Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications

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Mesenchymal stem (stromal) cells (MSCs) are multipotent cells with the ability to differentiate into several cell types, thus serving as a cell reservoir for regenerative medicine. Much of the current interest in therapeutic application of MSCs to various disease settings can be linked to their immunosuppressive and anti-inflammatory properties. One of the key mechanisms of MSC anti-inflammatory effects is the secretion of soluble factors with paracrine actions. Recently it has emerged that the paracrine functions of MSCs could, at least in part, be mediated by extracellular vesicles (EVs). EVs are predominantly released from the endosomal compartment and contain a cargo that includes miRNA, mRNA, and proteins from their cells of origin. Recent animal model-based studies suggest that EVs have significant potential as a novel alternative to whole cell therapies. Compared to their parent cells, EVs may have a superior safety profile and can be safely stored without losing function. In this article, we review current knowledge related to the potential use of MSC-derived EVs in various diseases and discuss the promising future for EVs as an alternative, cell-free therapy.

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INTRODUCTION

Regenerative medicine focuses on the restoration of lost, damaged, or aging cells and tissues in the human body. Ferrari *et al.*[1] demonstrated the value of a stem cell-based regenerative treatment for muscular dystrophies using bone marrow (BM)-derived myogenic progenitor cells. Since then numerous stem cell types have been investigated for use in tissue regeneration in both animal models and human clinical studies, with varying degrees of success.

Mesenchymal stem (or stromal) cells (MSCs) have emerged as a potential solution for tissue repair and wound healing.[2] MSCs are multipotent, nonhematopoietic adult stem cells, which can be isolated from BM, umbilical cord,[3,4] placental or adipose tissue. MSCs have the potential to differentiate into osteoblasts, chondrocytes, and adipocytes[5] as well as endothelial, cardiovascular, and neurogenic cell types and are gaining credibility as a therapeutic agent because of their *ex vivo* expansion capacity and ethical acceptability.[6] More recently, it has been discovered that, in addition to their direct role in tissue regeneration, MSCs have potent anti-inflammatory and/or immunosuppressive properties.[7] Extensive research and clinical trials are currently underway for the use of MSCs as regenerative agents in many diseases including spinal cord injury, multiple sclerosis, Alzheimer's disease, liver cirrhosis and hepatitis, osteoarthritis, myocardial infarction, kidney disease, inflammatory bowel disease, diabetes mellitus, knee cartilage injuries, organ transplantation, and graft-versus-host disease (http://www.clinicaltrials.gov; accessed November 2014).

PARACRINE ACTIONS OF MSCs

González *et al.*[8] studied the contact-dependent mechanism of human adipose-derived MSCs in regulating inflammatory cytokines. In their study, they determined that human adipose-derived MSCs and macrophages both produce high levels of interleukin-10 (IL-10) only after cell-to-cell contact is maintained.[8]

Although potentially triggered by cell-to-cell contact events, the regenerative potential of MSC therapies has been found—at least in part—to be mediated via paracrine actions.[9] For example, the paracrine effect of MSC-conditioned medium (CM) was observed to protect cardiomyocytes by interfering with the mitochondria-mediated apoptotic pathway. In this study, application of MSC-CM to cardiomyocytes exposed to hypoxia/reoxygenation reduced apoptosis through inhibition of the release of cytochrome C from mitochondria and reduction of caspase-3 activation.[10] Similarly, renoprotective effects of human umbilical cord blood-derived MSCs (hUCB-MSCs) in streptozotocin-induced diabetic rats was reportedly mediated through paracrine action.[11] In this case, the authors studied the effects of hUCB-MSC-CM on transforming growth factor (TGF)-β1-activated rat renal proximal tubular epithelia (NRK-52E) cells and observed attenuated expression of TGF-β1, α-smooth muscle actin, collagen I, and heat shock protein-47 mRNA and increased expression of E-cadherin and bone morphogenic protein-7 mRNA, thereby preventing diabetes kidney disease.[11]

Although it was initially believed that the potential of MSCs to differentiate into various cell types plays a crucial role in their therapeutic effects, the mechanism of action of transplanted MSCs does not predominantly include differentiating into a specific cell

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type for promoting or repairing the tissue damage in most disease settings.[12–14] Several studies have demonstrated the predominance of short-lived paracrine mechanisms among the therapeutic actions of MSCs. In one such study, Toma *et al.*[15] injected human MSCs (hMSCs) tagged with β-galactosidase into the left ventricle of immunodeficient mice. The majority of hMSCs were found in the spleen, lung, and liver, 4 days after injection. They also reported that only 0.44% of the injected hMSCs survived and, with time, they were morphologically indistinguishable from the surrounding cardiomyocytes. Other studies on systemically administered MSCs have also reported that <1% of the administered cells survive for more than 1 week and that the benefits of MSC therapy could be attributed to their secreted factors.[16–18]

In acute kidney injury (AKI), the protective effect of MSC administration was not attributed to MSCs differentiating into a tubular or endothelial cell phenotype, but to enhanced regulation of anti-inflammatory and organ-protective mediators such as IL-10, basic fibroblast growth factor, TGF-α, and B-cell lymphoma 2 (Bcl-2), reflecting primarily the paracrine function of MSCs.[19] Tögel *et al.*[20] reported the paracrine nature of cytoprotection in the immediate vicinity of administered MSCs in AKI. The authors demonstrated the production of renotropic factors—hepatocyte growth factor, and insulin-like growth factor 1 —that are known to decrease apoptosis and stimulate proliferation of renal epithelial cells.

Although these studies, and many others, provide strong evidence for the potency of MSC-secreted factors in mediating tissue repair and regeneration, the precise mechanisms by which MSCs act in a paracrine fashion are not fully understood. In addition to secreting an array of soluble factors, it has also been recognized that MSCs release large numbers of extracellular vesicles (EVs). Thus, it is of interest to consider the possibilities that the complex paracrine regenerative actions of exogenously administered MSCs and other stem cells communicate by transferring information and regulatory genes mediated, to some degree, by released EVs[9,21,22] and that EVs derived from cultured MSCs have the potential to constitute a safe, effective cell-free therapy.

