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Mesenchymal stem cell-derived extracellular vesicles for renal repair

Arash Aghajani Nargesi, MD, MPH[1], **Lilach O. Lerman, MD, PhD**[1], and **Alfonso Eirin, MD**[1]

[1]Division of Nephrology and Hypertension, Mayo Clinic, Rochester, MN, United States

Abstract

Transplantation of autologous mesenchymal stem cells (MSCs) has been shown to attenuate renal injury and dysfunction in several animal models, and its efficacy is currently being tested in clinical trials for patients with renal disease. Accumulating evidence indicates that MSCs release extracellular vesicles (EVs) that deliver genes, microRNAs and proteins to recipient cells, acting as mediators of MSC paracrine actions. In this context, it is critical to characterize the MSC-derived EV cargo to elucidate their potential contribution to renal repair. In recent years, researchers have performed high-throughput sequencing and proteomic analysis to detect and identify genes, microRNAs, and proteins enriched in MSC-derived EVs. The present review summarizes the current knowledge of the MSC-derived EV secretome to shed light into the mechanisms mediating MSC renal repair, and discusses preclinical and clinical studies testing the efficacy of MSC-derived EVs for treating renal disease.

Keywords

mesenchymal stem cells; extracellular vesicles; microvesicles; exosomes; kidney

Introduction

Acute kidney injury (AKI) is a common disorder defined by an abrupt loss in renal function that remains an important challenge in developed and developing countries. AKI is responsible for approximately 1.9% of all hospitalizations in the United States [1], and the incidence of severe dialysis-requiring AKI is estimated to be nearly 30 per 100,000 person per year [2]. Moreover, AKI is associated with increased short term mortality and long term risk of Chronic kidney disease (CKD) and other complications [3].

CKD is the progressive loss of renal function that affects over 200 million people worldwide [4], and is associated with increased morbidity and mortality rates [5]. According to the most recent reports of the Global Burden of Disease Study, CKD was ranked as the 25th leading cause of death in 1990, but rose to the 18th place in 2010 [6]. Similarly, total mortality for CKD rose by 31.7% from 937,000 deaths in 2005 to more than 1,234,000

Correspondence: Alfonso Eirin, MD, Division of Nephrology and Hypertension, Mayo Clinic, 200 First Street SW, Rochester, MN, 55905. eirinmassat.alfonso@mayo.edu, Phone: (507)-538-9941, Fax: (507)-266-9316.

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deaths in 2015 [7]. Furthermore, the prevalence of end-stage renal disease (ESRD), the most severe stage of CKD, is estimated to be 8-16% worldwide [5]. Thus, given the continuously rising incidence and prevalence of both diabetes mellitus and hypertension, the most common risk factors for developing CKD, continue to rise, mortality attributable to CKD is predicted to increase in the next decade [8].

Both AKI and CKD also impose a severe economic burden. Costs associated with AKI represent approximately 5% of overall hospital expenses [9]. Similarly, in many developed countries, more than 2-3% of the total annual health-care budget is allotted for the care of patients with ESRD [10]. According to the U.S. Renal Data System, Medicare spent over $29 billion, or 5.9% of its total annual budget, for treatment of patients with ESRD in 2009 [11], but the annual expenditure for patients in stages 2 to 4 of CKD was about $49 billion in 2011 [12].

Current therapeutics options for patients with AKI and CKD are limited. Management of AKI is mostly conservative and there are few measures to change its course and prevent its progression to CKD [13]. Treatment of the underlying cause and preventive measurements, such as blood pressure and glucose control, is the cornerstone in the management of patients with CKD. However, dialysis and kidney transplant are the only viable therapeutic options for patients reaching ESRD [14]. Therefore, the economic burden of both AKI and CKD, the grave prospect of its rising incidence and prevalence, and the limited therapeutic options underscores the need for developing novel interventions to prevent its progression to ESRD and the costs of dialysis or organ transplantation.

Over the last couple of decades, the field of regenerative medicine emerged as a novel promising strategy to modulate the progression of AKI and CKD. Mesenchymal stem/stromal cells (MSCs) have attracted much attention over other stem/progenitor-cell types, due to their self-renewal capacity, multi-lineage differentiation, immunomodulatory properties, and potential for tissue repair. According to the International Society for Cellular Therapy, the minimal criteria for defining MSCs include the evidence of plastic adherence in culture, expression of CD73, D90, and CD105 and lack expression of CD14, CD34, and CD45 surface markers, and the ability to differentiate in vitro into adipocytes, chondrocytes and osteocytes [15]. MSCs are present in many adult tissues and can be easily isolated from different sources, including the bone marrow, adipose tissue, and umbilical cord, becoming an ideal candidate for cell-based therapy [16].

In recent years, several experimental studies have uncovered protective roles of MSCs for both AKI and CKD [17, 18]. For example, a single intravenous injection on MSCs attenuates sepsis-associated AKI and improves survival in mice [19]. Likewise, in rats with partial nephrectomy, an experimental model of CKD, injection of MSCs in the tail vein preserves renal function and attenuates renal injury [20]. In agreement, we have previously shown in swine renovascular disease that a single intrarenal injection of adipose-tissue derived MSCs with or without renal revascularization ameliorates renal injury and improves function in the post-stenotic kidney, underscoring the therapeutic potential of MSCs for preserving the post-stenotic kidney [21-23].

According to the US National Institute of Health database (ClinicalTrials.gov), more than 30 clinical trials worldwide are currently testing the safety and efficacy of MSCs to treat patients with renal-related diseases. A phase-I clinical trial in patients with AKI following cardiac surgery demonstrated that administration of allogenic MSCs into the suprarenal aorta confers early and late protection of kidney function [24]. In addition, several clinical trials are testing the safety and efficacy of autologous and allogenic MSCs to treat CKD, including diabetic nephropathy, renovascular disease, and Lupus nephropathy, among others [25]. Taken together, preclinical and clinical data have illustrated the enormous potential therapeutic value of MSCs to prevent renal injury and promote functional recovery. However, challenges remain in clinical applications as reports have indicated that delivery of live-replicating cells may promote tumor growth, malformation, or microinfarctions [26], underscoring the need of safe and effective alternatives for cell-based therapy.

