA and chondroitin sulfate incorporating MSC-CM achieved corneal wound healing. Additionally, injection of MSC-CM encapsulated in a platelet-rich plasma hydrogel ameliorated renal healing and function in an acute kidney injury model, while MSC-CM delivered in synthetic PLGA nanoparticles coated with red blood cell membranes enhanced liver regeneration. Several studies have demonstrated the potential of biomaterial-based delivery of MSC-EVs for repair in indications such as peripheral nerve injury, epidural fibrosis, and incisional hernia (Table 2). For example, EV-functionalized polyethylene glycol hydrogels significantly enhanced hepatic regeneration in a chronic liver failure model compared to bolus EV injections by attenuating inflammation, apoptosis, and fibrosis. In this section, we present developments to date of acellular biomaterials functionalized with MSC-CM or MSC-EVs targeting in situ repair. It must be noted that it is expected that bulk MSC-CM contains various EV subsets, even if certain studies focus only on the functional effects of the soluble secretions within MSC-CM. The most common target organ systems for CM- and EV-functionalized biomaterials are presented here as representative examples.

5.1. Cardiovascular

MSC-secreted soluble and vesicular fractions have shown promise in cardiovascular regeneration, and recently biomaterials have demonstrated the ability to effectively deliver MSC-CM and EVs for repair in preclinical models of these
diseases. For instance, the 2D synthetic nanoclay laponite, which permits absorption of growth factors through electrostatic interactions, was mixed with GelMA and MSC-CM to form a nanocomposite hydrogel that enhanced angiogenesis and inhibited apoptosis in vitro.[215] This biomaterial/CM combination was subsequently evaluated in an MI rat model and found to enhance angiogenesis and reduce infarct area.[155] MSC-CM encapsulated in synthetic micro-particles cloaked with MSC cell membrane achieved heart regeneration in a mouse MI model.[216] EV-laden peptide hydrogels showed superior therapeutic performance compared to bolus EV injection in a rat MI model, in terms of increasing angiogenesis, and reducing fibrosis, apoptosis, and inflammation.[57] Similarly, intramyocardially injected EVs entrapped in an alginate hydrogel showed improved retention in the heart and enhanced reparative potency compared with bolus EV injections in a rat MI model.[108] Finally, in a rat model of hyperlipidemia, MSC-EVs loaded onto a PCL vascular graft promoted vascular regeneration, likely by immunomodulation as M1 to M2 macrophage polarization was observed both in vitro and in vivo.[217] 

5.2. Skeletal

Significant data suggest that MSC-secreted factors may be an alternative acellular therapy to MSC transplantation for the regeneration of bone defects. MSC-CM reduced oxidative stress of aged bone marrow mesenchymal stromal cells (BMSCs) in vitro and reversed age-related osteoporotic bone loss when delivered encapsulated in silk fibroin hydrogels.[31] MSC-CM administered in a collagen sponge reduced inflammation (TNF-α) and enhanced regeneration in a dose-dependent manner in periodontal defects in rats[218] and in dogs,[64] while also promoting bone healing in rat calvaria defects.[32] Interestingly, bone regeneration by MSC-CM delivered in an agarose gel was superior to that following transplantation of MSCs in the same carrier in calvaria defects.[31] Similarly, CM delivered in human blood plasma hydrogels achieved comparable healing to that of direct stem cell therapy in rabbit mandibular bone defects.[31] Enhanced recruitment of endogenous MSCs to the injury site by delivery of MSC-CM laden biomaterials was demonstrated and suggested to play an integral role in ultimate bone healing.[31,79] The enhanced secretion of tropic factors in