EXTRACELLULAR VESICLES

EVs were first clearly described by Pan and Johnstone in 1983.[23] Initially, the release of EVs was thought to represent a disposal mechanism by which cells eliminate unwanted proteins and other molecules. After years of subsequent research, however, EV release has emerged as an important mediator of cell-to-cell communication that is not only involved in normal physiological process but also plays a role in the development and progression of diseases. Among the subtypes of EV, the most numerous, referred to as exosomes, have a diameter of 40–100 nm, can be isolated by centrifugation at 100,000 ×*g* and can be concentrated at the interface of 0.8 and 2.7M sucrose layers. Preparations of EVs, typically a mixture of exosomes and other subtypes, can be isolated from all types of body fluids including blood, urine, bronchoalveolar lavage fluid, breast milk, amniotic fluid, synovial fluid, pleural effusions, and ascites.[24] EVs can also be isolated from culture supernatants of many cell types, including T-cells, B-cells, dendritic cells, platelets, mast cells, epithelial cells, endothelial cells, neuronal cells, cancerous cells, and, as we describe in detail later, MSCs.[25–37]

BIOGENESIS OF EVs

The modes of biogenesis for exosomes and microvesicles (MVs) are completely distinct and are described in this section.

Exosome biogenesis

Although the term "exosome" has been frequently used to describe all vesicles released by cells into the extracellular milieu, it is now known that there are multiple different types of EV. The major EV subtypes that are currently recognized are listed along with their basic characteristics in **Table 1**. Because of lack of specific markers it is very difficult to distinguish between different subtypes of vesicles within mixed preparations as they have overlapping composition, density, and size. Therefore, the International Society for Extracellular Vesicles suggested that the term EVs be used preferentially to describe preparations of vesicles from body fluids and cell cultures.[38]

Exosomes are EVs of endosomal origin. The endosomal sorting complex required for transport and its associated proteins are involved in the formation of multivesicular bodies (MVBs) and intraluminal vesicles (ILV).[39] Exosome membranes are enriched in lipids such as cholesterol, ceramide, and sphingolipids that are involved in the budding of ILVs into MVBs.[40,41] As was first described during reticulocyte differentiation, ILVs are released from cells as a consequence of MVB fusion with the plasma membrane and, once released, are then termed as exosomes.[23,42] Tan *et al.*[41] further confirmed the endosomal origin of MSC-derived exosomes by detecting the components of lipid rafts. **Table 2** provides additional details about proteins involved in MVB and exosome biogenesis. Exosomes may subsequently be internalized by other cells via direct membrane fusion, endocytosis or cell-type specific phagocytosis.[43–45] **Figure 1** illustrates the intracellular sources, release and uptake mechanisms associated with exosomes and other major subtypes of EV.

Microvesicle biogenesis

MVs result from outward budding and fission of plasma membrane. Membrane budding initiated by the activity of aminophospholipid translocases to translocate phosphatidylserine to the outer

Table 1 Different types of vesicles derived from various fluids and CM

Vesicles	Size (diameter)	Sucrose gradient	Origin
Exosomes	40–100 nm	1.13–1.19 g/ml	Luminal budding into MVBs; release by fusion of MVB with cell membrane
Microvesicles	50–1,000 nm	1.04–1.07 g/ml	Outward budding of cell membrane
Apoptotic bodies	1–5,000 nm	1.16 and 1.28 g/ml	Outward blebbing of apoptotic cell membrane

MVB, multivesicular body.

Table 2 Proteins associated with exosome biogenesis

Function	Proteins	References
MVB biogenesis	ESCRT-0, -I, -II, and -III; Vps4, VTA1, ALIX, Tsg101, CHMP4, ARF6, clathrin, and PLD2	127–137
Exosome cargo	Vps4, Vps27, Tsg101, ALIX, HRS, Hsc70, Hsp90, 14-3-3 epsilon, and PKM2	39,138–141
MVE docking	RAB27a, RAB35	142,143
Exosome trafficking	RAB2B, RAB9A, RAB5A, RAB27B, syndecan, syntenin, ALIX, RAP1B, RHO	58,144–146
Exosome release	Slp4, Slac2b, DGKα kinase, TfR, VAMP7, VAMP3, PLD2	144,147–151
Fusion of MVBs	SNAP receptors (SNAREs; v-SNAREs, t-SNAREs)	152–154

ALIX, ALG-2-interacting protein X; ARF6, ADP-ribosylation factor 6; CHMP4: charged multivesicular body protein 4; DGKα, diacylglycerol kinase α; ESCRT, endosomal sorting complex required for transport; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; Hsc70, heat shock cognate 70 kDa protein; Hsp90, heat-shock proteins; MVB, multivesicular body; MVE, multivesicular endosomes; PLD2, phospholipase D2; PKM2, pyruvate kinase M2; RAB27a, ras-related protein Rab-27A; RAP1B, Ras-related protein Rap-1B; RHO, rhodopsin; SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; Slac2b, synaptotagmin-like homolog lacking C2 domains b; Slp4, synaptotagmin-like protein 4; t-SNAREs, target SNAREs; TfR, transferrin receptor; Tsg101, tumor susceptibility gene 101; Vps4, vacuolar protein sorting 4; VTA1, vesicle (multivesicular body) trafficking 1; VAMP7, vesicle-associated membrane protein 7; v-SNAREs, vesicular SNAREs.

membrane.[46–48] ADP-ribosylation factor 6 plays an important role in enabling MV budding by stimulating phospholipase D activity, which in turn facilitates extracellular signal-regulated kinase activation.[49,50] Contractile protein myosin light chain kinase 2 (which contracts cytoskeleton) is phosphorylated by extracellular signal-regulated kinase, which in turn stimulates serine phosphorylation of myosin II that ultimately triggers the release of MVs.[46,50–52]

REGULATION OF EV BIOGENESIS

Earlier literature has shown that MSCs release EVs differently depending on external stimulation suggesting that this process is likely to be regulated by cross-talk between MSCs and their surrounding microenvironment.[53,54] For example, hypoxia or inflammatory conditioning of MSCs has been shown to regulate protein packaging into EVs and to affect their functional properties.[53,54] Several pathways, which may be relevant to the microenvironment in which MSCs reside, have been reported to regulate biogenesis and secretion of EVs. Tumor suppressor-activated pathway 6 is found to regulate EV formation[55] and is transcriptionally regulated by p53 thereby enhancing EV production.[56,57] An alternative cross-talk pathway was suggested by Baietti *et al.*[58] who described that syndecans interact with syntenin to regulate intraluminal budding of endosomal membrane domains containing CD63 and ALIX.