Although MSCs may contribute to repopulating injured renal tissue by engrafting into renal tubular and endothelial cells [27], their regenerative effects are primarily exerted by their paracrine function [28]. In addition to the release of cytokines, chemokines, and growth factors, these cells produce and secrete extracellular vesicles (EVs), membrane microparticles that transfer mRNAs, microRNAs, and proteins to recipient cells [29]. Previous studies have shown that MSC-derived EVs transfer enhances proliferation, inhibits apoptosis, decreases inflammation, and promotes angiogenesis by altering the gene expression profile of their target cells [28, 30]. Therefore, delivery of MSC-derived EVs may be an attractive cell-free therapy for renal disease. The present review summarizes the current knowledge of the MSC-derived EV secretome to shed light into the mechanisms mediating MSC renal repair, and discusses preclinical and clinical studies testing efficacy of MSC-derived EVs in treatment of renal disease.

MSC-derived EVs

EVs released from MSCs are phospholipid bilayer-enclosed structures which can be visualized by electron microscopy techniques. Generally, EVs appear as membrane surrounded particles emerging from the MSC surface on transmission electron microscopy (Fig. 1), with “cup-like” morphology on negative staining (Fig. 2). Independent of their cell of origin, EVs can be classified by their size in exosomes and microvesicles. Although exosomes (with a diameter of 30-120nm) are generally smaller than microvesicles (ranging from 100nm to 1μm), the main distinction between these EV subgroups reside in their primary mechanisms of biogenesis [31]. Exosomes arise from endosomal compartments, known as multi-vesicular bodies, and are released upon their fusion with the cell membrane [32, 33], whereas microvesicles are formed by outward budding and fission of the plasma membrane in a process dependent on calcium and the cytoskeleton [32, 33]. MSC-derived EVs express characteristics of their parental MSCs, including the surface markers CD44, CD73, CD90, and CD105, as well as specific EV surface markers, such as CD9, CD63, and CD81 [34-36]. Importantly, MSC-derived EVs contain a vast number of mRNAs, microRNAs and proteins, which mediate the paracrine effects of MSCs by modulating several cellular pathways in recipient cells [30].

Genes enriched in MSC-derived EVs

Accumulating evidence indicates that the mRNA cargo of MSC-derived EVs is not merely a reflection of the mRNA pool in their parental MSCs. A defined set of mRNAs are selectively packed in EVs (Table 1 and Figure 3). Characterization of the transcriptome of human bone marrow MSCs and their relative-derived EVs using real-time quantitative polymerase chain reaction (RT-qPCR) arrays revealed that EVs contain a wide range of mRNAs involved in transcription (e.g. *TCFP2, RAX2, IRF6*), cell cycle regulation (e.g. *SENP2, RBL1, CDC14B*), immune regulation (e.g. *IL1RN, MT1X, CRLF1*), extracellular matrix remodeling (e.g. *COL4A2, IBSP*), cytoskeleton (e.g. *DDN, MSN, CTNNA1*), and cell differentiation (e.g. *RAX2, EPX, SCNN1G*) [37]. In another study, RT-qPCR detected a selected pattern of transcripts in EVs versus their parental bone marrow-derived MSCs, including important members of the human insulin signaling pathway, such as Insulin-like Growth Factor-1 receptor (IGF1R) [38]. We have previously characterized the mRNA cargo of EVs from porcine adipose-tissue derived MSCs using high-throughput RNA sequencing (RNA-seq), identifying selective EV enrichment for distinct classes of RNAs. Functional annotation enrichment analysis of genes packed in EVs revealed mRNA for transcription factors (e.g. *MDFIC, POU3F1, NRIP1*) and genes involved in angiogenesis (e.g. *HGF, HES1, TCF4*) and adipogenesis (e.g. *CEBPA, KLF7*) [39]. EVs also contain Golgi apparatus genes (*ARRB1, GOLGA4*) and genes involved in transforming growth factor (TGF)-β signaling (*TGFB1, TGFB3, FURIN*, and *ENG*), whereas mitochondrial, calcium signaling, and cytoskeleton genes are selectively excluded from EVs, possibly because these genes remain sequestered in organelles or intracellular compartments. Thus, these findings indicate that MSC-derived EVs contain a selective cargo of genes with potential to alter the phenotype of recipient cells and exert tissue trophic and reparative effects.

microRNAs upregulated in EVs

In-vitro studies using RT-qPCR and RNA-seq have shown that a selective group of microRNAs are upregulated in EVs compared to their parent MSCs (Table 2 and 3). For instance, miR-24 was consistently detected in MSC-derived EVs and may mediate regenerative effects of EVs in renal [40] and cardiac [41] cells after ischemia. This microRNA has the potential to modulate both apoptosis [42] and vascular inflammation [43], ameliorating tissue injury in animals treated with MSC-derived EVs. Likewise, miR-29 is preferentially included in MSC-derived EVs [44]. This microRNA has been associated with improved repair of cardiomycoytes in a model of myocardial infarction [41]. miR-29 can also regulate the expression of the anti-apoptotic gene *MCL-1* [45], and modulate inflammation by suppressing the expression of *ZFP36* [46], which encodes for a protein that regulates tumor necrosis factor (TNF)-α production [47]. In addition, we and others have shown that several members of let-7 family, microRNAs highly conserved across different species [48], are packed in MSC-derived EVs [39, 49]. These microRNAs have the potential to modulate cell cycle and proliferation [50, 51], inflammation [52], cellular repair [53], and osteogenic differentiation [54].

Importantly, the microRNA cargo of MSC-derived EVs depends on the tissue source of MSCs. Characterization of the microRNA content of EVs released from bone marrow-derived and adipose tissue-derived MSCs indicated that despite similarities in the most

represented microRNAs, the relative microRNA proportions are different between EVs obtained from different MSC populations, implying that post-transcriptional regulation might differ between bone marrow and adipose tissue MSC-derived EVs [55]. Furthermore, expression of the miR-21 has been reported to be downregulated in MSC-derived EVs in several studies [41, 56], but increases in EVs derived from bone marrow MSCs under stressful conditions, such as serum deprivation [57]. Overall, these studies demonstrate that EV-derived microRNAs are capable of fine-tuning numerous pathways in recipient cells and contribute to the biological actions of MSC-derived EVs.