Figure 4. EV-functionalized biomaterials. EVs can be immobilized in biomaterials via crosslinking or by EV-binding sites, and administered to injured tissue in order to induce healing and regain function. Natural ECM biomaterials such as collagen and hyaluronan allow binding of EVs via integrins and CD44 receptors, respectively, while the porosity and degradation rates of synthetic biomaterials can be tightly tuned to control EV release. Biomaterials typically permit higher EV retention rates and improved regenerative effects compared with bolus EV delivery. Created with BioRender.com.
| Injury (species)                    | MSC-CM source | CM collection/processing                                                                 | Biomaterials                      | In vitro/In vivo observations                                                                 | Ref. |
|----------------------------------|---------------|------------------------------------------------------------------------------------------|----------------------------------|------------------------------------------------------------------------------------------------|------|
| Corneal wounds (rat)             | hBMSC         | Serum-free media, debris cleared by centrifugation, frozen (liquid N₂), lyophilized      | Hyaluronic acid (HA) and chondroitin sulfate (CS) hydrogel | In vitro, MSC-CM embedded in HA/CS enhanced proliferation of corneal epithelial cells. In vivo, CM in HA/CS hydrogels promoted wound healing in corneas following either mechanical injury or after alkaline burns, to a greater extent compared with either MSC-CM or HA/CS alone, in part mediated by CD44 receptor upregulation and stimulation by HA gel | [208]|
| Volumetric muscle loss (rat)     | rASC          | Serum-free media, debris cleared by centrifugation, concentrated 10x, filtered (0.22 µm), frozen (–80 °C) | Type I collagen hydrogel         | In vitro, mouse M2 macrophage-CM enhanced migration and angiogenesis of HUVEC. In vivo, MSC-CM-embedded hydrogel promoted repair in muscle defects, by enhanced angiogenesis and myogenesis, and decreased inflammation (M2 macrophage polarization) compared with the hydrogel alone. MSC-embedded gels showed superior healing to MSC-CM gels | [35] |
| Endometrial injury (rat)         | hBMSC         | Serum-free media, debris cleared by centrifugation, filtered (0.22 µm), frozen (–80 °C), lyophilized | HA hydrogel                      | In vitro, MSC-CM enhanced tube formation and proliferation of HUVEC, growth of human endometrial epithelial cell line AN3CA, and migration of endometrial epithelial cells. In vivo, HA gel allowed sustained release of CM components and enhanced endometrium healing | [21] |
| Skin wounds (mouse)             | hSDMSC        | Serum-free media, filtered (0.22 µm), concentrated (10 kDa), frozen (–80 °C)              | Carrageenan (CG) or poly(vinyl alcohol) (PVA) hydrogels | In vitro, MSC-CM promoted tube formation by HUVEC. In vivo, MSC-CM gels enhanced angiogenesis compared to untreated wounds, but was not different to MSC-CM without carrier. MSC-CM w/o hydrogels did not improve wound closures | [234]|
| Skin wounds (rat)               | hUCMSC        | Serum-free media, debris cleared by centrifugation, filtered (0.22 µm), lyophilized, stored frozen (–80 °C) | Hydrogel—composition not provided | In vitro, MSC-CM promoted proliferation of HUVEC. In vivo, MSC-CM-laden hydrogel enhanced angiogenesis, proliferation, wound healing, and reduced scar formation in radiation-induced skin wounds, with superior performance when compared with an EGF-laden hydrogel positive control | [233]|
| Renal ischemia (rat)             | hPSC          | Serum-free media, filtered (0.22 µm), concentrated 10x (2 kDa), frozen (–80 °C)            | Porcine platelet-rich plasma (PRP) and thrombin | In vitro, MSC-CM inhibited apoptosis and promoted proliferation in human primary renal cells and HUVEC. In vivo, injection of MSC-CM encapsulated in PRP gel ameliorated renal function and cell viability after acute kidney injury, to a greater extent compared to MSC-CM or PRP alone | [209]|
| Myocardial infarction (rat)      | hASC          | Serum-free media, debris cleared by centrifugation, then lyophilized                     | Laponite/gelatin hydrogel        | In vitro, hASC spheroid CM exhibited enhanced proliferation, migration, and tube formation of HUVEC. In vivo, spheroid CM-laden hydrogel reduced infarct area and enhanced angiogenesis | [155]|
| Myocardial infarction (mouse)    | hBMSC         | Serum-free media, debris cleared by centrifugation, then lyophilized                     | Polylactic-co-glycolic acid (PLGA) microparticles | In vitro, MSC-CM encapsulated in PLGA microparticles cloaked in MSC membrane fragments increased cardiomyocyte proliferation and contractility. In vivo, injection of CM-containing microparticles directly into the heart after MI reduced infarct area and enhanced angiogenesis | [216]|
| Acute liver failure (mouse)      | hBMSC         | Conditioned media filtered (0.22 µm), frozen (–80 °C), and lyophilized                   | PLGA nanoparticles (NPs)          | In vitro, MSC-CM in PLGA NPs enhanced proliferation of liver cells. Uptake of NPs by M1 macrophages was inhibited by cloaking NPs with red blood cell (RBC) membrane fragments. In vivo, RBC fragment-cloaked NPs containing MSC-CM enhanced liver cell proliferation and inhibited cell apoptosis, thereby promoting liver regeneration and enhancing survival in a carbon tetrachloride-induced liver failure mouse model | [210]|

