Biochimie 207 (2023) 33–48

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Versatility of mesenchymal stem cell-derived extracellular vesicles in tissue repair and regenerative applications

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ARTICLE INFO

Article history:
Received 9 August 2022
Received in revised form
29 October 2022
Accepted 21 November 2022
Available online 23 November 2022

Keywords:
Mesenchymal stem cells
Extracellular vesicles
EVs
Clinical trials
Regenerative medicine
Tissue repair
Cellular therapies

ABSTRACT

Mesenchymal stem/stromal cells (MSCs) are multipotent somatic cells that have been widely explored in the field of regenerative medicine. MSCs possess the ability to secrete soluble factors as well as lipid bound extracellular vesicles (EVs). MSCs have gained increased interest and attention as a result of their therapeutic properties, which are thought to be attributed to their secretome. However, while the use of MSCs as whole cells pose heterogeneity concerns and survival issues post-transplantation, such limitations are absent in cell-free EV-based treatments. EVs derived from MSCs are promising therapeutic agents for a range of clinical conditions and disorders owing to their immunomodulatory, pro-regenerative, anti-inflammatory, and antifibrotic activity. Recent successes with preclinical studies using EVs for repair and regeneration of damaged tissues such as cardiac tissue, lung, liver, pancreas, bone, skin, cornea, and blood diseases are discussed in this review. We also discuss delivery strategies of EVs using biomaterials as delivery vehicles through systemic or local administration. Despite its effectiveness in preclinical investigations, the application of MSC-EV in clinical settings will necessitate careful consideration surrounding issues such as: i) scalability and isolation, ii) biodistribution, iii) targeting specific tissues, iv) quantification and characterization, and v) safety and efficacy of dosage. The future of EVs in regenerative medicine is promising yet still needs further investigation on enhancing the efficacy, scalability, and potency for clinical applications.

Contents

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https://doi.org/10.1016/j.biochi.2022.11.011

Abbreviations

2D	two dimensional
3′UTR	3′ untranslated region
3D	three dimensional
5′UTR	5′ untranslated region
Aβ	myloid beta
AFC	alveolar fluid clearance
AKI	acute kidney injury
AML	acute myelogenous leukemia
ANP	atrial natriuretic factor
APC	antigen presenting cell
ApoBD	apoptotic body
ApoEV	apoptotic-cell derived extracellular vesicle
ARDS	acute respiratory distress syndrome
AT	adipose tissue
B-reg	B regulatory cell
BALF	bronchoalveolar lavage fluid
BM	bone marrow
BMI	body mass index
BNP	brain natriuretic peptide
BPD	bronchopulmonary dysplasia
CDS	coding exons
CFUF-F	colony forming units-fibroblastic
cGvHD	chronic graft vs host disease
CM	conditioned media
CS/Exo	scaffold laden with umbilical cord-derived MSC-exosomes
CTP	cardiac-targeting peptide
CX3CL1	X3-X motif chemokine ligand-1
DC	dendritic cells
DFL	dermal fibroblasts
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
ER	endoplasmic reticulum
ESCRT	endosomal sorting required for transport
EV	extracellular vesicle
FBS	fetal bovine serum
fluoMDS	fluorescence-based microfluidic diffusion sizing
GMP	good manufacturing practice
h	human
hADSCs-EVs	human adipose-derived stem cell-derived extracellular vesicles
HA-Gel	hyaluronic acid hydrogel
HCEC	human corneal endothelial cell
HG	high glucose
HLSC	human liver stem cell
HSC	hematopoietic stem cells
HSP	heat shock protein
hucMSCs-EVs	human umbilical cord mesenchymal stem cells-derived extracellular vesicles
h-UC-MSCs	human umbilical cord mesenchymal stem cells
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IL-1RA	interleukin 1 receptor antagonist
ILV	intraluminal vesicles
iNOS	inducible nitric oxide synthase
IRI	ischemia reperfusion injury
ISCT	International Society for Cellular Therapy
ISEV	International Society for Extracellular Vesicles
lncRNAs	long non-coding RNAs
LPS	lipopolysaccharide
LTB4	leukotriene b4
LTC4	leukotriene C4
miRNA	microRNA
MISEV	Minimal Information for Studies of Extracellular Vesicles
mRNA	messenger RNA

MHC-II	major histocompatibility complex class II
MI	myocardial infarction
MNC	mononuclear cell
MSC	mesenchymal stem sell
MSC-exo Klotho	MSC that overexpressed Klotho
MSCGATA-4-DE	mesenchymal stem cell overexpressing GATA-4-derived exosomes
mtDNA	mitochondrial DNA
MVB	multivesicular bodies
MWCO	molecular weight cut off
NASH	non-alcoholic steatohepatitis
ND	neurodegenerative disorder
nHP	nanohydroxyapatite/poly- ε-caprolactone
NK	natural killer
NOS2/iNOS	nitric oxide synthase
PD-L1	programmed cell death 1 ligand 1
PEG	polyethylene glycol
PGE2	prostaglandin E2
PL	platelet lysate
PM	plasmamembrane
PVD	portal vein diameter
RBPs	RNA binding proteins
rRNA	ribosomal RNA
RPM	rotations per minute
RSL3	RAS-selective lethal 3
SASP	senescence associated secretory phenotype
SEC	size-exclusion Chromatography
scl-cGVHD	scleroderma chronic graft vs host disease
SHED Cells	human exfoliated deciduous teeth stem cells
siRNA	small interfering RNA
snoRNAs	small nucleolar RNAs
snRNAs	small nuclear RNAs
SOCS3	suppressor of cytokine signaling 3
SRT501	resveratrol
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
TGFβ	transforming growth factor
TIRFM	total internal reflection fluorescence microscopy
TLR-2	toll like receptor-2
tRNA	transfer RNA
TSG6	tumor necrosis factor stimulated gene-6 protein
UC	umbilical cord
UCB	umbilical cord blood
VEGF	vascular endothelial growth factor

1. Introduction

The need for allogeneic or autologous grafts for tissue repair has grown as life expectancy has increased, as have age-related degenerative illnesses and organ/tissue dysfunction. However, employing such approaches carries the risk of immune-mediated rejection, possibility of disease transmission, and chronic immunosuppression treatments, as well as the limited sources of autologous tissues. With regards to such limitations, the need for new therapeutic approaches for tissue regeneration has led researchers to tissue engineering and regenerative medicine [1,2]. Because of their potential to self-renew and specialize into diverse cell lineages, mesenchymal stem cell (MSC)-based therapies are gaining popularity in regenerative medicine. MSCs maintain tissue homeostasis and facilitate process of replacing damaged cells with healthy ones in injured tissues. The two major types of stem cells are embryonic and somatic. The former coming from the inner cell mass of blastocysts while somatic stem cells come from either perinatal or postnatal sources. Hematopoietic stem cells (HSC) and mesenchymal stem cells (MSCs) are two types of somatic stem cells [3]. MSCs have been documented to display a range of pro-regenerative and tissue reparative functions chiefly mediated by secreted proteins as well as extracellular vesicles (EVs) – lipid bound vesicles secreted by the cells. In this review, we discuss the attributes of MSCs and EVs, their known functions, successes in preclinical and clinical studies, challenges associated with clinical translation, and scope for future research and advancements in the field of autologous and allogeneic regenerative therapies.

2. Human mesenchymal stem or stromal cells

Adult mesenchymal stem cells (MSCs) are multipotent somatic stem cells that possess the ability for self-renewal and multilineage differentiation useful for cell-based therapies. Under different culture techniques, MSCs are able differentiate into cells that form bone, cartilage, muscle, fat, and other connective tissues [4]. A unique aspect of these cells is their ability to also differentiate into non-mesenchymal cells such as astrocytes, oligodendrocytes, and neurons [5]. While MSCs can be derived from various tissues, the most common sources are bone marrow (BM) and adipose tissue (AT). The isolation process for MSCs from BM is a highly invasive procedure, therefore isolation from other tissues have been investigated; however, the quality of non-BM harvested cells remains a concern. Other equivalent sources of MSCs include adipose tissue, placenta, and umbilical cord blood (UCB). In a study conducted by Kern et al., a comparative analysis of mesenchymal stem cells from BM, UCB and AT showed that both BM and AT are still the most reliable sources of MSC isolation, while UCB had an isolation efficacy of maximum 63% [6]. Thus, bone marrow-derived MSCs continue to remain as the gold standard in the development of MSC-based therapies.

The International Society for Cellular Therapy (ISCT) has defined a set of guidelines to define human MSCs: MSC must be plastic-adherent when maintained in cell culture, must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules, and must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* [7a and]. Other adherent cells such as macrophages, endothelial cells, lymphocytes and smooth muscle cells may contaminate the early BM preparations during MSC isolation by plastic-adherence. Morphologically, MSC cultures are heterogeneous, varying in shape and size. Populations are heterogeneous between species and cultures, and express various nonspecific markers. It is thought that this variability arises from differences in isolation methods, tissue and species of origin, and culture conditions [8]. Even with a defined structure for identification of MSCs, it is difficult to use morphologic or phenotypic criteria alone to identify these cells. Therefore, there is a heavy reliance for induced *in vitro* differentiation of MSCs towards bone, fat, and cartilage, *in vivo* ectopic bone formation upon transplantation, and immunomodulation, to correctly identify MSC donors or populations with potential for tissue regenerative or modulatory applications.