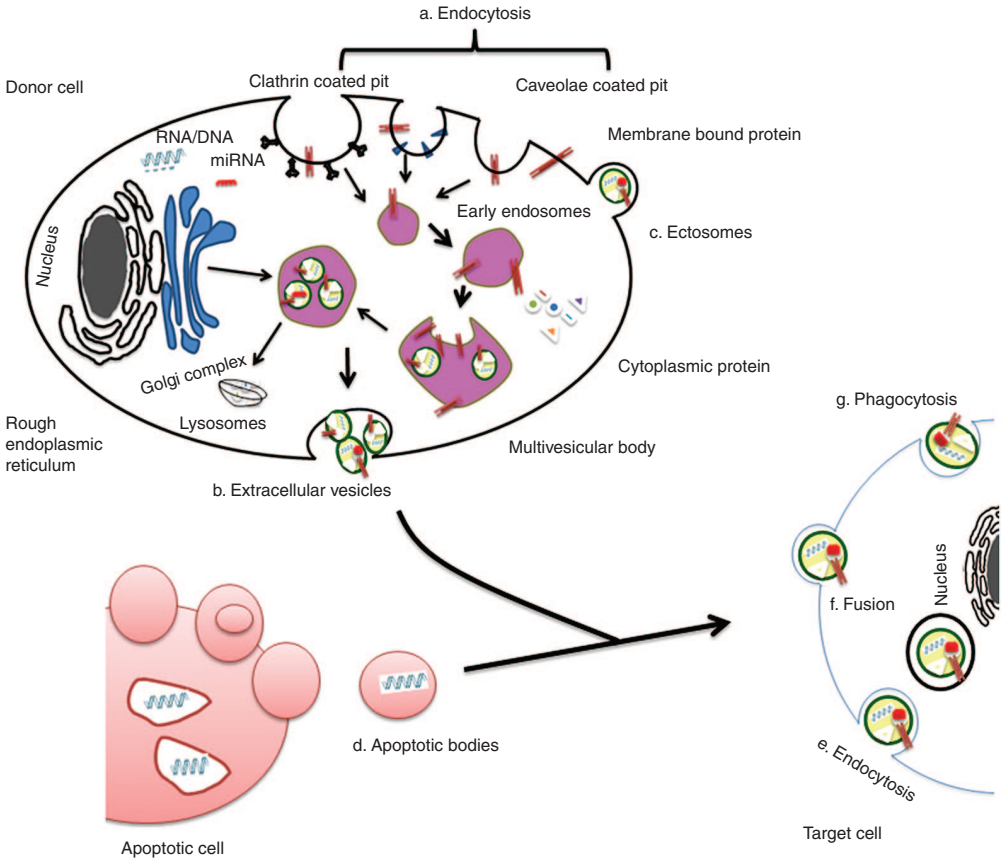

Figure 1 EVs origin and internalization. Origin of EVs are generally via (**a**) endocytosis or inward budding of plasma membrane that consist of lipid rafts and is mediated by clathrin-dependent or caveolae-dependent pathway, This gives rise to (**b**) early endosomes leading to the formation of numerous ILVs within a membrane maturing to MVBs. Finally MVBs fuse with plasma membrane releasing ILVs as exosomes. (**c**) Ectosomes are vesicles shed from the cell surface and (**d**) apoptotic bodies are also known as apobodies and are released by cells undergoing apoptosis. EVs are internalized by the target cells through several pathways including (**e**) endocytosis, (**f**) fusion, and (**g**) phagocytosis.

THERAPEUTIC EFFECTS OF MSC-DERIVED EVs (MSC-EVs)

As described earlier, EVs facilitate cell-to-cell communication via the transfer of functionally relevant biomolecules[59,60] (see **Table 3**) and thus, may be harnessed for therapeutic purposes in a similar fashion to their parent cells. From a translational perspective, EVs derived from MSCs have shown encouraging therapeutic effects in various animal models (see **Figure 2**), and their isolation from MSCs is potentially sustainable and reproducible. Furthermore, in comparison to whole cell-based therapies, MSC-EVs may offer specific advantages for patient safety such as lower propensity to trigger innate and adaptive immune responses[61] and inability to directly form tumors. For example, it has been shown that MSC-derived EVs induced anti-inflammatory cytokines as well as triggering apoptosis in activated T-cells.[62] MSC-EVs also carry mRNAs encoding immunoregulatory mediators including cytokine receptor-like factor 1, interleukin 1 receptor, and metallothionein 1X.[63]

In the remaining sections and in **Table 4**, we examine the evidence to-date for beneficial effects of MSC-EVs in several important disease areas and discuss some of the future needs and challenges that may be of critical importance to their successful clinical translation.

Table 3 Molecular composition of EVs

Source of exosomes	Protein content
Endosome-associated proteins	Rab GTPase, SNAREs, Annexins, flotillin, ALIX, Tsg101
Membrane proteins	CD63, CD81, CD82, CD53, and CD37
Lipid raft protein	Glycosylphosphatidylinositol-anchored proteins and flotillin
RNA	Structural RNAs, tRNA fragments, vault RNA, Y RNA, and small interfering RNAs

EV, extracellular vesicle; SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; Tsg101, tumor susceptibility gene 101.

MSC-EVs in cardiovascular disease

The CM obtained from hMSCs was shown by Timmers *et al.*[64] to have the potential to reduce myocardial infarct size by 60% in a porcine model of cardiac ischemia/reperfusion (IR) injury. In this same study, fractionation of the CM revealed that the cardio-protective effect was confined to the fraction containing products >1,000 kDa (100–220 nm). In a mouse model of myocardial infarction, Lai *et al.*[32] then directly demonstrated that the active, cardio-protective component of MSC-derived CM is, in fact, the EVs. In this study, administration of purified MSC-EVs reduced infarct size by ~40%.