Table 1. MSC-CM delivered via biomaterial scaffolds for tissue repair in representative examples in various injury models.
| Injury (species) | MSC-CM source | CM collection/processing | Biomaterials | In vitro/In vivo observations | Ref. |
|-----------------|----------------|---------------------------|--------------|-----------------------------|------|
| Periodontal defects (rat) | hPDLSC          | Serum-free media, cleared by centrifugation, filtered (0.22 µm), concentrated (10 kDa), stored (4 °C) | Collagen sponge and fibrin glue (BOLHEA) | In vitro, MSC-CM sponge inhibited IFN-γ-induced TNF-α expression in RAW264.7 murine macrophages. In vivo, MSC-CM enhanced regeneration of periodontal defects in a dose-dependent manner while suppressing the inflammatory response (TNF-α expression) | [218] |
| Bone defects (rat) | rBMSC           | Serum-free media, debris cleared by centrifugation | Polyactide-co-glycolide (PLGA) membrane | In vitro, CM-loaded PLGA increased proliferation and osteogenic differentiation (ALP) of rBMSCs. In vivo, CM/PLGA implants enhanced healing in calvaria bone defects | [220] |
| Bone defects (rat) | rBMSC           | Hypoxia, serum-free media, debris cleared by centrifugation | Fibrinogen/thrombin gel (GREENPLAST) | In vitro, hypoxia-CM reduced miR-221 expression in rBMSCs, which led to enhanced ICAM-1 expression and cell migration compared to normoxia-derived CM. In vivo, hypoxia-CM encapsulated gel promoted bone regeneration in calvaria defects by stimulation of endogenous MSCs | [219] |
| Osteoporosis (rat) | hUCMSC          | Serum-free media, debris cleared by centrifugation, filtered (0.22 µm), lyophilized, frozen (−80 °C) | Silk fibroin hydrogels | In vitro, MSC-CM promoted rBMSCs proliferation in a dose-dependent manner, and reduced senescence biomarkers and oxidative stress of aged rBMSCs, while promoting osteogenic, over adipogenic differentiation. In vivo, MSC-CM encapsulated silk hydrogels injected into the bone marrow of aged rats significantly increased bone mass | [51] |
| Periodontal defects (dog) | hMSC            | Serum-free media, stored fresh (4 °C), or frozen (−80 °C) | Atelo-collagen sponge (TERUPLUG) | In vitro, MSC-CM promoted migration and proliferation of dog BMSCs and periodontal ligament cells. In vivo, MSC-CM laden biomaterial enhanced bone regeneration in a one-wall intrabony periodontal defect | [64] |
| Periodontal defects (rat) | hMSC            | Serum-free media, stored fresh (4 °C), or frozen (−80 °C) | Atelo-collagen sponge (TERUDERMIS) | In vitro, MSC-CM enhanced migration and proliferation of rBMSCs and rat periodontal ligament cells, and tube formation by HUVECs. In vivo, MSC-CM laden biomaterial enhanced bone regeneration, angiogenesis, and mobilization of endogenous MSCs to the periodontal defect | [79] |
| Bone defects (rat) | hMSC            | Serum-free media, stored fresh (4 °C), or frozen (−80 °C) | Atelo-collagen sponge (TERUDERMIS) | In vitro, MSC-CM enhanced migration and osteogenic gene expression of rBMSCs. In vivo, MSC-CM entrapped sponge-enhanced bone regeneration in critical-sized calvaria defects | [80] |
| Maxillary sinus cavities (rabbit) | hMSC           | Serum-free media, stored fresh (4 °C), or frozen (−80 °C) | Beta-tricalcium phosphate (β-TCP) particles (OSferion) | In vitro, MSC-CM enhanced migration and proliferation of rabbit BMSCs. In vivo, MSC-CM-soaked biomaterial led to earlier angiogenesis and bone regeneration in a sinus floor elevation model but no overall increase in bone formation after 8 weeks | [221] |
| Mandibular bone defects (rabbit) | hASC            | Hypoxia, serum-free media, debris cleared by centrifugation, filtered (0.22 µm), frozen (−20 °C) | Human blood plasma hydrogels | In vitro, hypoxia-CM comprised significantly higher concentrations of angiogenic and osteogenic soluble secretions compared to normoxia CM. In vivo, MSC-CM loaded hydrogel, and likewise ASC transplantation, increased bone formation in defects compared with hydrogel controls | [32] |
| Bone defects (rat) | hBMSC           | Serum-free media, stored fresh (4 °C), or frozen (−80 °C) | Agarose hydrogel | In vitro, MSC-CM promoted rBMSCs proliferation, migration, and osteogenic gene expression. In vivo, MSC-CM-loaded gel promoted healing in calvaria defects more than MSC transplantation. MSC-CM mobilized rBMSCs (administered via the caudal vein) to the defect site | [31] |
Table 2. MSC-EVs delivered via biomaterial scaffolds for tissue repair in representative examples in various injury models.

| Indication                  | EV source | EV isolation and tracking methods | Biomaterial                          | In vitro/in vivo observations                                                                 | Ref. |
|-----------------------------|-----------|----------------------------------|--------------------------------------|-------------------------------------------------------------------------------------------------|------|
| Hyperlipidemia (rat)        | hPMSC     | Luminescent EVs from Gluc-lactadherin MSCs. Isolated by UC. Labeled with CM-DiI | Electrospun poly (ε caprolactone) (PCL) | In vitro, DiI-labeled EVs were taken up by macrophages and induced M1 to M2 polarization and increased anti-inflammatory gene expression. In vivo, EV-loaded PCL vascular graft (100 µg EVs) implanted into an abdominal artery in a hyperlipidemia model inhibited thrombosis and calcification, increased vascular regeneration and the switch from M1 to M2 macrophage. | [217]|
| Myocardial infarction (rat) | hUCMSC    | EVs isolated by differential centrifugation, labeled with PKH26 dye | GHRPS (His-d-2-methyl-Trp-Ala-Trp-d-Phe-Lys) added to the N-termini of peptide amphiphile, mixed with NapFF | In vitro, EVs protected the rat cardiomyocyte cell line H9C2 from oxidative stress induced by H2O2. In vivo, EV-laden hydrogel (×2 injections of 20 µg EVs/20 µL gel) improved myocardial function by increasing angiogenesis, reducing fibrosis, apoptosis, and inflammation in an MI model. | [57]|
| Myocardial infarction (rat) | rBMSC     | EVs isolated by UC and labeled with DiR dye | Alginate hydrogel | Intramyocardial injection of EVs (80 µg/100 µL gel) enhanced EV retention, repair, and functional outcome in the heart compared to bolus EV injection, by inhibiting apoptosis, promoting anti-inflammatory macrophage phenotype, and enhancing angiogenesis. | [202]|
| Epidural fibrosis (rabbit) | hUCMSC    | EVs isolated by UC | Bacterial cellulose membrane | In vitro, EV-functionalized membrane showed biocompatibility by using L929 fibroblasts. In vivo, EV-functionalized membrane inhibited epideral fibrosis and peridural adhesions post-laminectomy. | [213]|
| Peripheral nerve injury (rat) | hGMSC     | Quick exosome isolation kit (Hieff™) | Chitin conduits | In vivo, EVs promoted Schwann cell proliferation and dorsal root ganglion neurite outgrowth. In vivo, EV-laden chitin conduit (10µg EVs/conduit) regenerated nerve fibers, and restored muscle and motor function of rats with severed sciatic nerve. | [212]|
| Incisional hernia (mouse)   | mBMSC     | Differential centrifugation and ultrafiltration (3 kDa) | Fibrin glue (fibrinogen/thrombin). Polypropylene surgical meshes | In vivo, fixation of surgical meshes with EV in a fibrin glue (2000 µg EVs/400 µL glue) for the treatment of incisional hernia showed healing and immunomodulation by expression of genes for anti-inflammatory cytokines and increased presence of anti-inflammatory M2 macrophage phenotype, to a greater extent than MSC transplantation. | [214]|
| Chronic liver failure (rat) | ESMC      | EVs isolated by UC and labeled with PKH26 | Clickable polyethylene glycol (PEG): thiol and maleimide derivatives of PEG formed a hydrogel by a click reaction | During injection, PEG macromers were mixed with MSC-EVs to form EV-encapsulated PEG hydrogels that gradually released EVs in a constant manner and reduced apoptosis and inflammation and ameliorated tissue damage, to a significantly greater extent compared to bolus EV injection. | [59]|

h: human, m: mouse, r: rat, BMSC: bone marrow mesenchymal stromal cell, ASC: adipose tissue-derived stromal cells, SDMSC: skin-derived MSC, UCMSC: umbilical cord MSC, PDLSC: periodontal ligament MSC, HUVEC: human umbilical vein endothelial cell, PSC: placental stem cell, TNF-α: tumor necrosis factor-α, IFN-γ: Interferon gamma.
### Table 2. Continued.