Proteins enriched in EVs

Previous studies have described the biological signature of MSC-derived EVs from a proteomics perspective. Kim et al profiled the proteome of human bone marrow MSC-derived EVs and identified 730 proteins packed in EVs. Among them were MSC surface markers (e.g. CD44, CD73 and CD105) and proteins involved in pathways related to MSC self-renewal (e.g. platelet-derived growth factor receptor-β, insulin-like growth factor-2, and TGFβ induced) and differentiation (TGFβ, mitogen-activated protein kinases (MAPK), and peroxisome proliferator-activated receptor (PPAR) signaling pathways) [58]. Mass spectrometric analysis of human embryonic MSC-derived EVs detected a significant number of proteins enriched in EVs, including the pro-angiogenic proteins angiopoietin, hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF), as well as proteins that modulate apoptosis (caspase-14), inflammation (interleukin (IL)-10), and fibrosis (matrix metalloproteinase-3, TGFβ-1, TGFβ-2) [59]. In agreement, our recent proteomic studies in porcine MSC-derived EVs detected almost 5,000 proteins included in EVs, 128 exclusively detected in EVs, and 563 only expressed in MSCs [60]. Functionally, proteins enriched in EVs were involved in a wide range of biological activities, including angiogenesis (e.g. VEGF and angiopoietin-related protein-4), apoptosis (e.g. netrin-1), inflammatory response (e.g. TNF-inducible gene 6 protein), and extracellular matrix remodeling (e.g. matrix metalloproteinase-19 and TGFβ-1), whereas proteins excluded from EVs were mostly nuclear proteins, such as those involved in nucleotide binding and RNA splicing. Lastly, a recent study that characterized the proteomic profile of human bone marrow MSC-derived EVs under hypoxic conditions identified 1,927 proteins packed in EVs. Functional analysis revealed high expression of pro-angiogenic proteins and proteins associated with inflammation, TGFβ signaling, and Wnt signaling pathways [61]. Collectively, these studies identified a significant number of proteins that could contribute to the therapeutic efficacy of MSC-derived EVs.

MSC-derived EVs for renal repair

Experimental studies

Recently, several studies evaluated the potential of MSC-derived EVs to regenerate injured renal cells in experimental AKI and CKD (Table 4). Results from these studies suggest that EVs exert their trophic and reparative effects by shuttling their cargo of genes, microRNAs, and proteins to recipient cells in the kidney, attenuating renal injury and improving its recovery competence.

Several biological effects of EVs on the kidney are mediated by their cargo of mRNAs. In an in vitro model of cisplatin-induced AKI, Tomasoni et. al. demonstrated that co-incubation of damaged proximal renal tubular epithelial cells with MSC-derived EVs, which are selectively enriched with *IGF1R* mRNA, enhanced cell proliferation and repair, suggesting that the transfer of this gene to tubular cells is an important mechanism by which MSCs confer renoprotective effects in experimental AKI [38]. Similarly, Bruno and colleagues have shown that a single intravenous administration of MSC-derived EVs improved mouse survival after injection of a lethal dose of cisplatin, whereas multiple EV injections further decreased mortality, and preserved renal structure and function [62]. Administration of MSC-derived EVs up-regulated the expression of the anti-apoptotic genes *BCLX*, *BCL2*, and *BIRC8*, but down-regulated the expression of the pro-apoptotic genes *CASP1*, *CASP8*, and *LTA* in cisplatin-treated human tubular epithelial cells, suggesting that modulation of programmed cell death may contribute to MSC-derived EV-induced renal repair. Indeed, RNase treatment of EVs abrogated EV-induced in vitro proliferation and resistance to apoptosis, implying that the mRNAs shuttled by EVs activate a transcriptional program of repair in recipient cells [37]. In line with this observation, EVs released from kidney-derived MSCs pre-incubated with RNase failed to ameliorate TGF-β1-induced peritubular capillary rarefaction and tubulo-interstitial fibrosis in mice with unilateral ureteral obstruction (UUO) [63]. Likewise, in rats with gentamycin-induced AKI, bone marrow MSC-derived EVs prevented an increase in serum creatinine and urea, attenuated necrosis, apoptosis, and inflammation, and increased cellular proliferation [64], effects that were blunted when EVs were co-incubated with RNase. Administration of MSC-derived EVs immediately after IRI protected rats from AKI by inhibiting apoptosis and stimulating tubular epithelial cell proliferation, and protected against later development of CKD after AKI [65]. Yet, pretreatment of EVs with RNase abrogated these protective effects, suggesting that the renoprotective effects of EVs are mediated partly by the transfer of mRNA to target cells.

Experimental studies have demonstrated that phenotypic changes induced by MSC-derived EVs may be also mediated by their cargo of microRNAs. Using an in vitro model of ischemia-reperfusion injury (IRI) induced by ATP depletion in renal proximal tubular epithelial cells, Lindoso et. al. found that incorporation of MSC-EVs in damaged cells modulated several microRNAs related to important processes in renal recovery [66]. EV-mediated transfer of miR-410, miR-495, miR-548c-5p, and let-7a down-regulated several coding mRNAs associated with apoptosis, cytoskeletal reorganization and hypoxia, such as *CASP3*, *CASP7*, *SHC1*, and *SMAD4*. In addition, transfer of miR-375, miR-584c-5p, and miR-561 was associated with decreased expression of *SHC1*, which encodes for a signaling adapter that contributes to cell death by inhibiting pro-survival pathways [67]. EV transfer of this set of microRNAs was also associated with decreased expression of *SMAD4*, which encodes for a protein implicated in TGF-β1-mediated fibrosis [68]. In agreement, in mice with UUO, MSC-derived EVs containing selective patterns of microRNAs attenuated renal dysfunction in-vivo and reversed TGF-β1-induced morphological changes in proximal tubular epithelial cells in-vitro [44]. Therefore, these observations suggest that the anti-fibrotic effects of EVs may be, at least in part, mediated by the transfer of microRNAs that regulate targets related to renal fibrosis.