Recent studies have focused on the immunomodulatory properties of MSCs with increasing evidence of MSC interaction with T-cells, B-cells, natural killer (NK) cells, macrophages, dendritic cells (DCs), neutrophils, and mast cells to mediate specific immune responses [9]. However, successful translation into clinical applications is often limited by poor post-transplantation survival, time-

consuming *in vitro* cell expansion technology, and the risks for potential alterations within the host microenvironment. Furthermore, some concerns have been raised about the potential of MSC-related tumorigenesis, although it has neither been proven nor verified that MSCs cause tumors when transplanted [10]. It has been shown previously that various soluble factors, including indoleamine 2,3-dioxygenase, nitric oxide, transforming growth factor beta (TGFβ), prostaglandin E2 (PGE2), and tumor necrosis factor stimulated gene-6 protein (TSG-6) as well as cell-cell communication are key players in the MSC immunomodulatory field [11,12]. MSCs release EVs with paracrine and anti-inflammatory effects. Owing to their trophic functions in the enhancement of tissue regeneration and modulation of immune responses, various aspects of MSC secretome are being investigated as an alternative for using whole cells in cell-based therapies [13,14]. As will be discussed in more detail in this review, the potential of EVs in regeneration and immunomodulation holds promise in the development of novel acellular or cell-free therapies for regenerative and immunotherapies.

3. Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are lipid bi-layered membrane-bound vesicles that are released from cells and contain cargo that consists of proteins and genetic material [15]. EVs have the ability to transport their cargo to other cells as a means of intercellular communication [15]. This information exchange between EVs and the recipient cell can influence recipient cell function. EV is a broad term that refers to particles naturally released from the host cell, has a lipid bilayer bound membrane, and do not replicate [16]. While there are many subtypes of EVs, use of EV as an all-encompassing term is supported by The International Society for Extracellular Vesicles (ISEV) due to the difficulty assigning an EV to a particular biogenesis pathway [16]. In the following section, an exploration on the biogenesis of some specific EV subtypes will be discussed.

3.1. EV biogenesis and subtypes

EVs have generally been classified by their sub-cellular origin. For example, EVs that can be created and released by budding from the plasma membrane are commonly also known as macrovesicles, ectosomes, or microparticles [17]. They exhibit a range of sizes from 100–1000 nm in diameter. They have the ability to directly slip into the extracellular matrix through budding of the plasma membrane [18]. Another common sub-type of EVs are exosomes. They range in size from 30 to 150 nm in diameter and are formed via inward budding of endosomal membranes, creating an accumulation of intraluminal vesicles (ILVs) inside multivesicular bodies (MVBs) [19], which are able to be secreted upon fusing with the plasma membrane (Fig. 1). MVBs are membrane bound organelles that contain intraluminal vesicles generated from invagination and budding of the limiting membrane. MVBs are late endosomes whose content can be degraded through fusion with lysosomes/vacuoles or released into the extracellular space after fusion with the plasma membrane (PM). MVBs exchange cargo with other organelles such as the Golgi apparatus, lysosomes, endoplasmic reticulum, and mitochondria [20,21]. MVBs are a part of an endosomal pathway that begins with the formation of the early endosome. Endocytic vesicles coming from the plasma membrane fuse together. Endosomes become MVBs when they accumulate intraluminal vesicles in their lumen [22]. The incorporation of molecules into intraluminal vesicles is mediated by the ESCRT complex [23], and they are then sorted toward three possible routes: recycling, exocytosis, or degradation [24].

The other subtype of EVs are apoptotic bodies (ApoBDs). During cell apoptosis, the release of apoptotic bodies follows the myriad of changes occurring to the dying cell. It has been shown that the blebbing of the plasma membrane could be driven by the cleaving of ROCK1 by caspase-3, but there are still gaps in knowledge about the mechanism of apoptotic body formation [18,25]. They are directly secreted into the extracellular matrix from the bleb formation in dying cells [18]. Their size has been cited from 50 nm to 5000 nm in diameter, but this range is not in agreement across literature [26]. Dying cells release vesicular ApoBDs which contain a wide variety of cellular components: micronuclei, chromatin remnants, cytosol portions, degraded proteins, DNA fragments, or even intact organelles. Owing to their complex and varied content, ApoBDs hold potential for use in cell therapy [27]. However, there is no criteria established yet for the identification of ApoBDs, making it difficult to draw accurate conclusions. Research surrounding the utility of apoptotic bodies require detailed investigations to better understand their roles and possible functions. It has been well documented that there is an issue describing distinct subtypes. Whether it stems from size overlap or differences in nomenclature, it is difficult to determine exactly what sub-type of EV is being described without the use of other markers. Until a more solid set of guidelines is in place, EVs will continue to be the all-encompassing term for the above-mentioned subtypes.

3.2. EV isolation and purification

EVs have been isolated from various biofluids including blood, urine, cerebrospinal fluid, lymphatics, tears, saliva and nasal secretions, ascites, and semen [28]. While there are no standard isolation methods, there are a few that exist for isolating EVs based on their size, immunoaffinity capture, and precipitation of exosomes [29]. The specific method of EV isolation can have a profound effect on membrane integrity and yield. MSCs in culture at 80%–90% confluency can be treated with EV-free media prior to EV collection [30]. The most commonly used isolation method is differential centrifugation, an ultracentrifugation technique [31]. Conditioned media from MSC cultures are subjected to stepwise low speed (500 *g*) centrifugation to pellet out cells, microvesicles (MVs), extracellular matrix components, and debris. Resultant supernatant is centrifuged at 10,000 *g* to remove apoptotic bodies (ABs) and undesired proteins. This is followed by retrieval via ultracentrifugation at 100,000–200,000 g and final washing of the pellet in phosphate buffered saline. The final EV product is obtained as pellet that can be resuspended for downstream applications [29]. Rate-zonal ultracentrifugation is another type of density gradient ultracentrifugation where samples are placed on the gradient density medium surfaces such as sucrose, followed by ultracentrifugation at 100,000 g. As a result, sample components migrate through the gradient density which separates the samples based on size and shape. When samples are mixed with self-generating gradient compounds such as caesium chloride and subjected to long period of ultracentrifugation, it allows for density-based distribution of components that form bands that match with the gradient density of the surrounding solution, a process termed isopycnic centrifugation. Banded EVs can be retrieved from the density zones between 1.10 and 1.21 g/mL by fractionation. Sequential filtration enables separation of cells and larger particles when first processed through a 100 nm normal filtration process. Undesired proteins are excluded via tangential flow filtration using a 100–500 kDa MWCO membrane; non-exosomal EVs are prevented from entering the filtrate using a track-etch membrane filter (with pore size of 100 nm) at low pressure [32]. Other successful methods of isolation include immunoaffinity capture and use of precipitation kits.

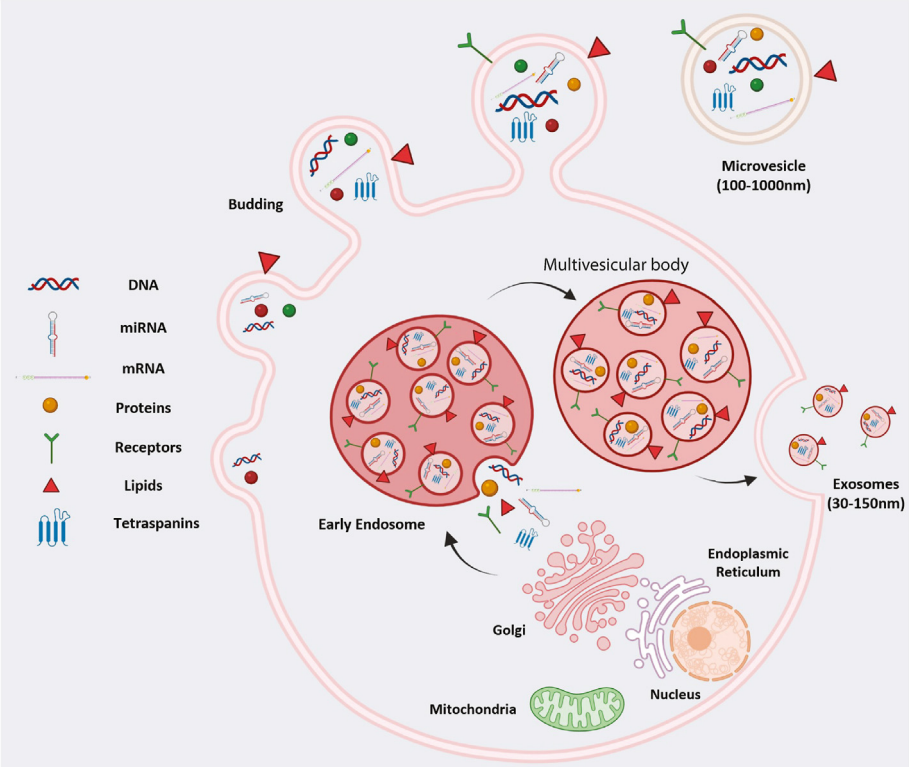

Fig. 1. EV biogenesis. Schematic of formation and release of EVs. Microvesicles have the ability to directly slip into the extracellular matrix through budding of the plasma membrane, while exosomes are formed via inward budding of endosomal membranes, creating an accumulation of intraluminal vesicles inside multivesicular bodies, and then released in the extracellular space.