| Indication                          | EV source                  | EV isolation and tracking methods                                           | Biomaterial                       | In vitro/in vivo observations                                                                                     | Ref.  |
|------------------------------------|----------------------------|-----------------------------------------------------------------------------|-----------------------------------|------------------------------------------------------------------------------------------------------------------|-------|
| Diabetic skin wound (rat)          | miR-126 over-expressing hSMSC | Ultrafiltration and UC on a sucrose cushion, labeled with DiI fluorescent dye | Chitosan hydrogel                 | In vitro, miR-126 EVs significantly enhanced proliferation, migration, and tube formation of human dermal microvascular endothelial cells compared to EVs from nonmodified MSCs. In vivo, miR-126 EVs promoted healing and angiogenesis in skin wounds when delivered in chitosan hydrogels | [190] |
| Diabetic skin wound (mouse)        | mASC                       | EVs isolated by UC                                                          | Pluronic F127, oxidized hyaluronic acid, and poly-ε-lysine hydrogel | In vitro, EV-encapsulated hydrogel promoted proliferation, migration, and tube formation ability of HUVEC. In vivo, EV-hydrogel (10 μg EVs/wound) reduced wound healing duration and enhanced angiogenesis in wounds | [58]  |
| Diabetic skin wound (rats)         | hGMSC                      | Size exclusion chromatography after ultrafiltration, labeled with Dio fluorescent dye | Chitosan/silk hydrogel            | In vivo, EV-incorporated hydrogel (150 μg EVs/wound) combination promoted cutaneous wound healing, with increased angiogenesis, collagen deposition, and nerve fiber density in an STZ-induced diabetes model | [235] |
| Skin wound (mouse)                | hUCMSC                     | EVs isolated by UC and labeled with PKH67 fluorescent dye                   | HydroMatrix hydrogel (Sigma Aldrich) | In vitro, MSC-EVs inhibited TGF-β1-induced α-SMA expression and myofibroblast differentiation by transferring miRNAs that target the TGF-β1/SMAD2 pathway. In vivo, injection of 100 μg EVs/200 μL hydrogel around each wound prevents α-SMA expression and scar formation, which was mediated by miR-21, miR-23a, and miR-145 in EVs | [236] |
| Hindlimb ischemia (mouse)         | hPMSC                      | Luminescent EVs derived from Gluc-lactadherin MSCs, isolated by UC          | Chitosan hydrogel                 | In vitro, hydrogels showed sustained release of EVs and enhanced retention of EV cargo, increased EVs proliferative, anti-apoptotic, and angiogenic (gene expression, migration, tube formation) effects on HUVEC. In vivo, an intramuscular injection of EV-loaded hydrogel (100 μg EVs/60 μL gel) increased angiogenesis (BLI of Vegf2-luc mice expressing firefly luciferase under the promotor of Vegf2) and accelerated ischemic muscle recovery compared to either free EVs or hydrogel alone | [56]  |
| Hindlimb ischemia (mouse)         | mBMSC                      | Iodixanol density gradient UC, Dio fluorescent dye                          | Matrigel                          | In vitro, EVs increased endothelial cell proliferation, migration, tube formation, and VEGFR1 and VEGFR2 expression in a dose-dependent manner. In vivo, intramuscular injections (≥2 per week) of 100 μg EVs in 100 μL gel suspension increased vascularization in the ischemic limb, to a greater extent than EVs alone, by slow release of EVs from the hydrogel | [229] |
| Hindlimb ischemia (mouse)         | hUCMSC over-expressing miR-675 | EVs isolated by UC and labeled with PKH26 fluorescent dye                  | Silk fibroin hydrogel             | In vitro, miR-675 inhibits the aging process by targeting the TGF-β1/p21 signaling pathway. In vivo, miR-675 EVs encapsulated in a silk fibroin hydrogel reduced the expression of TGF-β1 and enhanced blood perfusion in ischemic hindlimbs | [231] |
| Bone defects (rats)                | hiPS-MSC                   | Ultrafiltration followed by UC on a sucrose cushion, EVs labeled with Vybrant Dio fluorescent dye | β-TCP                             | In vitro, MSC-EVs increased proliferation, migration, and osteogenic differentiation (ALP) of hBMSCs, through activation of the PI3K/Akt signaling pathway. In vivo, EV-loaded β-TCP scaffolds (either 1 × 10¹⁰ or 1 × 10¹¹ EVs/scaffold) significantly augmented bone regeneration in critical-sized calvaria defects in a dose-dependent manner | [222] |
Table 2. Continued.