Subsequently, Arslan *et al.*[65] reported reduced infarct size following a single intravenous injection of MSC-EVs which could be attributed to the fact that EVs are internalized by target cells at the infarct site via endocytosis or phagocytosis. To further prove that intact MSC-EVs were required for therapeutic benefit, these authors demonstrated that homogenized EVs failed to reduce infarct size.[65]

Other studies have explored mechanisms by which the number and proangiogenic effects of EVs released by MSCs can be enhanced.[66] For example, in a study of placental MSCs, under

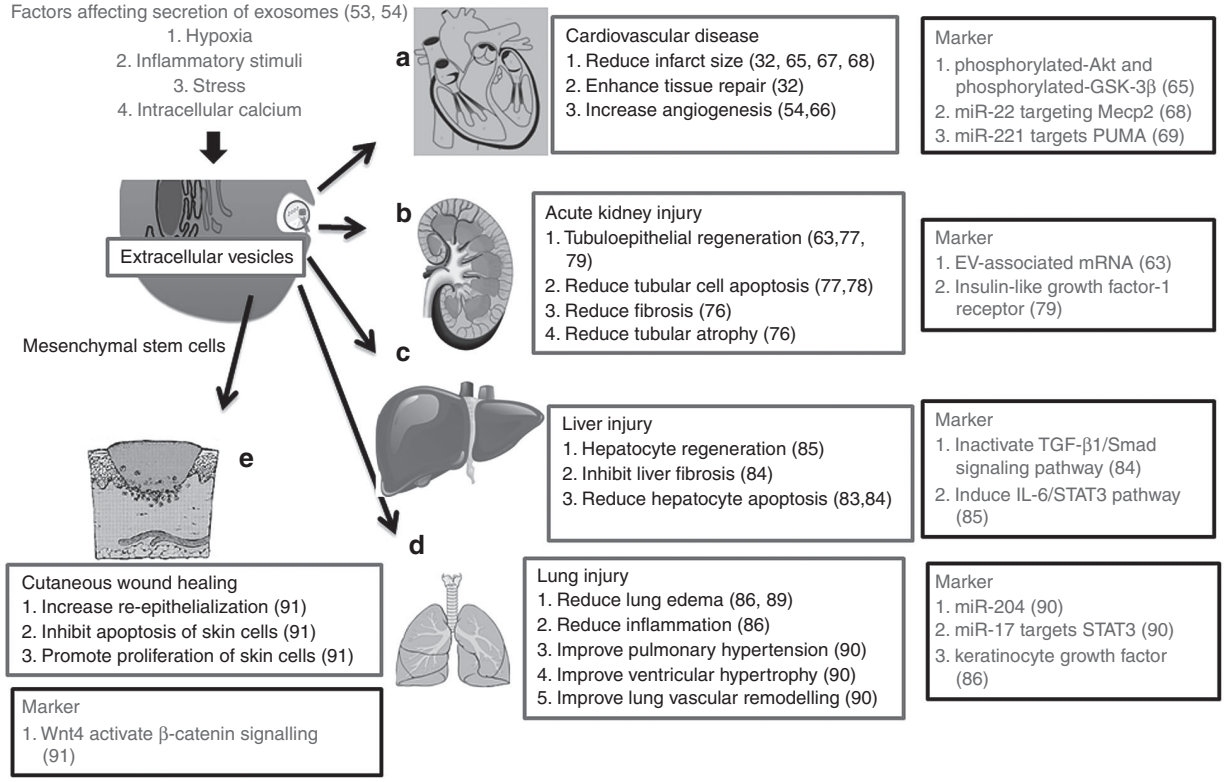

Figure 2 Potential clinical applications of EVs. Therapeutic benefits and mechanisms of action of MSC-derived EVs in: (**a**) various heart conditions, (**b**) kidney injury, (**c**) liver injury, (**d**) lung injury, and (**e**) wound healing.

Table 4 Information of MSC-derived EVs in different studies

Conditions	Model/cause of injury	Origin of EVs/mode of administration	Amount (volume)	Therapeutic capacity	Reference
Myocardial infarction	Mice/heart excision and aortic root canulation	MSC/intravenously	20 µl unfractionated MSC-CM (10–220 nm), <1,000 kDa fraction (10–100 nm), >1,000-kDa fraction, or saline	>1,000 kDa fraction 1.Confer cardio-protection 2.↓Infarct size	64
Myocardial IR injury	Mouse Langendorff heart model/heart excision, aortic root canulation, and perfusion	Human ESC-derived MSC/intravenously	0.4 µg of F1 fraction protein; 3 µg CM protein	F1 fraction + CM protein 1.↓Infarct size	32
Myocardial infarction	C57Bl6/J mice/ temporary left coronary artery ligation	MSC/intravenously via the tail vein	0.4 µg/ml	MSC exosomes 1.↓Infarct size by 45% 2.Prevents left ventricular dilatation 3.Improves cardiac performance 4.↓Inflammation	65
Acute myocardial infarction	Wistar rats/permanent ligation of the left anterior descending coronary artery	Human BM MSCs / intramyocardial injection	MSCs (2×10^6 cells); MSC-EVs (80 µg)	MSC-Evs 1.↑Proliferation, migration, and tube formation of HUVECs 2.↓Infarct size 3.Improved cardiac function 4.Angiogenesis	67
AKI	Sprague–Dawley rats/ bilateral renal ischemia	hUCB-MSC/left carotid artery	MVs dissolved in 0.5 ml PBS; control MV; IFN-γ-treated MV	MSC-MVs 1.↑Formation of T-cells with Treg phenotype 2.Ameliorated kidney dysfunction and acute tubular necrosis	53
Renal injury	C57BL6/J mice/5/6 subtotal Nx	Mouse MSC/injected through caudal veins	Nx + MSC group, 1×10^6/mouse, second day of surgery; Nx + MV group, 30 µg MV/mouse, day 2, 3, 5 after surgery	Nx + MSC and Nx + MV 1.Ameliorated renal injury 2.Prevent renal fibrosis 3.Preserved the remnant renal function	76
Chronic kidney disease	Sprague–Dawley rats/ IR injury	BM-MSCs; human fibroblasts/ intravenously	30 µg	MSC-MVs 1.↓Apoptosis tubular cells 2.↑Tubular cell proliferation 3.Protect against chronic kidney disease 4.↓Accumulation of matrix in the glomeruli	77
AKI	SCID mice/ rhabdomyolisis-induced AKI	Human BM-MSCs / intravenous injection into the tail vein	15 µg of MSC-MVs; 15 µg human fibroblasts-MVs; 75,000 BM-MSCs in 150 µl saline	MSC-MV 1.↑*In vitro* proliferation 2.↑*In vitro* apoptosis resistance 3.↑Morphologic recovery of AKI *in vivo* 4.MVs accumulated within the lumen of injured tubules	63
AKI	SCID mice/cisplatin	BM-MSCs/tail vein	Single injection—100 µg; Multiple injection—50 µg (days 2, 6, 10, 14, and 18)	MSC-MVs 1.↓Mortality induced by cisplatin 2.Improved renal function 3.Inhibited apoptosis induced by cisplatin *in vitro*	78