Since EVs can be isolated from various tissues that each require their own conditions for growth, their culture requirements also vary greatly. Currently, there is no optimized protocol for harvesting EVs from a specific cell type but it has been shown that various aspects of cell culture can influence yield and cargo compositions of EVs [33]. In a study conducted by Patel et al., the cell culture parameters of MSC-derived EVs were explored. The authors documented that increasing MSC passage in culture does not lead to increased production rate or size decreases but does significantly decline EV bioactivity. This group also found that smaller seeding densities correlated with an increase in EV production, however this parameter remained independent of bioactivity, which showed no decrease even in highly packed cells [34]. The various isolation strategies have their own set of advantages and disadvantages, and as more knowledge of EVs continues to become known, these methods will continue to be improved upon.

3.2.1. Culture conditions for clinical grade EVs

MSC-derived EVs could be enhanced through modification of specific *in vitro* parameters during the culture of parental cells. MSC growth conditions can be controlled with physical properties, oxygen tension, and media preconditioning with soluble factors. These factors may influence both biogenesis and biological function as cells are sensitive and responsive to biochemical and biophysical cues from the culture environment [35]. MSCs can be cultured in two-dimensional (2D) and three-dimensional (3D) conditions. 2D systems include common polystyrene flasks, while 3D systems mainly consist of two-chamber or hollow-fiber bioreactors [36]. While 3D models exhibit a small RNA profile, they also resemble *in vivo* conditions for circulating EVs obtained from patients' plasma. However, the small RNA profile of 2D culture-derived EVs correlated better with their parental cells conventional cultures. Cells seeded at a low density and cultured under low oxygen tension are also associated with higher proliferation rates [37]. Preconditioning MSCs with exogenous stimuli such as thrombin, hypoxic environment, lipopolysaccharide (LPS), and hydrogen peroxide (H_2O_2) have been known to promote biogenesis of MSC-derived exosomes, modify bioactive cargo, and improve regenerative, wound reparative, and immunomodulatory potential of MSC-derived EVs [38–40]. Xeno-free media to support stem cell growth and characteristics are now available on the market, providing an alternative to fetal bovine serum (FBS) which is the commonly used growth supplement in mammalian cell culture [41]. Human platelet lysate (PL) has been proposed as an alternative for FBS in the ex-vivo expansion of MSCs. PL has been shown to increase cell proliferation without affecting the MSC immunophenotype, immunomodulatory potential, differentiation potential, and relative telomere length. Since regulatory authorities recommend avoiding animal components during cell expansion process, xenobiotic-free culture conditions have to be considered for EV production and HPL offers such a possibility [42]. Although, a definitive environment most ideal for manufacturing MSC-EVs is yet to be determined, recent reports by Mareschi et al. and Pachler et al. discuss the use of pooled human platelet lysate (pHPL) in achieving GMP-grade EVs with optimal efficacy, purity, and potency [43,44]. Production of MSC-EVs based on cell growth media containing exclusively human components in agreement with recommendations of the regulatory authorities is necessary for accelerating GMP compliant products from the manufacturing facility to the bedside.

3.2.2. Selection/screening of donors

Studies have also shown that there is variability of MSCs from donor to donor [45–47], with the possibility of affecting the quality

of MSC-EVs for therapeutic use. A study that examined the effect of gender, age, and *in vitro* culture found that MSCs from younger, female donors had higher proliferation/clonogenic capabilities [48]. Body mass index (BMI) has also shown to be a determining factor in MSC-EV potential. High-BMI BM-MSCs exhibited low osteogenic and diminished adipogenic differentiation, decreased proliferation rates, and increased senescence [49]. With advancing donor age, MSCs are characterized by a decline in CFU-F efficiency, decreased differentiation ability, reduced wound healing properties and increased secretion of a senescence associated secretory phenotype (SASP) [50]. The topic is debatable with recent findings from a multi-parameter analysis of biobanked bone marrow MSCs by Andrzejewska et al. revealing that no substantial association was identified between donor age or comorbidities and BMSC characteristics. In contrast, their analysis identified that *in vitro* aging and inflammatory cytokine stimulation significantly altered phenotypic and functional heterogeneity [51]. In the discussion of donors, it is also important to acknowledge differences in sourcing EVs from allograft versus autograft donors. While patient-derived (autologous) MSCs may circumvent issues surrounding undesired immune responses, factors such as donor comorbidities may preclude these cells from clinical use. The compelling advantage offered by allogeneic sources of MSCs is the ability to obtain healthy donor cells which can be expanded *in vitro* to generate clinically relevant numbers. Although MSCs were originally characterized to be immune-privileged, emerging evidence suggests that allogeneic MSCs can induce a strong immune response *in vivo* which may impact the intended application depending on the clinical indication being treated [50,52–54]. It has been shown that allogeneic dendritic cell-derived exosomes transferred immunogenic MHC molecules [55]. It remains to be understood whether MSC-derived exosomes contain MHC molecules given that MSCs are phenotypically defined to be negative for expression of MHC Class II [56], and whether they can be transferred to recipient cells and induce alloimmune responses.

3.3. Properties of MSC-derived EVs

All subtypes of EVs have surface molecules that target recipient cells. Once accepted by the target cells, EVs may induce signaling via three mechanisms: bind via specific receptor to the target cell, fuse to the target cell's plasma membrane, or be taken up by the target cell via endocytosis [57]. Through some of these mechanisms, MSC-EVs have the potential to alter the functions of target cells. Since the functionality of EVs are largely dependent on their parental cells and culture conditions, there are several biochemical and immune components that support the exchange of information to trigger desired cellular responses.

3.3.1. Nucleic acids

MSC-EVs carry microRNAs, which are noncoding mediators for RNA silencing and post-transcriptional gene expression [58]. These RNA-housing EVs are thought to be another pathway of cellular communication. There is evidence suggesting that there is a regulated mechanism of the intracellular sorting of mRNA contained in EVs [59]. In a previous work performed by H. Valadi et al., this phenomenon is solidified from the identification of different miRNAs (eg. let-7, miR-1, miR-15, miR-16, miR-181 and miR-375) in EVs. Their findings showed that the RNA cargo from EVs appear to be specifically packaged exclusively, and in certain cases, EV-specific [60]. Studies have identified subtypes of RNA through sequencing techniques. This has demonstrated that several extracellular complexes, such as RNAs and RNA binding proteins (RBPs) can also be found within/associated with EVs [61,62]. EVs can contain many RNA biotypes, including non-coding RNA types such as small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), long non-coding RNAs (lncRNAs), vault RNA, Y-RNA, transfer RNA (tRNA) and ribosomal RNA (rRNA) [63]. Complete mRNAs are ~150 and 60,000 nucleotides long and code for protein production. Their DNA sequence consists of coding exons and non-coding introns, preceded by a 5′ untranslated region (5′UTR) and followed by a 3′untranslated region (3′UTR), both of which are involved in regulating stability, localization, and translation of the mRNA [64]. The complexity of the cargo holds significance in the cell-to-cell transfer of RNA, allowing EVs to be used as potential delivery vehicles.

3.3.2. Proteins

EV proteins consist of membrane transport and fusion related proteins, heat shock proteins (HSPs) including HSP60, HSP70, and HSP90, tetraspanins (also termed four-transmembrane cross-linked proteins) including CD9, CD63, CD81, CD82, CD106, Tspan8, ICAM (intercellular adhesion molecule)-1, and MVBs-related proteins such as ALIX and TSG101, and other proteins like integrins, actin and myosin [65]. In bone marrow-derived MSC-EVs specifically, over 700 proteins have been identified in a proteomic analysis, and depending on the source tissue, fewer or more proteins were identified [66]. The protein composition of EVs is largely dependent on the state of the host cell when extraction occurs; the protein content varies depending on stress signals, microenvironment, and as previously stated, source tissue [66,67].

3.3.3. Lipids

The presence of a lipid membrane is a feature that makes EVs unique to other biological features of the cell. This membrane creates the backbone structure of the EV, as well as plays a role in EV biogenesis and cargo loading [68]. Unlike the protein and nucleic acid portions of EVs, the lipid content is not as reflective of source tissue, however the lipid content does vary depending on different cargo, biogenesis, or function [68,69]. EVs are typically cholesterol and sphingolipid rich, also carrying saturated, monosaturated, and polyunsaturated fatty acids [69].

3.3.4. Mitochondria

Mitochondria play a vital role in maintaining metabolic homeostasis. In recent years, the discovery of mitochondrial content within EVs has led to the identification of a number of biological functions of extracellular mitochondrial content, including outsourcing mitochondrial degradation, activating inflammation, and modulating metabolism. Three major types of extracellular mitochondrial content have been reported: free mtDNA, functional mitochondria, and mitochondrial content within EVs [70]. It has been reported that stem cells can transport mitochondria to other cells via microvesicles [71]. Mitochondrial components such as soluble proteins, mt inner membrane, and mtDNA are transported by exosomes. MSC-derived mitochondria within the cytoplasm are packed into vesicles containing the autophagy marker light chain 3, which migrate to the cell's periphery and merge with exiting germinating vesicles during transport. MSCs restore damaged cell functions by secreting EVs that transport mitochondria and other components by (i) processing depolarized mtDNA through vesicles to complete mitochondrial quality control [72] and (ii) healthy cells secrete mitochondria to restore bioenergetics of damaged cells [73]. Mitochondrial cargo within EVs have been shown to stimulate a pro-inflammatory response when exposed to lipopolysaccharide (LPS) [74]. However, the mechanism by which these anti-inflammatory factors are secreted and whether they also participate in immune activation remains unknown. Given that EVs play a critical role in the intercellular transport of mitochondria and their components, further research on the direct evidence of the

biological activity of microvesicles-transported mitochondria is needed.