| Indication                  | EV source | EV isolation and tracking methods | Biomaterial | In vitro/in vivo observations                                                                 | Ref. |
|-----------------------------|-----------|----------------------------------|-------------|------------------------------------------------------------------------------------------------|------|
| Bone defects (rats)         | hBMSC     | EVs isolated by UC and labeled with PKH67 fluorescent dye | HyStem-HP hydrogel (thiol-modified hyaluronan and thiol-modified heparin) | In vitro, MSC-EVs induced osteogenic differentiation of human osteoblasts. miR-196a, miR-27a, and miR-206 were enriched in EVs, with miR-196a having the most potent osteogenic effects. In vivo, EV-laden hydrogels (100 μg per defect) accelerated bone healing in critical-sized calvaria defects | [224] |
| Bone defects (mouse)        | hASC      | UC and labeled with PKH26 fluorescent dye | Poly(lactic-co-glycolic acid)/polydopamine (PLGA/pDA) | In vitro, EVs enhanced proliferation, migration, and osteogenic differentiation of hBMSCs. In vivo, EV-loaded scaffolds (250 μg EVs) promoted repair of a critical-sized calvaria defect and enhanced recruitment of endogenous MSCs | [204] |
| Femoral fracture (rat)      | hUCMSC    | UC and labeled with PKH67 fluorescent dye | HyStem-HP hydrogel (thiol-modified hyaluronan and thiol-modified heparin) | In vitro, EVs enhanced proliferation, migration, and tube formation of HUVEC; mediated by upregulation of HIF-1α and VEGF gene expression. In vivo, EVs (100 μg) mixed with hydrogels were injected near the fracture site and enhanced angiogenesis and promoted bone healing | [223] |
| Bone defects (rats)         | hASC over-expressing miR-375 | UC and labeled with PKH26 fluorescent dye | Thiol-modified hyaluronan, hydroxyapatite, and thiol-modified heparin | In vitro, miR-375 EVs increased osteogenic differentiation of BMSCs compared with naive MSC-EVs. In vivo, miR-375 EVs encapsulated in the hydrogel (50 μg mL⁻¹) enhanced bone formation in calvaria defects to a greater extent than naive MSC-EVs | [226] |
| Osteochondral defect (rabbit) | BMSC     | Ultrafiltration followed by UC on a sucrose cushion, EVs labeled with Vybrant DiO fluorescent dye | Porcine cartilage ECM/GeMA | In vitro, EVs attenuated human chondrocyte degeneration (induced by IL-1β), and rescued mitochondrial function after damage (by rotenone). In vivo, EV functionalized 3D-printed ECM/GeMA scaffold (200 μg EVs/200 μL gel) induced M1 to M2 macrophage polarization and cartilage regeneration in osteochondral defects. EVs did not regenerate subchondral bone | [205] |
| Articular cartilage defect (rabbit) | hiPS-MSCs | UC, then ultrafiltration, labeled with DiI fluorescent dye | Hydrogel composed of o-nitrobenzyl alcohol moieties-modified hyaluronic acid and gelatin | In vitro, chondrocytes and hBMSCs encapsulated in EV laden hydrogels maintain higher viability compared to cells encapsulated in gels without EVs. In vivo, EVs incorporated into hydrogel (5 × 10⁹ EVs per defect) integrated with native cartilage matrix and promoted superior healing in defects compared with injection of EVs without hydrogel | [227] |

h: human, m: mouse, r: rat, hPMSC: placenta-derived mesenchymal stromal cells (MSC), hUCMSC: umbilical cord MSC, BMSC: bone marrow MSC, GMSC: gingival MSC, ES-MSC: Embryonic stem cell-derived MSC, SMSC: synovium MSC, ASC: adipose tissue-derived MSC, HUVEC: human umbilical vein endothelial cell, GMSC: gingival MSC, PMSC: placenta-derived MSC, hiPS-MSC: induced pluripotent stem cell-derived MSCs.

Hyposxia culture described in the previous section appears to translate into therapeutic benefit when delivered within biomaterial constructs, whereby a fibrinogen/thrombin gel encapsulating hyposxia generated MSC-CM-increased bone healing compared to normoxia-generated CM in rat calvaria defects through stimulation of endogenous MSCs.[219] Synthetic biomaterials have also been utilized to deliver MSC-CM. A PLGA membrane with incorporated MSC-CM promoted modest, but significantly higher bone formation compared to biomaterial controls in rat calvaria defects[220] and β-TCP soaked with MSC-CM led to earlier angiogenesis and bone regeneration in a sinus floor elevation rabbit model, but no overall increase in bone formation at 8 weeks.[221] Recent research which employed EVs isolated from MSC-CM suggests therapeutic potential of EV-functionalized biomaterials for bone repair. EV-loaded β-TCP scaffolds promoted bone regeneration in critical-sized rat calvaria defects in a dose-dependent manner, with EV-mediated osteogenic differentiation mediated through activation of the PI3K/Akt signaling pathway.[222] A hyaluronan/heparin hydrogel loaded with MSC-EVs enhanced angiogenesis and promoted significant bone healing in a femoral fracture model, with EV-induced angiogenesis shown to be mediated by upregulation of HIF-1α and VEGF expression in endothelial cells.[223] In another study, MSC-EV laden hydrogels significantly accelerated bone healing in critical-sized calvaria defects in rats, with EV-shuttled miR-196a identified as having the most...
potent osteogenic effects in vitro.[224] EVs from osteogenically primed MSCs immobilized on a poly(lactic-co-glycolic acid)/polydopamine (PLGA/pDA) scaffold promoted the recruitment of endogenous MSCs and increased bone healing in a critical-sized mouse calvaria defect.[204] EVs from MSCs genetically modified to overexpress miR-375, previously shown to enhance the osteogenic differentiation of MSCs,[225] promoted bone healing in rat calvaria defects when loaded into hydrogels composed of thiol-modified hyaluronan.[226] Cartilage damage can occur due to trauma or wear and MSC secreted factors may serve as a potential regenerative tool. An EV-laden hydrogel glue integrated with native cartilage matrix after delivery and promoted superior healing in rabbit articular cartilage defects compared with injection of EVs without hydrogel.[227] Furthermore, EV-functionalized 3D-printed porcine cartilage ECM/GelMA scaffolds induced M1 to M2 macrophage polarization and cartilage regeneration in an osteochondral defect rabbit model, while no beneficial effects of adding EVs to these constructs was observed in subchondral bone regeneration.[205]