Table 4 (Continued)

Table 4 Continued

Conditions	Model/cause of injury	Origin of EVs/mode of administration	Amount (volume)	Therapeutic capacity	Reference
AKI	CD1 nude mice/ intramuscular injection of glycerol	BM-MSCs/ intravenously	200 μg	MSC-EVs accumulate specifically in kidneys	80
Liver injury	C57BL/six mice/carbon tetrachloride (CCl_4)	MSCs/intrasplenic injection	0.4 μg (100 μl PBS)	MSC-EV 1.Reverse CCl_4-induced injury 2.↑Proliferation of hepatocytes 3.Up-regulated cell-proliferation markers 4.Induced hepatocyte-regenerative genes expression in liver tissue after CCl4-induced injury	85
Liver injury	Mice/CCl_4	hucMSCs/injected into livers	250 μg (330 μl PBS)	hucMSC-Ex 1.CCl_4-induced liver fibrosis significantly alleviated 2.Inhibit epithelial-to-mesenchymal transition 3.Ameliorate CCl_4-induced liver fibrosis	84
ALI	C57BL/six mice/ endotoxin from *E. coli*	hMSCs/intravenously, external jugular vein or intratracheal	30 μl of MVs released by 1.5×10^6 serum starved MSCs; 750,000 MSCs	MSC-MVs 1.↓Influx of inflammatory cells 2.↓Edema 3.Transfer of KGF mRNA	86
ALI	HPH mouse/HPH	hWJMSC/jugular vein, tail vein	0.1 and 10 μg	Exosome treatment 1.Suppress hypoxic inflammation 2.Inhibits lung vascular remodeling 3.Prevents hypoxic pulmonary hypertension	90
Skin deep second-degree burn wound	Sprague–Dawley rats/ injured with 80°C water for 8 seconds to create 16 mm diameter wound	hucMSC/subcutaneous	200 μg exosome (200 μl PBS); 1 × 106 cells (hucMSC and HFL1)	Exosome treatment 1.↑Cell proliferation 2.↑Re-epithelialization 3.Inhibits heat stress-induced apoptosis *in vitro* 4.Prompt wound healing	91
Multiple myeloma (MM)	SCID mice/N/A	BM-MSCs (healthy subjects, relapsed/ refractory MM patients/implanted subcutaneously)	3×10^6 cells/tissue-engineered bones; 1 μg exosomes	MM BM-MSC-derived exosomes 1.↑MM cell growth *in vitro* 2.↑Tumor growth *in vivo* 3.↑BM homing	109
Angiogenesis, tumor growth	BALB/c nu/nu mice /N/A	Human BM-MSC, human lung fibroblast/ subcutaneous injections	SGC-7901 cells alone (1×10^6); SGC-7901 cells (1×10^6) mixed with MSCs (1×10^6); SGC-7901 cells (1×10^6) mixed with MSC exosomes (200 μg/ml)	SGC-7901 cells mixed with exosomes 1.↑Tumor growth 2.↑Proliferation of tumor cells *in vivo* 3.↑Tumor angiogenesis	110
Angiogenesis	BALB/c mice	Mouse BM-derived MSCs/subcutaneous injections	100 μg (100 μl PBS); 2×10^5 4T1 cells mixed with 100 μg of MSC-derived exosomes or 2×10^5 4T1 cells mixed with 200 μg of MSC-derived exosomes	MSC-derived EVs 1.↓VEGF expression in 4T1 cells 2.↓Angiogenesis *in vitro* and *in vivo* 3.↓Tumor growth *in vivo*	111

Table 4 (Continued)

Table 4 Continued

Conditions	Model/cause of injury	Origin of EVs/mode of administration	Amount (volume)	Therapeutic capacity	Reference
Bladder tumor growth	BALB/c nu/nu mice	hWJMSC/ subcutaneous injection	1×10^7 T24 cells; 1×10^7 T24 cells mixed with 1×10^7 hWJMSCs; 1×10^7 T24 cells mixed with 200 μg protein hWJMSC-MVs; 200 μg protein hWJMSC-MVs.	hWJMSC-EVs + hWJMSCs 1.↓ Significantly tumor size 2.↑ Apoptosis	92
Hepatoma growth	SCID mice	HLSCs/intratumor injection	100 μg of EVs (20 μl)	HLSC-derived EVs 1.↓Significantly tumor size 2.↑Apoptosis	94
Breast cancer	CB-17/Icr-scid/scidJc1 mice	BM MSC	BM2 cells (20,000) treated with 3 μg of BM-MSC-derived EVs were then injected in mammary fat pad (100 μl injections of PBS containing 1×10^5 BM2 cells)	BM-MSC-derived EV-treated cells 1.↓Proliferation 2.↓Tumor formation	96

Up arrow (↑) indicates increased and down arrow (↓) indicates decreased activity.
AKI, acute kidney injury; ALI, acute lung injury; BM, bone marrow; CM, conditioned medium; EV, extracellular vesicle; HLSCs, human adult live stem cells; HLSC, human adult liver stem cell; HPH, hypoxia-induced pulmonary hypertension; HUVEC, human umbilical vein endothelial cells; hWJMSC, human umbilical cord Wharton's jelly MSC; IR injury, ischemia/reperfusion injury; KGF, keratinocyte growth factor; MSC, mesenchymal stem (stromal) cell; MV, Microvesicle; Nx, nephrectomy; PBS, phosphate-buffered saline; SCID, severe combined immunodeficient.