Renal oxidative stress and inflammation may be also modulated by MSC-derived EVs. Renal expression of the NADPH oxidase (NOX)-2 is up-regulated in rats with IRI, but not in those treated with intravenous MSC-derived EVs [69]. Importantly, this intervention not only mitigates oxidative stress, but also reduces apoptosis and enhances renal cell proliferation, suggesting that post-transcriptional regulation of NOX2 in renal recipient cells may be implicated in MSC-derived EVs-induced renal repair. In rats with IRI, MSC-derived EVs alleviated renal inflammation and improved renal function by suppressing the expression of C-X3-C motif ligand-1 (CX3CL1), a potent chemo-attractant protein for macrophages that also promotes interstitial fibrosis [70]. Interestingly, MSC-derived EVs were enriched with miR-16, miR-15b and miR-15a, all of which target *CX3CL1*, suggesting that post-transcriptional modulation of *CX3CL1* is an important mechanism by which MSC-derived EVs mitigate inflammation and renal injury in ischemic AKI. Furthermore, in rats with glycerol-induced AKI, treatment with human bone marrow MSC-derived EVs increased the expression of genes involved in fatty acid metabolism and downregulated the expression of those that modulate inflammation, matrix-receptor interaction, and cell adhesion [40]. However, global down-regulation of microRNAs enriched in MSC-derived EVs halted the renal regenerative effects of these particles, suggesting that EV-mediated transfer of microRNAs is implicated not only in preventing injury, but also in the healing properties of MSC-derived EVs.

Proteins enriched in MSC-derived EVs are important contributors to the renal reparative potency of MSCs. Studies in rats with cisplatin-induced AKI have shown that EVs derived from human umbilical cord MSCs attenuated tubular cellular oxidative stress, apoptosis, necrosis, and renal dysfunction [71]. Notably, MSC-derived EVs promoted cell proliferation, and subsequently renal repair through activation of several protein members of the extracellular signal regulated kinase (ERK)1/2 pathway, a subfamily of the MAPKs involved in relaying extracellular signals into intracellular responses [72]. In line with these observations, studies in rats subjected to IRI have shown that MSC-derived EV delivery upregulated ERK 1/2 protein expression, as well as the expression of the pro-angiogenic factor HGF, promoting tubular cell differentiation and regeneration [73]. Similarly, treatment with bone marrow MSC-derived EVs conferred protection against IRI in rats by decreasing the expression of several pro-inflammatory cytokines including IL-1β and TNF-α [74]. Notably, this intervention decreased the expression of the pro-apoptotic protein caspase-3, in parallel with decreased levels of serum creatinine and blood urea nitrogen (BUN) levels.

The renal anti-inflammatory potential of MSC-derived EVs is often parallel with robust anti-fibrotic properties. He and colleagues previously demonstrated that administration of bone marrow MSC-derived EVs through the caudal vein of mice with subtotal nephrectomy attenuated renal interstitial lymphocyte infiltrates [75]. Importantly, these anti-inflammatory effects were associated with decreased tubular swelling and necrosis, and tubulo-interstitial fibrosis. We have recently shown that intra-renal injection of MSC-derived EVs attenuated renal inflammation and fibrosis, and improved medullary oxygenation and renal function in pigs with coexisting metabolic syndrome and renovascular disease. Interestingly, these renoprotective effects were blunted in pigs treated with IL-10 depleted EVs, suggesting that some of the salutary effects of EVs are mediated by the cargo of this anti-inflammatory cytokine [76]. Also, MSC-derived EVs have been shown to protect rats against IRI by

modulating the expression of proteins involved in inflammation (TNF-α, NF-κB, IL-1β, MIF, PAI-1, Cox-2), oxidative-stress (NOX-1 and NOX-2), apoptosis (Bax, caspase-3, PARP), fibrosis (SMAD3, TGF-β), and angiogenesis (CD31, vWF, angiopoietin) [77], suggesting a critical role of proteins packed into MSC-derived in the renal regenerative potential of these particles. Clearly, the anti-fibrotic effects of EVs outweigh potential pro-fibrotic effects that might result from activation of the MAPK pathway.

Taken together, accumulated evidence suggests that the reno-protective effect of MSC-derived EVs is in part mediated by a selective three-component cargo (mRNAs, microRNAs and proteins). Upon release from EVs, mRNAs can be translated, increasing the protein content of recipient cells. microRNAs can inhibit the expression of multiple target genes, suppressing protein translation, whereas proteins packed in EVs can directly exert an immediate biochemical effect in recipient cells. Importantly, interactions among the different types of molecular cargo of EVs may regulate the transcriptional control of cellular function in recipient cells. In a recent comprehensive integrated analysis of the mRNA and microRNA transcriptomes and proteome of porcine MSC-derived EVs, we have found that mRNAs and microRNAs enriched in EVs are predicted to interact and control the activity of transcription factors, whereas EV proteins are capable of modulating multiple cellular phosphorylation pathways. Hence, interactions among mRNA, microRNA, and proteins may be an important mechanism driving MSC-based repair [78]. However, additional studies are needed to elucidate the molecular mechanisms by which genes, microRNAs, and proteins packed in MSC-derived EVs exert tissue trophic and reparative effects.