5.3. Muscular

Scar formation can occur after muscle injury caused by trauma or surgical interventions,[228] and the MSC secretome can potentially promote muscle regeneration. For example, in a model of volumetric muscle loss, MSC-CM delivered in a collagen hydrogel exhibited immunomodulatory activity and led to enhanced muscle regeneration.[65] Compared with MSC-EVs alone, EV-laden hydrogels attenuated fibrosis, showed superior angiogenesis, and enhanced structural and functional muscle healing after ischemic injury.[56] In another study, injection of Matrigel-encapsulated MSC-EVs, found to be enriched with VEGF and miR-210-3p, enhanced angiogenesis and muscle recovery in an ischemia model by activating VEGF receptors in endothelial cells.[229] Interestingly, delivery of miR-675, previously shown to promote muscle regeneration,[230] by EVs from miR-675 overexpressing MSCs encapsulated in a silk fibroin hydrogel prevented ischemia-induced vascular dysfunction in muscles by inhibiting the TGF-β1/p21 pathway.[231]

5.4. Integumentary

Cutaneous wounds resulting from burns or delayed healing, as in the case of diabetic ulcers,[232] can potentially be treated with MSC secreted factors. For instance, an MSC-CM-loaded hydrogel promoted angiogenesis and wound healing in a rat model of radiation-induced skin wounds, with superior performance in vitro and in vivo compared with an EGF-laden hydrogel positive control.[215] Another study observed that MSC-CM embedded in a natural seaweed-derived material, Carrageenan (CG), or a synthetic poly(vinyl alcohol) (PVA) polymer, enhanced angiogenesis in vitro and in vivo, although MSC-CM without a biomaterial carrier showed no difference from untreated controls.[214] MSC-EVs delivered in a chitosan/silk hydrogel[233] and in a self-healing, anti-bacterial polypeptide hydrogel[235] promoted cutaneous wound healing in diabetic rat models. One study demonstrated that an EV-laden hydrogel reduced myofibroblast accumulation and scar formation in skin wounds, and showed that EV-mediated inhibition of myofibroblast differentiation in vitro was facilitated by RNA and not protein EV cargo. EVs were enriched in miR-21, miR-23a, miR-125b, and miR-14 and blocking these abrogated the beneficial effects of MSC-EVs on reducing scar formation in vivo.[236] In a different study, chitosan hydrogels functionalized with EVs from MSCs transduced to over-express miR-126 promoted healing and angiogenesis in skin wounds of a Sprague Dawley diabetic rat model.[190]

6. Future Challenges for the Clinical Translation of MSC Secretome Delivery

To date, the clinical evaluation of MSC-CM and MSC-EVs is limited, but significant trials are required to establish the efficacy of this approach. Several clinical trials and case studies have addressed the potential of MSC-CM, and report safety and potential efficacy, including for multiple sclerosis,[38] skin wound healing,[237] hair follicle regeneration,[238] and psoriasis.[239] Another current trial is listed with the goal of evaluating MSC-CM for healing in chronic skin ulcers (phase 1, NCT04134676). While these clinical approaches are using bolus CM administration, one recent clinical report found that MSC-CM derived from allogenic BMSCs delivered via biomaterials successfully regenerated bone tissue in eight patients requiring bone augmentation prior to dental implant placement. CM incorporated into atelocollagen sponges led to faster resorption and denser bone matrix compared with CM-soaked β-TCP.[36] There are also a few clinical studies using MSC-EVs targeting regeneration, although none yet delivered via a biomaterial. Allogenic MSC-EVs used to treat chronic kidney disease were reported to be safe, well tolerated, and enhanced healing.[240] A further clinical trial (phase 1, NCT03437759) using MSC-EVs was launched to determine their potential for treating refractory macular holes, with preliminary results suggesting safety and efficacy of EV administration in these cases.[241] Finally, a trial has been listed whose goal is to assess the safety and efficacy of allogenic MSC-EVs enriched with miR-124 on functional improvements in patients with acute ischemic stroke (NCT03384433). While this is yet a very small cohort of clinical studies, they provide a strong rationale to further evaluate the therapeutic potential of MSC-EVs for clinical use when coupled with the growing preclinical evidence. However, there are many obstacles to overcome prior to clinical translation of the MSC-secreted EVs and soluble factors, including determining the optimal tissue source of MSCs, dosage, and route of administration, understanding the bioactive components and mechanisms of action, and achieving scalability and GMP-grade products.

6.1. Cell Source

Similar to what has been reported in regard to the effectiveness of MSCs, with some tissue sources preferable for certain indications (e.g., umbilical cord MSCs for immunomodulatory tasks[33] and BMSCs for bone regeneration[25]), the tissue...
of origin also impacts the effectiveness of the MSC secretome. First, there is a significant difference in the levels of soluble factors and EVs secreted by MSCs according to their tissue of origin\cite{243,242} and MSCs secrete EVs enriched in distinct miRNA and tRNA species\cite{243} and proteins\cite{243} according to their tissue of origin. It has been demonstrated that the degree to which EVs are taken up by cells and their bio-distribution in vivo vary greatly depending on the EV cell of origin.\cite{52} Tissue specificity of EVs in terms of the parent MSC tissue source, as well as the injured tissue targeted for healing, is an important aspect of MSC-EVs that needs to be fully investigated in order to deliver EVs with optimal regenerative effects. Passage number of MSCs is also important since it impacts the bioactivity of secreted EVs, with reduced angiogenic potential observed with increasing passage number.\cite{244} Additionally, EVs from cells obtained from aged animals exhibit altered miRNA profiles and functional effects in vivo compared with those from young animals.\cite{245} While this review focuses on the potential of MSC-EVs in regenerative medicine, it must be noted that the bulk secretome or isolated EVs derived from other cell sources such as cardiospheres, iPSC-derived cardiomyocytes, mononuclear cell fraction of the bone marrow, and endothelial progenitors have also shown therapeutic benefit in tissue regeneration when delivered via biomaterials scaffolds.