hypoxic conditions, Salomon *et al.*[54] observed 3.3- and 6.7-fold increases in EV release in the presence of 1% and 3% O_2 when compared with placental MSCs maintained at 8% O_2. The resulting placental MSCs-derived EVs induced a significant, dose-dependent increase in tube formation by placental microvascular endothelial cells when compared with vehicle-treated cells.[54] It was speculated that the increased proangiogenic effect of MSC-EVs derived under hypoxic conditions may be conferred by transcriptional activities of the hypoxia inducible factor family of proteins.[54]

Following on from the above result, Bian *et al.*[67] isolated EVs from MSCs cultured under hypoxic conditions. In an *in vitro* angiogenesis assay, MSC-EVs at a concentration of 80 μg/ml, promoted human umbilical vein endothelial cell migration and tube formation that was comparable to that induced by vascular endothelial growth factor (VEGF). *In vivo* studies confirmed that intramyocardial injection of hypoxia-conditioned MSC-EVs significantly improved cardiac function and reduced myocardial infarct size with similar potency to that observed in a whole-cell MSC-treated group.[67]

Micro-RNAs associated with MSC-EVs also play an important role in cardio-protection. For instance, it was found that cardiac remodeling following myocardial infarction is regulated by miR-22-loaded EVs via targeting of methyl CpG binding protein 2.[68] Similarly, the level of miR-221 is significantly higher in MSC-EVs when compared with their parent MSCs, and this miRNA was shown to enhance cardio-protection by reducing the expression of p53 upregulated modulator of apoptosis.[69]

MSC-EVs in AKI

AKI is a major cause of morbidity and mortality among hospitalized patients and is most commonly caused by IR injury, exposure to nephrotoxic compounds, and severe volume loss or obstruction to urine flow.[70] It has been well established in animal models of renal IR and other forms of kidney injury that systemic or localized administration of MSCs results in amelioration of AKI.[71–73] MSCs downregulate proinflammatory cytokines in T-cells and consequently induce regulatory T-cells (T-regs) in the spleen.[71] Anti-inflammatory and immunoregulatory properties of MSCs have become one of the important mechanistic approaches to the treatment of AKI. A broad range of growth factors, cytokines, and chemokines secreted from MSCs have been identified including hepatocyte growth factor, insulin-like growth factor 1, VEGF, IL-1, IL-4, IL-5, IL-6, keratinocyte-derived chemokine, chemokine (C-X-C motif) ligand 16 (CXCL16), chemokine (C–C motif) ligand 2 (CCL2), CCL3, chemokine (C-X3-C motif) ligand 1 (CX3CL1), and CCL5.[20,74] In experimental models, mediators such as these have been associated with enhanced cell proliferation and reduced cell apoptosis, identifying MSCs as uniquely providing multimodal therapeutic effects in AKI.[75]

Similar to MSCs, MSC-EVs are capable of modulating T-cell as well as innate immune cell functions.[53] To date, there are few reported studies that directly compare the effect of MSCs and MSC-EVs in the setting of AKI. However, in a study involving mouse 5/6 subtotal nephrectomy (Nx)—a model of chronic kidney disease–He *et al.*[76] reported that both MSC- and MSC-EV-treated mice showed strikingly similar benefits including reduced fibrosis and interstitial lymphocyte infiltration and reduced or absent tubular atrophy when compared with the untreated control group.

In the rat model of renal IR, Gatti *et al.*[77] found that intravenous injection of 30 μg of MSC-EVs prevented AKI. The administered EVs were shown to transiently accumulate within glomeruli and injured tubules in association with increased proliferation and reduced apoptosis of tubular epithelial cells.[77] This study also reported that the protective effect was specific to MSC-EVs as fibroblast-EVs were ineffective. Similarly, Bruno *et al.*[63] also reported that human BM-derived MSC-EVs accelerated renal morphologic and functional recovery in glycerol-induced AKI in immunodeficient mice by inducing proliferation of tubular cells. In this study, they also reported that the effect of MSC-EVs on the recovery of AKI was similar to that of hMSCs.[63]

The effects of human MSC-EVs were also studied in severe combined immunodeficient (SCID) mice with AKI induced by the chemotherapeutic agent cisplatin.[78] In this study, MSC-EVs significantly improved the survival (40% at day 21) by improving renal function and morphology, but were unable to prevent chronic tubular injury (see **Table 4**). Multiple injections of MSC-EVs, however, further decreased mortality in association with normal histology and renal function.[78] MSC-EVs were found to upregulate antiapoptotic genes, including B-cell lymphoma-extra large, *Bcl-2* and baculoviral IAP repeat containing 8, and downregulating cell apoptosis genes including, Caspase-1 (Casp1), Caspase-8 (Casp8) and lymphotoxin α in cisplatin-treated human tubular epithelial cells.[78] Renoprotection was also conferred by horizontal transfer of insulin-like growth factor-1 receptor via BM–MSC–EV.[79]

Grange *et al.*[80] studied the biodistribution of intravenously injected MSC-EVs in an AKI mouse model. They observed the specific accumulation of EVs at the site of injury as compared to healthy mice receiving the same quantity of MSC-EVs.[80] Overall, of the disease areas studied, AKI, caused by a variety of clinically relevant insults, represents one of the most convincing examples of a distinct therapeutic benefit of systemic MSC-EV injection.[78,80,81]

MSC-EVs in liver disease

MSCs have been shown to be of benefit in a range of acute and chronic liver disease models and clinical translation of this work is currently underway in a number of centers.[82] For example, injection of MSCs into the portal vein has been reported to protect the liver in a rat model of hepatic IR injury after partial hepatectomy. In this study, MSC administration was shown to reduce hepatocyte apoptosis and enhance liver regeneration.[83]