Clinical studies

Promising results from these experimental studies provided the impetus to apply MSC-derived EVs to address clinical needs of patients with renal disease. However, to date only one clinical trial investigated the safety and therapeutic efficacy of MSC-derived EVs in patients with kidney disease [79]. This single-center, randomized, placebo-controlled, phase II/III clinical pilot study recruited 40 patients with stage III-IV CKD (eGFR between 15-60mg/ml/min), who were randomized to receive either placebo or two doses (first intravenous and second intra-arterial) of MSC-derived EVs, one week apart. After a 12-month follow-up, EV-treated patients exhibited a significant improvement in renal function (improved eGFR and decreased serum creatinine, BUN, and albuminuria). Clinical improvement paralleled changes in plasma levels of several immune inflammatory markers, including TNF-α, TGF-β1, and IL-10. Although kidney biopsy specimens obtained 3 months after therapy did not show significant histologic changes, expression of Ki67 (a marker of regeneration) and the number of CD133 cells (possessing capabilities of clonal expansion and repair) were both upregulated in kidney samples [79]. These observations suggest that MSC-derived EVs are safe and can ameliorate the inflammatory immune reaction and improve the overall kidney function in patients with CKD. Nevertheless, additional studies are needed to confirm these results and provide further insight into the role of MSC-derived EVs in improving renal function in patients with CKD.

Conclusions and future perspectives

In this review, we summarized the evidence available from several studies in animal models of AKI and CKD, identifying a potential for MSC-derived EVs for preservation of renal structure and function. These studies suggest that EVs contribute to renal repair by virtue of their unique gene, microRNA, and protein cargo, which possess potent pro-regenerative properties. However, several aspects need to be carefully considered before moving toward clinical applications.

Both the route of MSC-derived EV delivery and the fate of these membrane particles post-transplantation may affect their efficiency for kidney repair. Experimental studies in murine AKI and CKD models suggest that administration of MSC-derived EVs in the caudal vein is feasible, safe, and effective to confer important reno-protective effects [64, 65, 75]. Likewise, we have recently shown in swine coexisting metabolic syndrome and renovascular disease that a single injection of MSC-derived EVs into the renal artery preserves the structure and function of the post-stenotic kidney [76]. Retention of MSC-derived EVs in the stenotic kidney of treated pigs peaked at 2 days and decreased thereafter, remaining at approximately 2% by 4 weeks after intra-renal injection. Moreover, injected EVs were detected in higher proportions in other organs, including the liver, lung, and spleen. Notably, we found that EVs engrafted in renal proximal and distal tubular cells, as well as in macrophages. In line with this, mice with glycerol-induced AKI, the biologic action of MSC-derived EVs required their CD44- and β1-integrin-dependent incorporation into tubular cells [37]. Further studies are needed to elucidate the precise molecular mechanisms underlying incorporation of EVs on damaged renal cells.

Fewer studies have explored whether multiple MSC-derived EV injections are associated with more pronounced improvements in renal function compared to a single delivery. In mice with AKI, multiple intravenous injections of MSC-derived EVs exerted superior pro-survival and reno-protective effects compared to single-dose regimens [62]. Contrarily, the need for several MSC-derived EV injections was not confirmed in the remnant kidney CKD mouse model [75]. Possibly, the remnant kidney cannot accommodate increased EV engraftment. Additional studies are warranted to identify the most appropriate therapeutic regimen and doses of EVs.

It is also reasonable to speculate on whether administration of MSC-derived EVs would confer more efficient renoprotection compared to delivery of their parent MSCs. Studies in murine models of AKI suggest that administration of MSC-derived EVs recapitulate the beneficial effect in kidney repair of their parent MSCs [74, 75]. Furthermore, MSC and MSC-derived EV delivery has shown similar potential to decrease fibrosis, interstitial lymphocyte infiltrates, and tubular atrophy, and preserve the remnant renal function in mice with 5/6 nephrectomy [75]. A single administration of EVs derived from MSCs immediately after induction of UUO mimicked the effects of their parent cells in protecting mice against renal failure [44]. Yet, EVs were superior to MSCs in some respects, suggesting that the former confers additional renoprotective effects. In a recent study, Lin and colleagues investigated the efficacy of MSC, MSC-derived EV, and combined MSC and MSC-derived EV therapy on protecting the kidneys from acute IR injury [77]. They found that MSCs and

MSC-derived EVs were comparably effective for decreasing inflammation and oxidative stress, and preserving kidney function. However, combined MSC and MSC-derived EV therapy was superior to either one alone in reducing proteinuria and preserving kidney function after acute IR injury. Taken together, these studies suggest that MSC and MSC-derived EVs exhibit a comparable and potentially additive effect on reducing renal injury and dysfunction. Nevertheless, further experimental research is needed to select the most appropriate regenerative therapy to improve the damaged kidney.

Employing EVs as a therapeutic tool in large scales faces practical challenges. Currently, there are no standard effective methods for EV mass production, and billions of MSCs need to be cultured in vitro to harvest only few micrograms of EVs [80]. Evidence supports that increasing intracellular calcium levels [81, 82] and modulation of ex-vivo culture conditions (thermal stress [83], hypoxia [84], radiation [85], etc.) might potentiate EV production. Likewise, sulfhydryl-blocking agents, which alter cytoskeletal function, may also enhance the rate of EV release [86].

The quality of harvested EVs, with regard to their cargo and membrane composition, also needs to be carefully controlled and standardized. The ability to MSCs to suppress the immune response raises concerns of immunologic dysfunction [87], and EVs, at least partially, share some membrane characteristics with their parent MSCs. Human bone marrow MSCs [88] and EVs [89] express MHC class I molecules. Thus, using EVs derived from a patient's own MSCs should be sufficient to avoid an immunogenic response [80]. However, EVs derived from different donors may harbor different membrane markers, which may alter their therapeutic efficacy.

Last, but not least, co-morbid conditions, such as aging, smoking, obesity, hypertension, and diabetes may compromise MSC functionality and vitality [90]. Furthermore, progressive accumulation of several uremic toxins, including advanced glycation end products, p-cresylsulfate, and indoxyl sulfate may impair the renoprotective properties of autologous MSCs in patients with CKD [91]. Therefore, whether these factors impact the genetic and protein cargo of MSC-derived EVs and their potential to repair the kidneys needs to be evaluated carefully prior to wide clinical application.

In summary, MSC-derived EVs currently emerge as a promising approach to repair damaged kidneys. However, clinical data supporting the use of MSC-derived EVs in patients with renal disease is limited to a single clinical trial in patients with CKD. No doubt, additional experimental and clinical studies are needed to further explore the mechanisms of MSC-derived EV reno-protection, and develop adequate protocols to treat patients with renal disease.