6.2. Obtaining Purified MSC Secretomes

Elucidating the bioactivity of the MSC secretome is vital for clinical translation, and requires reproducible and effective purification processes. However, given that the commonly employed isolation methods such as UC without a density gradient often lead to various nonvesicular contaminants, the isolation method must be considered when attributing EV effects. As such, in many cases it is not possible to exclude contributing contaminants to EV-associated functions. Furthermore, this confounds characterization of the protein content of EVs and their functional effects, and therefore it is essential to characterize clean EV preparations in order to accurately identify the roles of each factor. ISEV recommends using protease and RNase treated quality controls in order to confirm they are within EV cargo.\cite{117} It is unlikely that the mode of action of MSC trophic factors arises from a single protein or RNA, and it was recently observed that EVs and soluble molecules secreted by MSCs act synergistically to promote muscle regeneration.\cite{251} Cell culture medium is another important aspect to consider when determining the contents and functional effects of the EVs. Most published studies using fetal bovine serum (FBS) in the culture medium utilize prolonged UC to deplete the FBS of EVs; this technique was also reported to deplete human platelet lysates of EVs.\cite{225} However, a substantial quantity of exogenous EVs is reported to remain when using this technique, which then contaminate EVs secreted from cells in culture. The use of serum-free, chemically defined media may bypass this issue, but contaminating miRNA in serum-free media supplements was recently observed to co-purify with EVs.\cite{254} Advancements in EV isolation and separation techniques, such as asymmetric-flow field-flow fractionation (AF4), permit isolation of distinct EV populations,\cite{255} revealing unique protein, lipid, DNA, and RNA profiles in subsets of EVs, with each EV subset having different in vivo bio-distribution patterns.\cite{256}

6.3. Scalability, Dosing, and Targeting

A scalable and GMP-compliant manufacturing process is required for clinical translation of the MSC secretome. Scalability of EVs is a major challenge since the yield of EVs from MSCs is relatively low. As such, various alterations in cell source and culture methods have been implemented to overcome this hurdle. Immortalized cells, such as MSCs genetically modified to overexpress c-Myc could provide an unlimited supply of EVs.\cite{256} Currently, hollow fiber bioreactors have been shown to vastly increase the yield of cell-secreted EVs,\cite{257} while tangential flow filtration can concentrate large quantities of cell-conditioned media for EV enrichment.\cite{258} The method of EV isolation has important implications for the purity and functionality of EVs, as well as their scalability, as reviewed recently.\cite{107} With the advent of new technology, EV purification techniques that efficiently provide high EV yields will be possible. Determining the optimal dosage of the MSC secretome required to elicit a therapeutic response is imperative for each intended application since dose escalation studies have demonstrated that accurate dosing of MSC-CM is vital for adequate periodontal tissue healing,\cite{218} while adequate MSC-EV dosage was needed to achieve enhanced angiogenesis\cite{171} and bone regeneration.\cite{222} It is also relevant to note that a significant enhancement in survival rates was found with a multiple dose EV regime, as contrasted to a single dose in a murine model of acute kidney injury.\cite{48} However, the enhanced retention rates and controlled release of EVs by biomaterial carriers may obviate the need for multiple doses.\cite{96-98} Uptake of EVs by cells is facilitated by ligand-receptor coupling and surface lipids, and in addition to the natural components of EVs such as integrins and tetraspanins (Figure 4), EVs can be modified with artificial surface components to enhance cell targeting and uptake, such as engineering the parent cells to express specific surface fusion proteins.\cite{259} Glycosylphosphatidylinositol-anchored targeting moieties,\cite{260} or the addition of targeting moieties fused via the phosphatidylinerse-binding C1C2 domains of lactadherin.\cite{261} Further to this, EVs can be modified to contain certain cargo that permits targeting to desired locations, e.g., EVs loaded with iron oxide nanoparticles that can be manipulated and spatially controlled under a magnetic field.\cite{262}

6.4. Biomaterial Design Considerations for MSC Secretome Administration

Ideally, controlled release of the MSC secretome from functionalized biomaterials will aid in recruitment of endogenous stem or immune cells, provide a mechanically and chemically favorable environment for regeneration, and promote tissue revascularization, all the while degrading and being replaced by newly formed tissue. Biomaterial characteristics such as chemistry, pore size, and degradability must be optimized to control the retention and release profile of EVs from the biomaterial carrier. For instance, nanoporosity is typically required to retain EVs, but
macroporosity may be desirable for cell infiltration and angiogenesis. Synthetic biomaterials offer several advantages in this regard since they can be fabricated to have tunable architecture at multiple size scales, and variable degradation rates. Tunable release of the MSC secretome can also be achieved by employing materials that permit binding of growth factors and EVs. Many decellularized ECM-derived hydrogels retain the ability to bind growth factors via sulfated glycosaminoglycans and allow for prolonged delivery and enhanced performance. Further-