Fewer studies have addressed the potential benefits of MSC-EVs in chronic liver disease models. In one such study, human umbilical cord-MSC (hucMSC)-EVs were shown to specifically localize to the liver and to alleviate liver fibrosis in carbon tetrachloride (CCl_4)-induced injury by reducing hepatocyte apoptosis and hepatic lobule destruction.[84] MSC-EV administration suppressed epithelial to mesenchymal transdifferentiation via reduced TGF-β1 expression and Smad2 phosphorylation.[84] Other i*n vivo* studies have shown that MSC-EVs promote hepatocyte regeneration after CCl_4-induced injury by inducing the IL-6/STAT3 pathway and cell cycle progression.[85] In this case, the authors validated the direct hepatoprotective effects of MSC-EVs using the cell lines TAMH (an immortalized mouse hepatocyte line derived from transgenic MT42 male mice overexpressing TGF-α), THLE-2 (an immortalized primary human hepatocyte) and HuH-7 (a human hepatocarcinoma cell line) exposed *in vitro* to acetaminophen and hydrogen peroxide.[85] Increased cytoprotection compared to control-treated cells was observed following treatment with 0.1 µg/ml MSC-EVs. Thus, both *in vivo* and *in vitro* studies have confirmed that MSC-EV therapy has the potential to promote liver regeneration following acute injury by directly enhancing hepatocyte survival and proliferation[85] (see **Table 4**).

MSC-EVs in lung diseases

Endotoxin-induced acute lung injury (ALI) in mice results in increased lung protein permeability causing an inflammatory response in the alveoli that is commonly used as a model of human ALI associated with severe pneumonia or sepsis. In this model, it has recently been shown by Zhu *et al.*[86] that administration of MSC-EVs decreased the influx of total inflammatory cells into the lung by 36% and influx of neutrophils by 73%. The suppression of lung inflammation was accompanied by reduced protein permeability, thereby preventing the formation of pulmonary edema. From a mechanistic perspective, keratinocyte growth factor (KGF) has been shown to reduce lung edema and inflammation in various ALI models.[87,88] Lee *et al.* [89] reported that hMSCs produced KGF and that its secretion as a paracrine soluble factor mediated the restoration of alveolar fluid clearance *in vivo*. Thus, Zhu *et al.* [86] hypothesized that MSC-EVs transfer KGF mRNA to the injured alveolar epithelium and to verify this, they transfected the MSCs with KGF-specific small interfering RNA before isolating EVs. In keeping with this mechanism, the therapeutic effect of EVs from KGF-depleted MSCs was reduced compared to that of control MSC-EVs.

In a mouse model of hypoxia-induced pulmonary hypertension, the injection of MSC-EVs resulted in a delayed pulmonary influx of macrophages and reduced production of proinflammatory mediators compared to injection of EVs-derived from mouse lung fibroblasts.[90] MSC-EVs, upon low dose multiple administration, also ameliorated pulmonary hypertension via increasing the levels of miR-204,[90] ventricular hypertrophy, and lung vascular remodelling.[90] The authors further tested the efficacy of two sequential injections of a higher dose of MSC-EVs and observed similar beneficial effects on early and later outcomes.[90] Finally, MSC-EVs have been found to suppress hypoxic activation of signal transducer and activator of transcription 3 (STAT3) by upregulating miR-17.[90]

MSC-EVs in cutaneous wound healing

In a recently reported study by Zhang *et al.* [91], the effects of locally injected hucMSC and hucMSC-EVs were studied in a rat deep second degree burn injury model. Using a range of histological and molecular indexes of healing, the authors found that injection of hucMSCs and hucMSC-EVs resulted in comparable and significant increase in re-epithelialization when compared with burn wounds that were treated with saline, human lung fibroblasts (HFL1) or HFL1-EVs. The epithelial healing effects were replicated *in vitro* in keratinocyte and dermal fibroblast cell lines in the form of increased cell proliferation and reduced apoptosis and were shown to be mediated by MSC-EV-delivered Wnt4 resulting in activation of β-catenin signaling and by activation of the AKT signaling pathway.[91] Although additional studies are needed to confirm these striking observations in other preclinical models, the results suggest that cutaneous injury and ulceration represent one of the most promising clinical translational avenues for MSC-EV preparations.

ANTITUMOR ACTIVITY OF MSC-EVs

MSCs have also been shown to have anticancer activities. Wu *et al.*[92] demonstrated that human umbilical cord Wharton's jelly MSC (hWJMSC)-derived EVs reduce the growth of T24 bladder carcinoma cells *in vitro* and *in vivo*. The authors reported that incubation of T24 cells with various concentration of hWJMSC-EVs

(0, 50, 100, 200 μg/ml protein) resulted in cell-cycle arrest and tumor cell apoptosis.[92] Similarly, Bruno *et al.*[93] reported inhibited cell-cycle progression and induced apoptosis in HepG2 (liver) and Kaposi's cells, and necrosis in Skov-3 (ovarian cell line) when treated with MSC-EVs.

In a study carried out using human adult liver stem cell (HLSC)-EVs, Fonsato *et al.*[94] reported induction of apoptosis in HepG2 hepatoma and primary hepatocellular carcinoma cells. Significant reduction in tumor growth was also observed in the presence of MV-HLSC in SCID mice inoculated with primary hepatocellular carcinoma cells.[94] The authors concluded that the antitumor effects of HLSC-EVs could be because of selective delivery of miRNAs—a mechanism that may also explain the potential antitumor effects of MSC-EVs in some settings.

MicroRNA-9 has been associated with drug resistance via increasing the expression of P-glycoprotein.[95] Munoz *et al.*[95] reported that anti-miR-9-Cy5 was transferred from MSCs to glioblastoma multiforme cells via EVs, blocking the increase of P-glycoprotein and reversing the chemoresistance. Ono *et al.*[96] reported that BM-MSC-EVs contributed to the dormant state of BM2 cells through EV-mediated transfer of miRNA.