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Abbreviations

AKI	Acute Kidney Injury
CKD	Chronic Kidney Disease
ESRD	End-Stage Renal Disease
MSCs	Mesenchymal Stem/Stromal Cells
EVs	extracellular vesicles
RT-qPCR	Real-time quantitative Polymerase chain reaction
RNA-seq	RNA sequencing
TGF	Transforming growth factor
TNF	Tumor necrosis factor
MAPK	mitogen-activated protein kinases
HGF	Hepatocyte growth factor
VEGF	Vascular endothelial growth factor
IL	Interleukin
IGF1R	Insulin-like Growth Factor-1 receptor
IRI	ischemia-reperfusion injury
NOX	NADPH oxidase
CX3CL1	C-X3-C motif ligand-1
ERK	Extracellular signal regulated kinase
BUN	Blood urea nitrogen

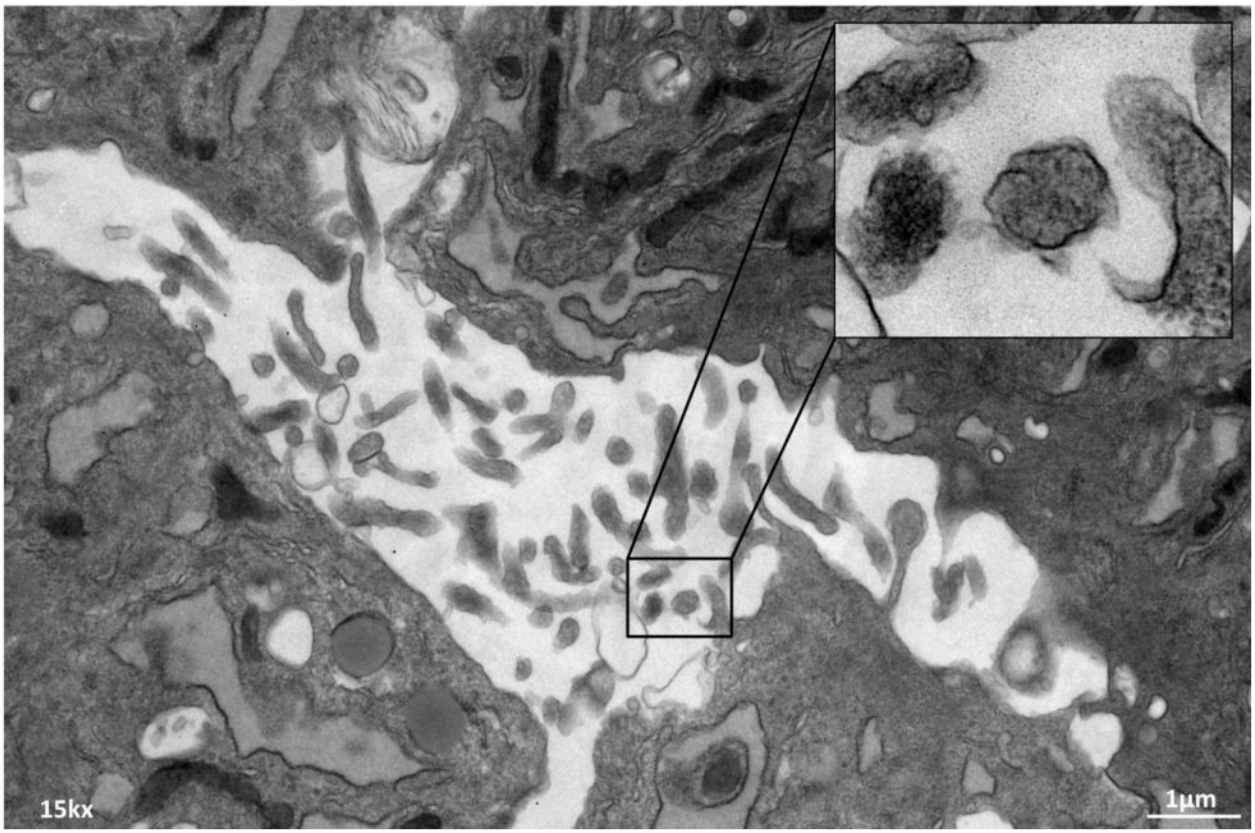

Figure 1.
Transmission electron microscopy of culture swine adipose tissue-derived mesenchymal stem cells (MSCs) showing release of multiple extracellular vesicles (EVs).