3.4. Major known immunomodulatory functions of MSC-EVs

3.4.1. Adaptive immune response

MSC-EVs have been shown to influence T and B lymphocytes and play a role in the adaptive immune response [58]. In a study done by Cosenza et al., MSC-EVs were shown to have immunosuppressive functions by decreasing T and B lymphocyte proliferation and induce T-regulatory cells [75], similar to parental cells. T-cell proliferation inhibition is aligned with the reduction or lack or pro-inflammatory cytokines (IL-2, IL-6, TNF-α, and IFNγ), and MSC-EVs were shown to increase the T cell number in a graft of rat renal transplant model while maintaining a TNFα reduction and no difference in IL-10 levels [76]. It has also been found that both $CD4^+$ and $CD8^+$ T cell activation was suppressed by MSC-EVs lacking β2-microglobulin [77]. B cells that function similar to T-regulatory cells have been coined as B-regs (B-regulatory cells). B-regs express regulatory factors (i.e. IL-10, IL-35, transforming growth factor (TGF)-β, and programmed cell death 1 ligand 1 (PD-L1)) that have suppressive effects on T cells [78]. Another group applied molecular characterization of MSC-EV content to explore how B cell activity is modulated by EVs, and found that MSC-EVs strongly modulate B cell activity and are capable of influencing typical immune effector cell activity through the proteins involved in PI3K-AKT signaling pathway as well as miRNA [79]. A related investigation showed that MSC-EVs did not inhibit T cells obtained from resting or primed MSCs, but a slight increase in $CD4^+$ cells was observed as compared to sorted T cells cultured alone, while resting EVs displayed a significant suppressive effect on B cell proliferation [80]. Together, these studies point towards the effectiveness of EVs in serving as immunomodulatory adjuvants useful for immunotherapies.

3.4.2. Innate immune response

In addition to the adaptive immune response, MSC-EVs have also been shown to affect the players in the innate immune system such as dendritic cells, natural killer cells, and macrophages. Macrophages can be affected by a variety of factors to change their phenotype and thus affect their function. Activated macrophages usually fall into two categories, M1-like macrophages and M2-like macrophages. Both M1 macrophages and M2 macrophages are closely related to inflammatory responses, among which M1 macrophages are mainly involved in pro-inflammatory responses and M2 macrophages are mainly involved in anti-inflammatory responses. M1 macrophages are characterized by an elevated ability to secrete cytokines such as IL-1β, TNF, IL-12, and IL-18. M1 macrophages express high levels of major histocompatibility complex class II (MHC-II), CD68, and CD80 and CD86 costimulatory molecules. They can up-regulate the expression of intracellular protein called suppressor of cytokine signaling 3 (SOCS3), and activate the inducible nitric oxide synthase (NOS2 or iNOS) generating NO [81]. M2 macrophages are characterized by a high expression of scavenging, mannose, and galactose receptors, as well as production of ornithine and polyamines [82]. M2 macrophages produce anti-inflammatory cytokines like transforming growth factor beta (TGF-β), IL-1 receptor antagonist (IL-1RA), and IL-10 [83]. EVs are thought to play a role in the macrophage phenotype regulations. When lipopolysaccharide (LPS)-treated macrophages were used as an *in vitro* model of UC-induced inflammatory response, MSC-EVs promoted the proliferation of macrophages exposed to LPS and reduced the levels of TNF-α, IL-6, and IL-12, increased IL-10 expression [84]. MSC-EVs affect dendritic cells as part of an immune response. Dendric cells (DCs) are a type of antigen-presenting cells (APC) that acts as the mediator between the innate and adaptive immune system [85]. Derived from blood monocytes or hematopoietic progenitor cells in bone morrow, DCs initiate the immune response by capturing, processing, and presenting antigens to native $CD4^+$ T cells [86]. After up-taking MSC-EVs, DCs had decreased levels of co-stimulatory markers and an altered cytokine pattern, and also were unable to activate antigen-driven inflammatory effector T-cell activation [87]. MSC-EVs have shown a possible enhancement in the release of TGF-β and IL-10 from CD11c+ DCs, which in turn can inhibit lymphocyte proliferation, while having no effect on the expression of MHC class II, CD86, CD83, and CD40 [86]. Natural killer (NK) cells are a type of white blood cells and are known for their ability for killing virus-infected cells as well as detecting for signs of cancer. MSC-EVs exert immunosuppressive effects on NK cells in various models. In rats, downregulation of the renal expression of C-X3-C motif chemokine ligand-1 (CX3CL1) and toll-like receptor-2 (TLR-2) was shown, as well as the transfer of various miRNAs, all inhibiting the $CD3^-CD161+$ NK infiltration [88]. Fan et al. demonstrated that MSC-EVs could inhibit proliferation, activation, and cytotoxicity of NK cells via regulatory molecule TGF-β [89]. While much data does exist for the role of MSC-EVs in the innate immune system, their role in NK cells specifically still has a need for more research to support existing data.

3.5. Potential of MSC-EVs in regenerative medicine

Due to the above-described characteristics, it is no surprise that there is great interest in researching the potential of MSC-EVs for therapeutic use. The effects of MSC-derived EVs for treatment of different diseases and damaged tissues have been explored in various studies and in some cases have made it to clinical trials. The following section will outline the findings that support the potential use of MSC-EVs in therapies across major organ systems, including cardiovascular, pulmonary, hepatic, pancreatic, musculoskeletal, skin, and corneal repair and regeneration (Fig. 2).

3.5.1. Cardiac disease and injury

Several microRNAs and proteins constituting the MSC-EV cargo are shown to mediate reparative and regenerative functions in cardiovascular repair. Through the enrichment of miR-210, MSC-EVs have been shown to improve angiogenesis and cardiac function in mice with myocardial infarction injury (MI); such improvements were accompanied by evidence of limited fibrosis in ischemic hearts in post-MI mice [90]. Similar work on human(h) umbilical cord (UC)-MSC-EVs delivered via intravenous injection after an MI event resulted in reduced cardiac fibrosis, promotion of angiogenesis and improved cardiac systolic function in rats [91]. Another study showed that EVs isolated from Akt-overexpressing h-UC-MSCs were highly efficient at repairing ischemic myocardium and promoting angiogenesis. The high levels of PDGF delivered to endothelial cells from Akt-EVs improved proliferation, migration, and formation of blood vessels [92]. Monguio-Tortajada et al. designed a 3D scaffold for *in vivo* delivery of MSC-EVs from porcine cardiac adipose tissue to the ischemic myocardium in a pig model of myocardial infarction. This study demonstrated that after six days post-operation, vascular density was increased, while macrophage and T cell infiltration in the damaged myocardium were reduced. Furthermore, the engineered scaffold was integrated into the post-infarcted myocardium [93]. In targeted hypoxic CMCs, MSCs overexpressing GATA-4-derived exosomes (MSCGATA-4-DEs) expressing anti-apoptotic miRNAs diminish apoptosis while preserving mitochondrial membrane potential [94]. It was shown that PTEN and BIM expression are downregulated by miR-19a, leading to the activation of AKT and ERK signaling pathways. On the other hand, JNK/caspase-3 activation is inhibited by targeting the

transcription factor SOX6. Mayourian et al. indicated that increased expression of the TGF-signaling pathway, pro-angiogenic VEGF, ANGPT-1, hypertrophic atrial natriuretic factor (ANP), and brain natriuretic peptide (BNP) is a result of hMSC-exo—mediated effects on cardiac contractility, with miR-21-5p playing a critical role [95]. Overall, MSC-EVs continue to be widely studied owing to their ability to be targeted and influencing cardiac protection and regeneration.

3.5.2. Lung disease and injury

Owing to the immunomodulatory and regenerative capacities of MSC-EVs, several preclinical and clinical studies have focused on evaluating MSC-EV potency for lung repair and regeneration. In an *E. coli* induced pneumonia model of mice and in an acute lung injury model, MSC-EVs administered to both models were able to promote higher leukotriene B4 (LTB4) levels in the bronchoalveolar lavage fluid (BALF). The treatment also decreased MRP1 mRNA expression, protein levels, and pump activity. MSC-EV suppression of MRP1 through transfer of miR-145 was able to reduce extracellular levels of leukotriene C4 (LTC4) and enhanced LTB4 production [96]. Through this process, there was an increase in bacterial phagocytosis through LTB_4/BLT1 signaling. Zhu et al. showed that IV delivery of MSC-EVs resulted in a decrease in the influx of white blood cells and neutrophils, MIP-2 cytokine, and total protein levels in the alveolus of mice with *E. coli* endotoxin [97]. It was also shown that there was an association between administration of EVs and reduction of extravascular lung water. Another study assessed the use of human lungs that were rejected from transplant to test the effect of MSC-EVs on alveolar fluid clearance (AFC). They found significant improvement in AFC via IV administration in a dose-dependent manner, along with prevention of tracheal pressure increases and increased lung compliance [98]. By inducing an M1-M2 shift in macrophages, MSC exosomes enhanced lung structure and function in mice with bronchopulmonary dysplasia, as indicated by decreased IL-6, TNFα, CCL5, and upregulated arginase 1 [99].