MSC-EVs FOR DRUG DELIVERY

EVs are natural transporters that may potentially reach a wide range of tissues following systemic administration, including the central nervous system as they have been reported to cross the blood–brain barrier.[97] As EVs consist of a bilayered lipid membrane with an aqueous core they may potentially be loaded with both hydrophilic and lipophilic drugs.[98] Furthermore, drugs could be either loaded into purified preparations of EVs[99] or applied to parent cells and incorporated during EV biogenesis.[100] Small molecules including siRNAs can also be loaded into the EVs either by electoporation or by chemical disruption.[97,101] Although little explored to date, MSC-EVs may constitute a particularly promising vehicle for drug delivery given their inherent ability to exert disease-modulatory effects and the extensive literature documenting *in vitro* modification of MSCs using genetic and nongenetic approaches. As an example, Pascucci *et al.*[102] observed that paclitaxel-treated MSCs mediated strong antitumorigenic effects because of their capacity to take up the drug and later release it in EVs. In this study, paclitaxel -treated MSC-EVs induced a dose-dependent inhibition of CFPAC-1 (human pancreatic adenocarcinoma) cell proliferation as well as 50% inhibition of tumor growth.

CLINICAL TRANSLATION OF MSC-EVs: UNRESOLVED ISSUES AND FUTURE PRIORITIES

Tumorigenesis and other potential adverse effects of MSC-EVs

Despite reported antitumor effects in some settings, there is also theoretical potential for whole cell MSC therapy to directly or indirectly induce cancerous tumors or to accelerate the progression of pre-existing cancers. Although this concern has not, thus far, been borne out in human clinical trials, subpopulations of MSC-like cells have been found in the tumor microenvironment of several human cancers including gastric adenocarcinoma[103] and osteosarcoma.[104] Furthermore, some animal model studies have demonstrated preferential migration of intravenously administered MSCs to tumors.[105,106] Although EVs clearly lack the potential to directly form tumors following *in vivo* administration, this does not imply that MSC-EV administration to human subjects is without any risk of promoting neoplasia. For instance, multiple myeloma (MM) cell proliferation has been shown to be increased in the presence of either autocrine or paracrine secretory factors of BM-MSCs.[107,108] Roccaro *et al.*[109] isolated EVs from BM-MSCs derived from both MM patients and healthy controls. In this study, the MM BM-MSC-derived EVs were found to promote MM tumor/cell growth, whereas normal BM-MSC-derived EVs inhibited the growth of MM tumor/cells both *in vitro* and *in vivo*. The MM BM-MSC-derived EVs were also found to induce cell dissemination and metastasis to distant BM niches.[109]

MSC-EVs have been found to modulate the tumor microenvironment, creating a niche for cancer cell metastasis and have been proven to mimic the effects of MSCs to promote tumor growth. Zhu *et al.*[110] showed that MSC-EVs co-implanted with SGC-7901 (human gastric cancer) cells increased tumor growth and angiogenesis when compared with SGC-7901 cells alone. However, Lee *et al.*[111] reported contradictory results suggesting that MSC-EVs suppress angiogenesis *in vitro* by downregulating the mRNA and protein levels of VEGF in tumor cells in a concentration-dependent manner. They speculated that this inconsistency could be because of different tumor types or MSC heterogeneity.[111]

Intravascular infusion of MSCs has been documented to cause embolism and death in experimental animals,[112] whereas MSCs inoculated into infarcted myocardium were reported to induce adverse cellular growth such as cardiac sympathetic nerve sprouting.[113] For adverse effects such as these, it appears likely that the risk associated with MSC-EV administration will be significantly lower or perhaps absent. However, as evidence of striking efficacy in a variety of disease settings now exists, it is incumbent on the research community to carefully evaluate the short- and long-term safety of biologically active EVs. Based on this limited information, it is clear that successful translation of MSC-EVs as a clinical therapy will require a significant amount of additional preclinical investigation of the interaction between MSC-EVs and tumor cells.

Large-scale EV production for clinical use

Although MSCs are relatively easy to expand using conventional tissue flasks and bioreactors, their growth in culture is finite and their biological properties may become altered with repeated passage. In order to facilitate large-scale MSC-EV production, new batches of MSCs will have to be periodically derived with significant impact on the costs of derivation, testing, and validation.[114] Strategies such as MSC immortalization by natural selection or by genetic modification or clonal isolation could be used to overcome this limitation although this would also raise specific safety issues.[115,116] Chen *et al.*[117] proposed a robust scalable manufacturing process for therapeutic EVs through oncogenic immortalization of human embryonic stem cell (ESC)-derived MSCs. As EVs are isolated from media conditioned by cells, MSC culture in serum-free media would be of specific value to limit extraneous biological activity within the final therapeutic product. Other approaches to enhancing the purity of MSC-EVs preparations

could include sequential centrifugation, filtration, and ultracentrifugation followed by sucrose density gradient to remove contaminating protein aggregates, cell debris, and genetic material.[118–120] To scale up the amount of EVs isolated, bioreactors could be used to culture the MSCs.[121] In this regard, a small number of studies have documented significant increases in EV yield from cells cultured in bioreactor systems when compared with conventional tissue culture flasks.[122] It will be important, however, to also determine whether bioreactor culture conditions result in alterations to EV protein and RNA content that may impact on therapeutic efficacy.[123,124] There are many challenges related to bioreactor culture including adequacy of oxygen supply, hydrodynamic shear stress, metabolic byproducts build-up, and pH balance.[125,126] One should also be mindful that the impacts of such parameters are likely to differ for different cell types.

CONCLUSION

As we have summarized in this article, EVs can be readily isolated from MSCs of various origin and MSC-EVs are now known to have striking therapeutic benefits in a range of animal disease models. In some cases, these effects have been clearly shown to be of equal potency to those observed with whole cell MSC administration. The mechanisms underlying the anti-inflammatory and proregenerative effects of MSC-EVs have not yet been fully elucidated and are likely to vary from one disease target to another. Nonetheless, the fundamental basis for MSC-EV therapeutic effects lies in their ability to transmit biological information—in the form of proteins, glycoproteins, lipids, and ribonucleic acids—from stem cells to injured cells.

MSC-EVs have theoretical advantages over intact MSCs as a medicinal product and may, in the future, gain preference over whole cells in the discipline of regenerative medicine. However, in order for the field to advance to widespread clinical use of MSC-EVs for common human diseases, a range of important questions regarding their definition, standardization, cost-effective production, optimal dosing, and, most importantly, safety must be methodically addressed and answered.

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