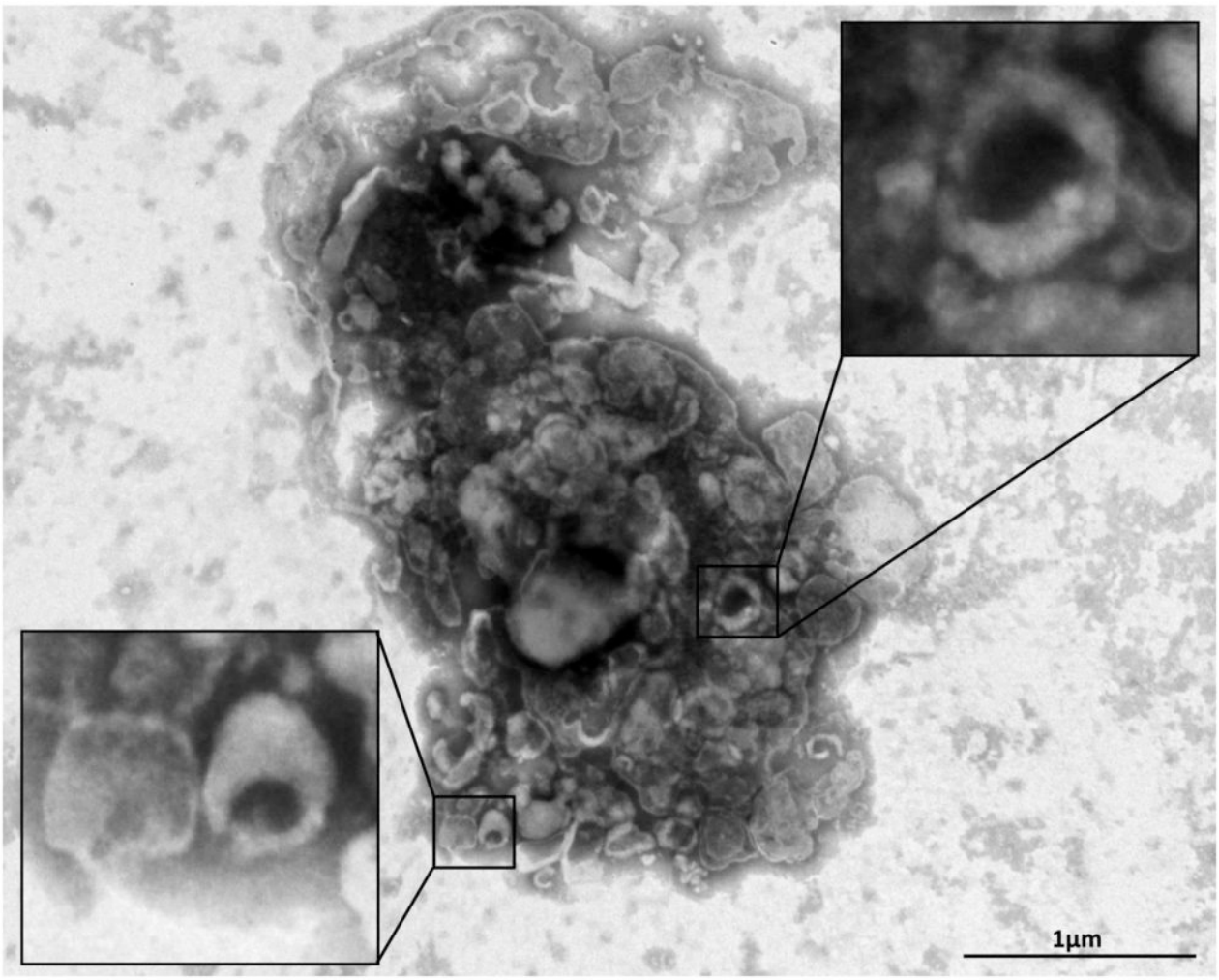

Figure 2.
Negative staining of MSC culture supernatants showing EV clusters with the classic “cup-like” morphology.

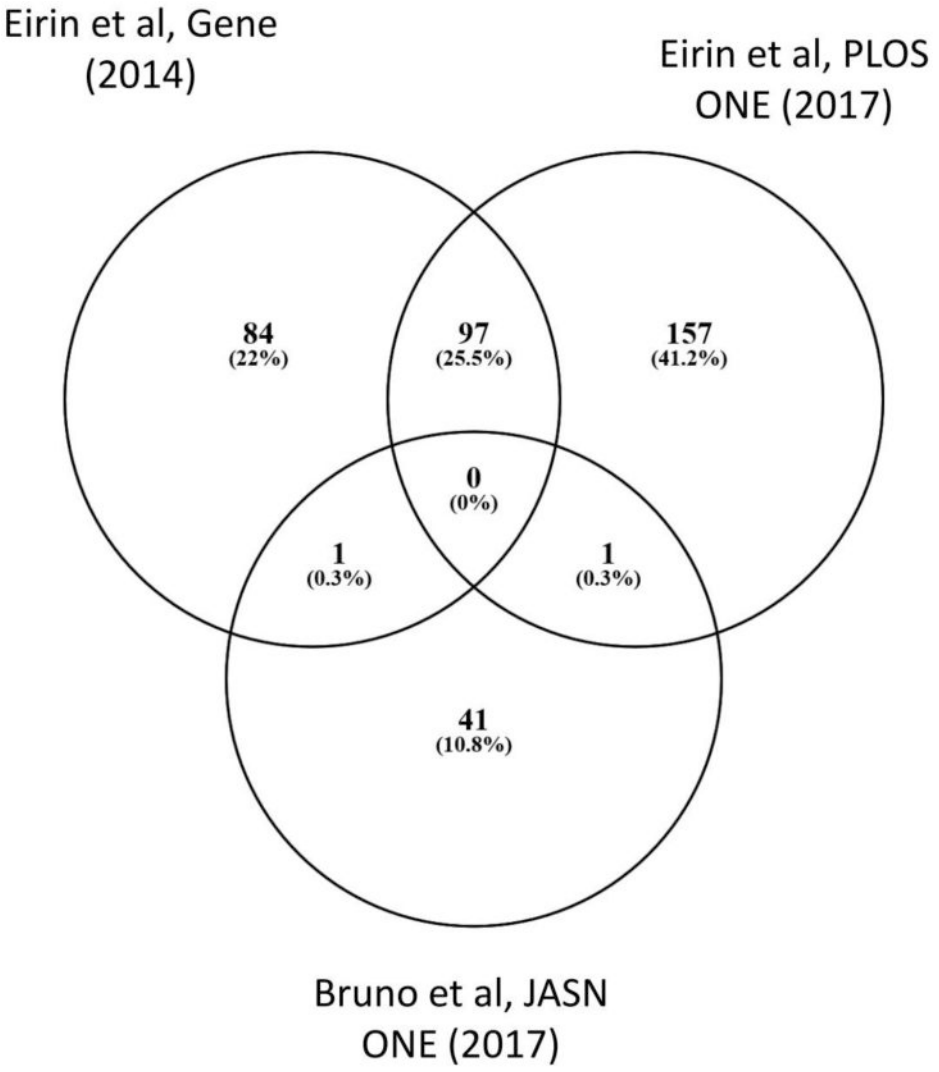

Figure 3.
Overlap of mRNAs enriched in MSC-derived EVs among studies.

Table 1

Evidence of mRNAs enriched in MSC-derived EVs.