Relevance to the recent pandemic: The COVID-19 pandemic has dramatically increased the number of sickness and deaths worldwide. Even with recently developed vaccines, treatment for the rapidly expanding virus remains limited [100]. Severe cases of COVID-19 may result in acute respiratory distress syndrome (ARDS) characterized by alveolar, endothelial cell damage, increased vascular cell damage, and poor pulmonary oxygenation [101]. In a study reporting EV therapy for ARDS in a large animal (pig) model, it was shown that EV administration led to reduction of inflammation, alveolar epithelial regeneration, and repair of the pulmonary endothelium. Based on these preliminary reports, we can expect that EVs could be a promising solution for reparation of lung tissue in severe COVID-19 cases [102].

3.5.3. Liver disease and injury

Cell-derived therapies for both acute and chronic liver disorders are continuously evolving in regenerative medicine. It is well known that MSCs migrate toward injured organs where they can provide tissue protection and promote regeneration. Various toxic, metabolic, and inflammatory stresses on the liver lead to progressive disorders characterized by inflammation, apoptosis, and necrosis of parenchymal cells. Acute liver failure and cirrhosis are some of the major disorders that can be ameliorated with the use of MSCs and MSC-EVs. In a rat liver cirrhosis model, Mardpour et al. injected MSC-EVs intrasplenically and found alleviated fibrosis and collagen density, accompanied by improved necrosis, caspase density, and portal vein diameter (PVD) [103]. In an acute liver failure mouse model, MSC-EVs that were injected intraperitoneally were shown to increase the survival rate among the treatment group and impacted the biochemical markers surrounding hepatic injury. After administration of D-galactosamine/TNF-α, histological examination revealed MSC-EVs had reduced hepatic inflammation and hepatocyte apoptosis *in vivo* [104]. Another study demonstrated that non-alcoholic steatohepatitis (NASH), one of the most common liver diseases, could be treated with human liver stem cells (HLSCs). EVs released by HLSCs in a NASH induced, immunocompromised mice model were shown to significantly improve liver function and reduce signs of liver fibrosis and inflammation [105]. A related study investigated the treatment of NASH and liver fibrosis with EVs from amnion-derived MSCs which were intravenously injected in rats that had been induced with NASH through a 4-week high-fat diet. EV treatment resulted in a significant decrease in the number of Kupffer cells in the liver of rats with NASH and the mRNA expression levels of inflammatory cytokines such as TNF-α, IL-1β and IL-6, and TGF-β [106]. Overall, MSC-EVs have demonstrated ability to reduce degree of hepatic injuries, promote antifibrosis, inhibit apoptosis and systemic inflammation in several models of liver disorders, thus emerging as a potent and viable treatment option for acute and chronic liver diseases.

3.5.4. Pancreatic Disease and injury

Several studies have shown that MSC-exosomes can be used to reduce inflammatory responses and treat inflammatory diseases. Therefore, MSC-derived exosomes have potential clinical value in the treatment of inflammatory diseases, especially pancreatitis. Wang et al. demonstrated that MSC-EVs overexpressing Klotho (proteins encoded by the KL genes in humans, noted for forming a unique endocrine system that regulates multiple metabolic processes) attenuated the severity of pancreatic inflammation in cerulein-stimulated AR42J cells [107]. EVs from MSCs that overexpressed Klotho (MSCs-exo Klotho) decreased the expression of IL-6 and TNF-α compared to that of the control group. Limited studies on pancreatic repair with MSC-EVs are documented in literature. Of note is a study using type 2 diabetes mellitus (T2DM) rat model wherein MSC-EVs were delivered via tail vein and blood glucose levels monitored every three days over the course of one month. MSC-EVs promoted significant improvement of hyperglycemia in the T2DM rats, with continued maintenance after the final injection [108]. In addition, increased insulin sensitivity, increased glucose uptake and metabolism, and inhibition of streptozotocin (STZ)-induced β-cell apoptosis were observed with MSC-EV treatment. In another study, type 1 diabetes mellitus (T1DM) mice were also treated with MSC-EVs. Their results showed increase in the potential of splenic MNCs for production of TGF-β, IL-4, and IL-10 as prominent anti-inflammatory cytokines with a significant decrease in the production of IL-17 and IFN-γ as leading inflammatory cytokines without any significant changes in the stimulation index of splenic mononuclear cells [109]. With the demonstrated potential to modulate the endocrine functions of pancreatic cells, MSCs and MSC-derived EVs are gaining significance in pancreatic repair and regeneration.

3.5.5. Bone disease and injury

Bone repair and regeneration using cell-based therapies and tissue engineering approaches have been investigated for almost three decades. MSCs and MSC-EVs have emerged as attractive candidates for enhancing the potency of biocompatible, osteoinductive scaffolds that are routinely applied in bone tissue engineering. As a notable example, MSC-EVs were found to enhance the proliferation and osteogenic capacity of older BM-MSCs *in vitro*, as well as post local injection, significantly promote new bone formation during distraction osteogenesis in an older rat model [110]. A similar study documents the effects of MSC-EVs in enhancing osteogenesis, angiogenesis and bone integration and healing in a

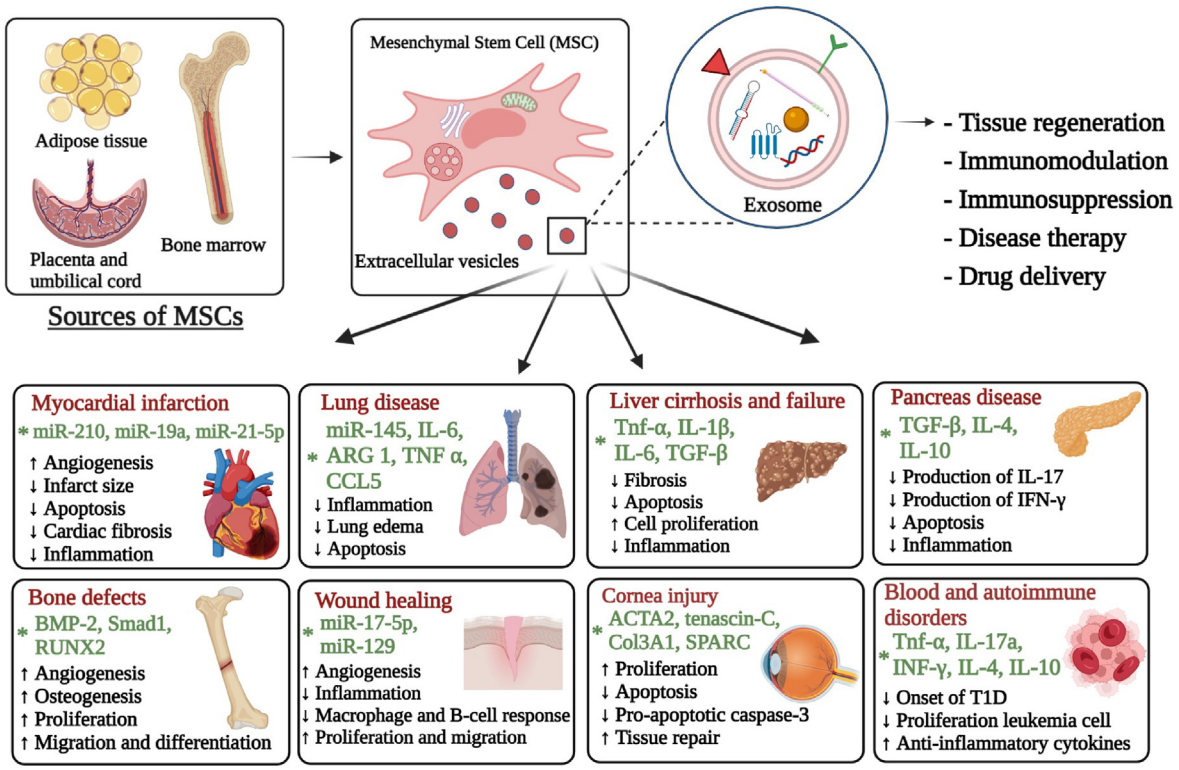

Fig. 2. Schematic of tissue repair and regenerative functions of MSC-EVs. EVs obtained from MSCs derived from multiple tissue sources have demonstrated potential to repair. Key players such as growth factors, microRNAs, interleukins and cytokines mediating regeneration and/or immunomodulation are notated (green) for tissue-specific applications.

rat femoral fracture model. Additionally, investigation at the molecular level showed upregulation of BMP-2/Smad1/RUNX2 signaling confirming the pro-osteogenesis process enhanced by MSC-EVs [111]. In a calvarial defect model, critical-sized defects in rat parietal bones treated with atelocollagen sponge scaffolds soaked with MSC-EVs resulted in increased migration, enhanced differentiation potential, improved osteogenesis and angiogenesis gene expression, and promoted bone formation *in vivo* [112]. Together, these studies validate the usefulness of MSC-EVs in promoting bone healing and treating bone defects across multiple models and species.