more, EVs bind to fibronectin and collagen via integrins, and to hyaluronic via CD44, thereby giving these natural ECM-derived biomaterials advantages for EV retention. In addition, EVs are negatively charged entities, and hence this could also be exploited to control their immobilization within biomaterials. The mode of delivery is also an important biomaterial design consideration. Injectable hydrogels offer the advantage of minimally invasive administration and could exploit advances in catheter technology for targeted delivery. However, implantable biomaterials could be more appropriate in indications requiring significant structural support. Since systemically injected EVs are cleared rapidly from the blood circulation and are distributed in the kidney, spleen, liver, lungs, and to a much lower extent in the heart, brain, and muscle, local delivery of EVs directly to the site of required regeneration would likely be optimal in many situations. Targeted delivery of the MSC secretome to desired tissues and organs, such as the heart example, in a minimally invasive manner presents a challenge. Recent advancements in catheter technology for local delivery of hydrogels to the heart could be exploited in order to overcome this hurdle. Furthermore, localized and repeated delivery of factors to the heart could be achieved by implantable and replasenable reservoirs.

7. Conclusions

It is widely considered that the therapeutic benefit of MSCs is mediated by trophic factors including soluble secretions and EVs, and the MSC-EV cargo of proteins and nucleic acids, in particular miRNAs, mediates healing via inhibition of apoptosis, inflammation, and fibrosis and by promoting angiogenesis. Several strategies have been developed to manipulate the MSC secretome via culture stimuli and genetic modification. Biomaterials have exhibited significant benefits for the delivery of EVs by enhancing their tissue retention and thereby improving their healing ability. However, for clinical translation to be achieved, improved EV characterization and isolation methods that can be used at large scales, and are free of culture media contaminating components are crucial.

Acknowledgements

This work was supported by the NIH (R01 DE013033). M.A.B. is funded through a Marie Sklodowska Curie Individual Fellowship PARAGEN H2020-MSCA-IF-2015-708711.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

exosomes, extracellular vesicles, functionalized biomaterials, hydrogels, mesenchymal stromal cells, tissue regeneration

Received: November 2, 2019
Revised: December 19, 2019
Published online: March 11, 2020

[1] M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonti, S. Craig, D. R. Marshak, Science 1999, 284, 143.
[2] M. A. Brennan, A. Renaud, F. Guilloton, M. Mebarki, V. Trichet, L. Sensebé, F. Deschaseaux, N. Chevallier, P. Layrølle, Stem Cells Transl. Med. 2017, 6, 2160.
[3] A. Erics, P. Conget, J. M. Minguell, Br. J. Haematol. 2000, 109, 235.
[4] M. Bari, L. Cava, M. Viganò, T. Montemurro, V. Boldrin, V. Parazzi, E. Montelatici, M. Costi, M. Moro, R. Giordano, L. Lazzari, Stem Cells Dev. 2015, 24, 104.
[5] Z. Miao, J. Jin, L. Chen, J. Zhu, W. Huang, J. Zhao, H. Qian, X. Zhang, Cell Biol. Int. 2006, 30, 681.
[6] J. Botelho, M. A. Cavacas, V. Machado, J. J. Mendes, Ann. Med. 2017, 49, 644.
[7] A. W. James, B. Péault, J. Orthop. Res. 2019, 37, 1221.
[8] A. I. Caplan, J. Cell. Physiol. 2007, 213, 341.
[9] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz, Cytotherapy 2006, 8, 315.
[10] M. A. Brennan, A. Renaud, J. Amiaud, M. T. Rojewski, H. Schrezenmeier, D. Heymann, V. Trichet, P. Layrølle, Stem Cell Res. Ther. 2014, 5, 114.
[11] L. C. Amado, A. P. Saliaris, K. H. Schuleri, M. St John, J.-S. Xie, S. Cattaneo, D. J. Durand, T. Fitton, J. Q. Kuang, G. Stewart, S. Lehrke, W. W. Baumgartner, B. J. Martin, A. W. Heldman, J. M. Hare, Proc. Natl. Acad. Sci. USA 2005, 102, 11471.
[12] E. Gómez-Barrena, P. Rosset, F. Gebhard, P. Hernigou, N. Baldini, H. Rouard, L. Sensebé, R. M. Gonzalez-Daganzo, R. Giordano, N. Padilla-Eguiluz, E. García-Rey, J. Cordero-Ampuero, E. Gómez-Barrena, P. Rosset, F. Gebhard, P. Hernigou, N. Baldini, H. Rouard, L. Sensebé, R. M. Gonzalez-Daganzo, R. Giordano, N. Padilla-Eguiluz, E. García-Rey, J. Cordero-Ampuero, E. Gómez-Barrena, P. Rosset, F. Gebhard, P. Hernigou, N. Baldini, H. Rouard, L. Sensebé, R. M. Gonzalez-Daganzo, R. Giordano, N. Padilla-Eguiluz, E. García-Rey, J. Cordero-Ampuero, E. Gómez-Barrena, P. Rosset, F. Gebhard, P. Hernigou, N. Baldini, H. Rouard, L. Sensebé, R. M. Gonzalez-Daganzo, R. Giordano, N. Padilla-Eguiluz, E. García-Rey, J. Cordero-Ampuero, E. Gómez-Barrena, P. Rosset, F. Gebhard, P. Hernigou, N. Baldini, H. Rouard, L. Sensebé, R. M. Gonzalez-Daganzo, R. Giordano, N. Padilla-Eguiluz, E. García-Rey, J. Cordero-Ampuero, E. Gómez-Barrena, P. Rosset, F. Gebhard, P. Hernigou, N. Baldini, H. Rouard, L. Sensebé, R. M. Gonzalez-Daganzo, R. Giordano, N. Padilla-Eguiluz, E. García-Rey, J. Cordero-Ampuero.