3.5.6. Skin disease and injury

The primary goal of wound healing is rapid wound closure and recovery with minimal scarring. MSCs have been known to improve healing by increasing the rates of healing, enabling the conversion of non-healing wounds to actively healing wounds, and promoting anti-scarring mechanisms [113]. Recognizing that many of the beneficial effects of MSCs in cutaneous wound healing are mediated through secreted proteins and EVs, it is envisioned that MSC-EVs are potent immune modulators which can be applied as therapeutic candidates for promoting skin tissue repair. In a scleroderma chronic graft vs host disease (cGvHD) mouse model, MSC-EVs were injected via tail vein and samples would be scored based on criteria surrounding the skin lesions. It was observed that fibrosis of scl-cGVHD mice was alleviated with treatment, as well as a reduction of macrophages and B cell response [114]. Dermal fibroblasts (DFL) were isolated from newborn mouse skin in order to test MSC-EVs ability to rejuvenate senescent dermal fibroblasts. By encapsulating MSC-EVs into a chitosan hydrogel and applying them to the DFLs *in vitro*, they found renewed senescent DFLs, increase in proliferation and migration, decrease in SA-β-gal activity, enhanced ECM protein synthesis, and overall positive effects on restoration of skin aging [115]. Wei et al. investigated the effect of hucMSC-EVs on angiogenesis in diabetic wound healing under high glucose (HG) settings. Their findings show that hucMSC-EVs can improve cutaneous wound repair in diabetic mice as hucMSC-EVs can boost the proliferation, migration, and angiogenic activities of HG-pretreated HUVECs. These therapeutic benefits are miR17-5p-dependent, since miR-17-5p inhibition dramatically reduces the hucMSC-EVs-induced effects, and agmirR-17-5p can partially mimic hucMSC-EVs effects. Furthermore, miR-17-5p has direct effects on the PTEN/AKT/HIF-1/VEGF signaling pathway [116]. Another study by Hu et al. used a rat model of T1DM with wounds surrounding the deep fascia to analyze the underlying molecular mechanism of resveratrol in T1DM via miR-129-containing EVs produced from MSCs. MSCs treated with resveratrol (SRT501) and the matching EVs (SRT501-EVs) increased the proliferative, migratory, and tube formation potentials of HUVECs, as well as the skin wound healing of T1DM mice. Resveratrol promotes wound healing in T1DM patients by downregulating TRAF6 and promoting miR-129 transfer through EVs, whereas TRAF6 was validated as a miR-129 target gene. Furthermore, inhibiting miR-129 reduced resveratrol's proangiogenic impact on HUVECs [117]. Research advancements in wound healing and skin regeneration have incorporated MSC-EVs in several translational studies that point towards development of promising acellular therapies.

3.5.7. Cornea Disease and injury

Human corneal endothelial cells (HCECs) were obtained from patients undergoing corneal transplantation. Once HCECs were adherent to a 96-well plate, concentrations of MSC-EVs were added to assess effects on induced damage to the cells. The results showed higher proliferation rate of HCECs as well as decrease in the total

number of apoptotic cells. After completion of a wound healing assay, HCECs also showed faster repair post MSC-EV treatment [118]. In another study where HCECs were obtained in the same manner as previously described, endoplasmic reticulum (ER) stress was induced either by serum deprivation or treatment with tunicamycin. After being treated with MSC-EVs, ER stress-related genes were found to be down-regulated, as well as reduced levels of pro-apoptotic caspase-3 in stress conditions [119]. In a mouse superficial stromal wound model, Hertsenberg et al. found that topical cornea stem cells-derived exosomes reduced corneal inflammation and scarring by lowering neutrophil infiltration through a TSG-6-dependent pathway and downregulating fibrotic markers such tenascin-C, ACTA2, COL3A1, and SPARC [120]. Research outcomes continue to support that MSC-derived EVs show promising therapeutic potential and could be considered as an option for cell-free treatment in ocular surface disease.

3.5.8. Autoimmune and blood disorders

MSC-EVs have been routinely tested in several pre-clinical models of blood and autoimmune disorders. The role of EVs within the leukemic microenvironment has also been investigated for its therapeutic potentials. Kasumi investigated the factors of MSC-EVs that would inhibit growth of nascent acute myelogenous leukemia (AML) and alter the population of leukemia cell proliferation. The cells were seeded and cultured in various concentrations of EVs and quantified to determine proliferation. MSC-EVs were shown to inhibit the proliferation of the AML cell in line *in vitro* via an apoptotic mechanism [121]. Ahmadvand Koohsari et al. found that administering HUCMSC-EVs intravenously to EAE mice models alleviated autoimmune encephalomyelitis [122]. In an animal model of MS, they found that the treatment's efficacy was linked to a drop in proinflammatory cytokines like IL-17a, TNF-α, and IFN-γ, as well as an increase in anti-inflammatory cytokines like IL-10 and IL-4, as well as a decrease in leukocyte infiltration. In a similar study, Jafarnia et al. claim that on a chronic model of MS, intravenous treatment of hADSC-EVs reduces induced EAE by decreasing T cell proliferative potency, mean clinical score, leukocyte infiltration, and demyelination [123]. In other autoimmune conditions affecting endocrine systems such as type 1 diabetes mellitus (T1DM), preclinical mouse models provide promising results with the potential for translation. TIDM was induced through an adoptive transfer of intravenously infused splenocytes isolated from 12-week-old mice and injected into a 7-week-old mice. MSC-EVs were injected, and mice were monitored for hyperglycemia twice a week. MSC-EV treatment resulted in significant delay of the onset of T1D and increase in the plasma amount of insulin in the adoptive transfer model [124]. Together, these studies indicate the clinical utility of MSC-EVs in ameliorating blood and autoimmune disorders.

3.5.9. Neurodegenerative disorders

Stem cell-based treatment for a wide variety of neurodegenerative disorders (NDs) have been studied and delivered promising results. Pre-clinical models of cerebrovascular disease, traumatic brain injury, and neurodegenerative diseases such as Parkinsons and Alzheimer's have been tested [125]. EVs exhibit reactive gliosis, neuronal death, pro-inflammatory signaling, as well as reduced cognitive, behavioral, and motor deficits. EVs are able to permeate the blood-brain barrier and their contents can be modified to deliver molecules of interest [126]. In a rat model of Alzheimer's disease (AD), bone marrow-derived MSC-EVs ameliorated symptoms via miR-29c-3p/BACE1 axis and Wnt/β-catenin pathway activation, together improving cognitive function [127]. It was reported that MSC-EV treated diseased rats showed noticeably reduced amyloid beta (Aβ) plaques and Aβ contents. In another mouse model of AD, animals treated with bone marrow-derived MSCs performed better in cognitive tests including object recognition test, Y-maze, and Barnes maze test, accompanied by reduced Aβ plaque load [128]. Another study utilizing a mouse model of Parkinson's disease demonstrated MSC-EVs delivering miR-181a-2-3p decreased apoptosis of dopamine neurons as well as down-regulated EGR1, significantly decreasing oxidative stress injury in the treated mice [129]. A recent study investigating EVs derived from human exfoliated deciduous teeth stem cells (SHED cells) on a Parkinson's disease rat model showed that animals treated with SHED-derived EVs had a reduction on gait impairment. Additionally, EVs reduced the number of unilateral 6-hydroxydopamine-induced contralateral rotations whilst also mediating the normalization of tyrosine hydroxylase expression in the substantia nigra and striatum [130]. Several studies have thus pointed out that EVs derived from MSCs and other adult stem cells have potential for treating Parkinson's disease.

In addition to research literature, we collected data on ongoing or completed clinical trials using MSC-EVs from clinicaltrials.gov (Table 1). There is a notable preference for MSC-derived EVs use in treating infections and diseases affecting the respiratory tract. There have not been many studies involving the musculoskeletal system, wounds and injuries, and pregnancy conditions. Nevertheless, the use of MSC-derived EVs as a form of therapy continue to be tested in multiple pathologies that are not classified in any of the categories shown. It can be concluded that MSC-EVs are generating interest among clinicians based on the number of trials that are being investigated.

3.6. Delivery strategies of MSC-EVs

The most used methods for delivery of EVs are systemic injection (intravenous or intraperitoneal) or localized delivery. However, systemic approaches result in rapid clearance of EVs from blood stream and/or accumulation in the liver, spleen, and kidneys, necessitating higher therapeutic doses to sustain the effects. Therefore, biomaterials of different forms (hydrogels, scaffolds, etc.) are being widely employed in order to deliver EVs locally, thereby prolonging the retention of EVs at target sites and ameliorate their therapeutic efficacy [131–133].

3.6.1. Systemic delivery, homing, and retention of EVs

In drug-delivery and cell-based therapy applications, when EVs are administered systemically, they are expected to reach desired target cells, release the cargo inside, and avoid routes that may destroy or recycle the cargo [134]. One of the key aspects involved in efficacious MSC-EV delivery is their homing to target tissues post-administration. Research indicates that MSC-EVs mostly home to the lung, liver, spleen, kidneys, and surface engineering of exosomes can induce and increase targeting ability [135–138]. Though the molecular mechanism behind such specific tissue tropisms is unknown, it is hypothesized that EVs express surface components/cell surface proteins to facilitate specific interaction with target cells. Several studies have generated new knowledge that the adhesion molecules on the surface of EVs dictate such tissue tropic effects; specifically, integrin presentation on EVs play a critical role in their homing patterns [139,140]. The physical size of EVs may also contribute to the localization of EVs, especially large-EVs to the lungs. The initial target site is the lungs, followed by redistribution to other organs; the high ‘entrapment’ in the pulmonary capillaries may thus explain the initial retention of EVs in the lungs. MSC-derived EVs express several adhesion molecules (CD29, CD44 and CD73), which enable their homing to the injured and inflamed tissues [141]. Additionally, the presence of specific integrins on EVs are also involved in targeting to the lung and liver [142,143].

Targeting small-EVs to the spleen requires the presence of CC chemokine receptor 7 (CCR7) on the EVs [144]. In the event of injury, host cells release certain biochemical cues that allow cell-cell communication and enable EVs to travel to site of injury or defect to mediate repair or healing.

Research involving labeling EVs to track their entry and retention in tissue have enabled understanding on the fate of EVs post-delivery. From a recent comprehensive compilation on reports discussing the biodistribution of EVs, we understand that EV retention was dependent on various factors namely route of delivery, concentration or number of EV particles administered, and time-periods of tracking [145]. In most reports, the liver, followed by the lung, was found to be the site of accumulation of EVs. Other tissues of EV retention were spleen, GI tract, and kidneys. The route of administration of EVs had some influence on the site of EV retention. Subcutaneous or intraperitoneal administration resulted in increased homing and retention of EVs in the pancreas and GI tract, whereas intravenous delivery of the same EV preparation resulted in preferential accumulation in liver and spleen [146]. In an acute kidney injury (AKI) model, administration of MSC-derived EVs showed retention in injured kidneys [147]; on the other hand, muscle cell-derived EVs (from C2C12 cells) were retained in the liver [146]. Taken together, we can understand that parental cell types and routes of delivery, determine the biodistribution of EVs in recipient tissues.

(i) Intravenous administration (IV) of MSC-EVs: The administration of EVs into recipient animals is commonly performed by IV administration into the tail vein. In a study by Haga et al. [148] the effects of intravenous injection of mouse bone marrow-derived MSC-EVs were investigated in a murine model with induced hepatic ischemia-reperfusion injury (IRI). Their results showed that intravenous administration of MSC-EVs 30 min before IRI could decrease the necrosis area, caspase-3 positive cells, and apoptotic cells, as well as decrease the serum aminotransferase levels. This approach proved to recuperate hepatic IRI through modulation of the inflammatory response. Lee et al. [149] reported that bone loss in osteoporosis mice model reduced after intravenous injection of adipose stem cell-derived EVs. It was shown that adipose stem cell-derived EVs include osteoprotegerin, miR-21-5p, and let-7b-5p, which prevent osteoclast differentiation and lower bone resorption, implying that ASC-EVs represent a promising cell-free osteoporosis therapy.

(ii) Intraperitoneal administration of MSC-EVs: An alternative method for administration of MSC-EVs is via the intraperitoneal route. In a study performed by Drommelschmidt et al., two repetitive doses of MSC-EVs were administered intraperitoneally to 3-day-old Wistar rats with preterm brain injury in order to assess the regenerative potential of MSC-EVs for developing central nervous system [150]. EV treatment significantly improved inflammation-induced cellular damage and is now explored as a novel candidate for prevention of neuronal cell death. Ai et al. used a hyperoxia-induced rat bronchopulmonary dysplasia (BPD) model to study the effect of different dosages of early intraperitoneal MSC-EVs on BPD [151]. The administration of MSC-EVs on the first day of culture considerably delayed the hyperoxia-induced transdifferentiation of AT2 cells. Hyperoxia increased the expression of WNT5a mRNA and protein, a major regulator of AT2 transdifferentiation, whereas MSC-EVs treatment reduced it.

Table 1
List of clinical trials (or studies) utilizing mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) to treat diseases classified in major categories of organ systems. A system/category can have multiple disease classifications. Studies were sourced from the clinicaltrials.gov database using key words 'MSC-EV' or 'MSC-EVs' or 'mesenchymal stem cell extracellular vesicles' on trials conducted between 2019 and 2022.

System/disease category	Number of Studies
Infections	48
Respiratory Tract Diseases	45
General Pathology and Symptoms	21
Digestive System Diseases	23
Skin and Connective Tissue Diseases	15
Abnormalities and Diseases Before or at Birth	13
Musculoskeletal Diseases	8
Wounds and Injuries	9
Pregnancy Conditions	3

3.6.2. Local delivery of MSC-EVs

(i) Biomaterial-based delivery with hydrogels: Significant therapeutic effects of intraperitoneal injection of MSC-EVs encapsulated with polyethylene glycol (PEG) on reversion of liver fibrosis in rat was observed by Mardpour et al. [152]. In vivo tracking proved that regardless of delivering through hydrogel or as free EVs, MSC-EVs could migrate to fibrotic liver and provide anti-fibrotic effects. However, higher anti-fibrotic, anti-apoptotic and regenerative effects were observed in sustained release system of MSC-EVs due to prolonged and gradual release of EVs, rather than the conventional bolus injection. Exosome ability and effective delivery of human umbilical cord MSC-exosome in pluronic F-127 hydrogel were reported in a study by Yang et al. [153] in order to cure chronic refractory wounds. The hUC-MSC-exosome encapsulated in pluronic F-127 hydrogel were topically administered to diabetic rat model and results demonstrated that the combination of pluronic F-127 and hUC-MSC-exosome resulted in a significantly faster wound closure rate, increased expression of CD31 and Ki67, improved granulation tissue regeneration, and upregulated expression of VEGF and TGFβ-1 when compared to hUC-MSC-exosome, pluronic F-127-only, or control treatment. For increasing retention of and short-lived therapeutic effects of MSC-EVs for treating myocardial infarction, Lv et al. incorporated MSC-EVs with sodium alginate hydrogel as a sustained delivery system [154]. In addition to enhancing retention in the heart, cardiac cell apoptosis was decreased; angiogenesis and polarization of macrophages was promoted in MSC-EVs-hydrogel treatment, thus enhancing the overall cardiac function in comparison to EVs treatment alone.

(ii) Biomaterial-based delivery with implantable scaffolds: Xin et al. developed a collagen scaffold laden with umbilical cord-derived MSC-exosomes (CS/Exos) using a local delivery method for treating intra-uterine adhesions in a rat endometrium-damage model. In vivo local transplantation of CS/Exos aided endometrial regeneration and collagen remodeling, as well as fertility restoration, according to their findings. In the regenerated endometrium, estrogen receptor alpha/progesterone receptor expression was enhanced. *In vitro* and *in vivo* experiments revealed a relationship between CS/Exos potency and M2 macrophage polarization, which was attributed to exosome-enriched miRNAs. In a recent study by Zhang et al. [155], the effect of microvessels for promoting bone tissue engineering was investigated. Umbilical MSC-derived exosomes were encapsulated in

hyaluronic acid hydrogel (HA-Gel) and combined with nanohydroxyapatite/poly-ε-caprolactone (nHP) scaffolds. In vivo regeneration of bone in rats with cranial defect proved that composite scaffolds seem to be promising which might be associated with sustained release of exosomes through HA-Gel. Skin wound healing has been studied using collagen chitosan scaffolds enriched with bone marrow-derived MSCs or their EVs on the back of rats with induced skin wound [156]. In contrast to the control group, the number of $CD68^{+}$ macrophages were reported to be higher in the EVs and bone marrow-derived MSCs. Furthermore, in enriched scaffolds-based treatments, greater collagen deposition and better collagen alignment were reported, which promoted wound healing and collagen remodeling with EVs.

3.7. Challenges in clinical applications

While MSC-derived EVs have demonstrated characteristics that show a strong potential for therapeutic success in preclinical studies, there are still numerous issues that must be solved before they are able to be used in clinical settings [157]. The current challenges include (i) a lack of large-scale production and isolation methods, (ii) biodistribution, (iii) the targeting and transfer mechanisms of EV to specific target sites, (iv) precise and rapid methods for accurate quantification and characterization of cargo, (v) determination of safety and efficacy of optimal dosage [158].

3.7.1. Scalability and isolation of MSC-EVs

A major bottleneck in EV biomanufacturing is scalability during development of a product to achieve clinically relevant doses. Current practices rely on invasive manipulations of cells and culture conditions, which can have deleterious implications on the final product and can incur high cost and operate at small scales, making them less viable for best-practice manufacturing. When MSCs are extensively sub-cultured and passaged to generate sufficient numbers for direct or derived (EVs) products, cells lose their clonal, differentiation, and trophic capacity [46,159]. Even with the promising therapeutic potential of EVs, the manufacturing processes that would allow the scalability and consistency of EV production are still lacking [160]. For preclinical and clinical testing, large scale manufacturing of EVs up to 1.4×10^{11} are required [161]. Studies have shown that three dimensional methods can improve differentiation and provide more potential for scalability [162]. To achieve large quantities of potent EVs for therapy, new methods of scalable manufacturing need to be developed [163]. Aside from difficulties in obtaining the amount of MSCs needed, isolation of the MSC-EVs is also a major concern. There is currently no standard protocol for isolation of EVs at large quantities for clinical use [158]. The five major isolation methods in research are (i) differential centrifugation (ii) density gradient ultracentrifugation, (iii) size-exclusion chromatography (SEC), (iv) precipitation, and (v) immune based capture [164]. SEC has shown to be one of the more effective methods in isolating and preserving EV biophysical and functional properties, although SEC alone cannot separate plasma EV from lipoproteins [165]. Key strategies to improve EV production include hypoxic culture conditions, serum starvation, and immortalization of MSCs – all of which have deleterious implications in cell integrity and functionality and operate at small scales, thus necessitating development of feasible methods to enhance manufacturing. Good Manufacturing Practice (GMP) laboratories that have met the specific standards and been approved by the FDA will be needed to produce the quantities of clinical grade MSCs and MSC-derived EVs. A criterion for staff organization, environment/equipment qualification and monitoring, raw materials management, technical manufacturing processes, and quality controls are also involved in GMP laboratory set-up. The current traditional methods for culture and proliferation of cells consists of two-dimensional systems, which are most likely difficult to scale up to the needed amount for clinical use. Mass productions of clinical grade MSC-EVs will require a large quantity of MSCs from donors. Pachler et al. have reported the development of media formulation prepared using pooled human platelet lysate (pHPL), free from animal-derived xenogenic additives and depleted of EVs which has been validated as GMP-grade [44]. Choice of reagents and media supplements in supporting a GMP-compliant process has to be evaluated and confirmed. Based on safety considerations, use of human material such as pooled human platelet lysate (pHPL) is favorable; however, scalability issues with acquiring sufficient amount of pHPL necessitate the development and use of chemically defined media.

In order to make EV therapeutics available for patients, it is critical to ensure scalable, reproducible and good manufacturing practice (GMP)-compliant manufacturing protocols while conforming with regulatory protocols and policies. An indispensable parameter in biomanufacturing of MSC-EVs is regulatory compliance prior to obtaining manufacturing licenses and market authorization. The regulatory protocols involve major release criteria such as purity, identity, quantity, potency and sterility which will need to be met before products can be released for clinical use. With optimized large-scale production and isolation techniques, MSC-EVs manufactured in GMP laboratories are expected to undergo extensive preclinical and clinical trials to ensure the safety and efficacy for approved clinical use.

3.7.2. Biodistribution

Biodistribution is an important aspect of EV therapy that must be considered when investigating their use as a therapeutic tool [158]. MSC-EVs take a variety of different pathways throughout the body, resulting in different final concentrations at target sites than originally intended to be delivered to the targeted tissue owing to less optimal biodistribution. Studies have shown that the factors that primarily impact distribution are administration route, cellular origin, concentration, and time [166]. EVs are also heterogenous and therefore can contain vesicles of a wide variety of sizes within a sample. A review by Kang et al. compared the biodistribution to different target organs of large versus small EVs administered by varying methods. The data in this review compared the EV localization for liver, spleen, lungs, kidneys, brain, heart, muscle, and GI tract target organs. A majority of studies have documented varying localization of EVs in the target organs over time [145]. For effective EV-based clinical therapies, a better understanding of their biodistribution is needed [167]. *In vivo* tracking can be used to determine and quantify the distribution of EVs [166]. The ideal method should be specific, have a high signal-to-noise ratio, and indicate EV half-life [168].

3.7.3. Targeting specific tissues

While the mechanism of therapeutic action of EVs remains unclear, EVs contain cargo including membrane proteins, cytoplasmic proteins, mRNAs, and microRNAs which can all be delivered to recipient cells. It is speculated that the therapeutic effect stems from the transfer of miRNAs to diseased and injured cells [169]. To target specific tissues, EV cargo can be engineered and manufactured to contain desired elements [170]. EVs can be loaded with therapeutic agents (drugs, targeting peptides, imaging contrast agents, etc.) which makes them further promising for applications in drug delivery and regenerative medicine. Targeting strategies include enhanced cellular uptake, organ tropism, and immunomodulation, all of which enhance EV accumulation to specific tissues. While there is evidence to support the modification

of EVs for specific cell targeting, there is still controversy as to what is the most safe and effective method for clinical use [166].

3.7.4. Quantification and characterization

There is currently no standardized method for rapid quantification and characterization of EVs. The International Society of Extracellular Vesicles proposed a set of guidelines for the field known as Minimal Information for Studies of Extracellular Vesicles (MISEV). These recommendations were developed to improve reliability and reproducibility of published EV results. EVs have a specific structure containing proteins, lipids, nucleic acids, and other biomolecules [171]. The latest guidelines, MISEV2018, states that EVs can be quantified through assays that identify each of these components individually, however none of these will generate values that will perfectly correlate with the amount of EVs within a sample. Common methods of quantification used in research include flow cytometry, BCA protein assay, Western blotting, fluorescence microscopy, and capillary electrophoresis. The issue with these methods is that they all are time and sample demanding [16]. A recent study has presented a microfluidic technique that integrates particle separation, sizing, biomarker detection, and quantification on one single microfluidic chip, with only few microliters of sample and minutes of operating time. This strategy combines a diffusion sizing technique with a multiwavelength fluorescence detection. It contains a nonreactive dye and allows different EV biochemical components to be individually tracked such as lipids, primary amines, and EV-specific proteins. Fluorescence-based microfluidic diffusion sizing technique (fluoMDS) can robustly measure the average size of the EVs and quantify the amount of free and bound antibodies without requiring additional secondary antibodies or washing steps [172]. While this technique cannot fully replace certain characterization techniques, it holds a great promise for future rapid technologies.

3.7.5. Engineered EVs

Extracellular vesicles can be modified and engineered to improve the yield, targeting efficiency, and content of beneficial cargos. Strategies for EV engineering includes genetic modification of EVs, molecular modification of the EV membrane and the loading of nucleic acids, as well as building of EV mimetic nanovesicles. Physical and surface level modifications can be made to enhance effectiveness. This includes EV loading and insertion of peptides/proteins into the EVs membrane [134]. There is also an established content modifications process which includes loading the EVs with proteins, lncRNAs, and miRNAs. Various techniques can be used to allow drugs to rapidly enter into the membrane, holding promise for therapeutical applications of EVs [161]. Genetic modification of exosomes to express cardiac-targeting peptide (CTP)-Lamp2b stabilized by attaching glycosylation sequences showed 15% increase in targeted delivery to heart in mice (*in vivo*), as well as increased uptake by HEK293 cells and H9C2 cell lines (*in vitro*) [173]. Polycationic membrane-penetrating TAT peptides to encapsulate siRNAs into EVs have also been evaluated. Total internal reflection fluorescence microscopy (TIRFM) showed siRNA co-localization, indicating success in delivering siRNA into EVs. Delivery of siRNA mixtures by engineered EVs effectively downregulated androgen receptor expression and induced LNCaP-AI cell apoptosis in cancer cell lines [174]. Encapsulation of curcumin (an anti-inflammatory agent) in EVs increased the survival rate and lowered inflammation in a mouse model of lipopolysaccharide-induced septic shock, suggesting that the specificity of EVs could be directed to allow accumulation of curcumin to the inflammation site to mediate anti-inflammatory outcomes [175]. Enhancement of tumor targeting and anti-tumor effects of EVs have been achieved by genetically engineering mouse immature dendritic cells to express Lamp2b with iRGD peptide [176]. Maintenance of physicochemical stability and functional stability is important during surface and genetic modification of EVs whilst also ensuring safety of the final EV product.

3.7.6. Safety and efficacy of dosage

Prior to use of EVs in a clinical setting, a safety profile must be established. While we know that many of the harmful effects of other cell-based therapies are absent in EV-based treatments, there are still several concerns to be considered [158]. A primary concern is the potential of MSC-EVs, owing to the immunomodulatory nature of MSCs, to inhibit anti-tumor immune response and possibly promote tumor growth [177]. Studies have shown that EVs immunomodulation effects are correlated to the source type and state of cell origin [178]. The immunogenicity depends on the expression levels of MHC-class I and II, as well as molecules such as CD80 and CD86 [179]. Along with concerns of the safety of EV treatment, the optimal dosage for maximal efficacy is yet to be determined [180]. The biological effects of EVs are concentration-dependent at the target site [181]. A study attempted to determine whether low or high doses of EVs would be more effective in regeneration of neuronal cells in stroke patients. Low, intermediate, and high doses were intravenously injected in a stroke induced rat model. It was concluded that a dose of at least 50 μg was needed to promote proliferation and differentiation of neuronal cells [182]. While these research findings are promising towards determination of optimal dosage, more studies investigating varying tissue target sites are needed for clinical use.

3.8. Conclusion

Overall, EVs secreted from MSCs exhibit therapeutic effects such as tissue repair and regeneration, suppression of inflammatory responses, immune system modulation, and a variety of other mitogenic and differentiation inducing functions. Despite the properties of MSC-EVs and their significant potential for therapeutic success in preclinical investigations, there are still several challenges that need to be addressed. From a practical standpoint, standard methodologies for EV isolation and large-scale production, high-throughput characterization, and effective administration require standardization and optimization in order to be applied as efficacious, safe, and potent cell-free therapies. Taken together, advancements in research underpinning the mechanism of therapeutic action of EVs, along with the development of preclinical models to test the therapeutic efficacy will accelerate novel discoveries on MSC-EVs towards achieving successful translation and clinical implementation.

Declaration of competing interest

The authors indicate no conflict of interest.

Acknowledgements

We acknowledge funding support from the National Science Foundation Future Manufacturing Grant (to RMS), Musculoskeletal Transplant Foundation Biologics (Junior Investigator Grant to RMS), Women's Giving Circle (to RMS), Undergraduate Summer Research Grant (TW), Honors College Research Grant (VM, ES), College of Engineering, and the Department of Biomedical Engineering, University of Arkansas.

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