Study	Source of EVs	mRNAs
Tomasoni et al [38], Stem Cells Dev, 2013	Mouse Bone-marrow MSCs	IGF-1R
Bruno et al [37], J Am Soc Nephrol, 2009	Human Bone-marrow MSCs	RAX2, OR11H12, OR2M3, DDN, GRIN3A, NIN, BMP15, IBSP, MAGED2, CEACAM5, COL4A2, SCNN1G, PKD2L2, HK3, EPX, CLOCK, IRF6, LHX6, RAX2, TCFP2, BCL6B, HMGN4,TOPORS, ESF1, POLR2E, ELP4,HNRPH2, SENP2, RBL1,CDC14B, S100A13, CEACAM5, CLEC2A, CXCR7, ADAM15, FUT3, ADM2, LTA4H, BDH2, RAB5A, CRLF1, IL1RN, MT1X, DDN, MSN, CTNNA1, COL4A2, IBSP
Eirin et al [39], Gene, 2014	Porcine adipose tissue MSCs	ABCC2, ACER2, ACTL10, ADAM8, ADAMTS13, ADAMTS5, AKAP5, AKAP9, ANKAR, ANKRD11, ANKRD12, ANKRD50, ARRB1, ASPN, ATP11A, AXIN2, B3GALNT1, BAZ2B, C11orf74, C1orf27, C6orf163, CACNA1G, CAPS2, CASP12, CCDC88A, CD83, CEBPA, CEP57L1, CLIC2, CLK4, CLMN, CLU, COL27A1, CREG1, CRISPLD2, DGKH, ENG, ERICH1, ERO1LB, F13A1, FBXO30, FGD4, FILIP1L, FMO4, FOXP3, FURIN, GDF1, GFPT2, GOLGA4, GPRIN1, GRM2, GUCY1A3, HES1, HINT3, HIVEP1, HSP70, HSP70, IFN-ALPHA9, IFNAR1, IFT57, IGSF3, INPP4B, ITGA11, ITM2B, IVNS1ABP, JAK, JARID2, JMJD1C, KAT6B, KBTBD11, KCNE4, KCNH6, KDM6B, KLF7, KLHL24, KRCC1, LACC1, LCOR, LDB2, LGALS1, LOC733612, LPAR6, LYRM2, MDFIC, MDM4, MGA, MKLN1, MPZL3, MXD1, MYH3, MYNN, N4BP2, NCALD, NDRG4, NFKBIZ, NIP7, NRIP1, OAF, PAIP2, PCDH7, PDCD4, PDGFRB, PEG3, PEX13, PI15, PLXNC1, PMAIP1, PPAP2B, PPP4R2, PRKAR2B, PROX2, PRR15, PRR5L, PRRX1, PTGER4, RAB39A, RAB5A, RAPGEF5, RARR, ES2, RBAK, RCSD1, REL, RFTN2, RGS1, RGS17, RICTOR, RIT1, RNF138, RNF19A, RUNX1T1, SAMD9, SAV1, SEMA5A, SEMA7A, SETBP1, SH2B3, SH3PXD2A, SIGLEC15, SIKE1, SKIL, SKOR1, SLC15A2, SLC25A36, SLC38A2, SLC47A2, SMAD5, SNAPC1, SNORA11, SNORA21, SNORD16, SNO, RD89, SPAG16, SSBP2, SUFU, SUMO1, TBK1, TBK1, TBK1, TCF4, TET2, TGFB1, TGFB3, TMEM55A, TMF1, TRPS1, TRPS1, TRPS1, UBE2H, UBN2, UFSP2, VANGL2, VPS37B, WDR43, WDR52, YPEL5, ZBED3, ZBTB1, ZFAND5, ZHX1, ZNF217, ZNF238, ZNF280D, ZNF461, ZNF568, ZNF608, ZNF667, ZSWIM6
Eirin et al [78], PloS One, 2017	Porcine adipose tissue MSCs	SNORD89, C6orf163, SKOR1, VPS37B, HINT3, ACTL10, ACER2, RNF19A, PROX2, NRIP1, TRPS1, TET2, CASP12, IGSF3, ANKRD12, ZSWIM6, RGS1, MDM4, KCNH6, CCDC88A, JARID2, BAZ2B, TRPS1, ZNF667, MYNN, CACNA1G, DGKH, INPP4B, FILIP1L, RGS17, KAT6B, ASPN, COL27A1, ZNF461, F13A1, PEG3, MYH3, PDCD4, GFPT2, CRISPLD2, ATP11A, FMO4, FGD4, ABCC2, UFSP2, CAPS2, RUNX1T1, SAMD9, SH2B3, GDF1, PRR5L, LCOR, N4BP2, ADAMTS5, SIGLEC15, SEMA5A, GOLGA4, NFKBIZ, KLHL24, C1orf27, SPAG16, UBN2, ANKAR, MXD1, ZBTB1, GPRIN1, B3GALNT1, AKAP5, ZHX1, ITM2B, WDR52, KBTBD11, REL, PRR15, RICTOR, KCNE4, PI15, FBXO30, SIKE1, RFTN2, JMJD1C, RBAK, SETBP1, ZNF217, ZNF568, TMF1, LPAR6, LDB2, PPP4R2, MGA, CLMN, LGALS1, PRRX1, LACC1, ZNF280D, SNORD16, RAB39A, PAPOLG, LYSMD2, ETV1, PPP3CA, ZNF131, BRWD1, ZFP30, GLIS3, RSPH9, SLC10A6, EFNA1, TRIP11, ITGB1, PPM1L, JMY, KLF7, LYST, C10orf118, CCNT2, ZNF260, MAP3K1, SENP1, PIGU, RING1, MLXIP, TBC1D15, CEP290, ZBED6, DDI2, FAM171B, ZCCHC6, ARRDC4, BMPR1B, ELK4, LARP1B, ARRDC3, NIPBL, MED13, SEMA6A, EFHB, TSPAN18, ZNF146, SOS2, OMD, STARD9, CARD14, MBTD1, ERBB2IP, SP4, LMBRD2, ZNF292, SLMO2, MYSM1, ERS1, NLRC3, DENND3, USP37, SAMD8, ZFP37, UBASH3B, OPRD1, SLC6A19, GCC2, ATP2A2, ARL5B, IKZF2, CACNB2, KCTD12, SMOC2, OLFML2B, PTAR1, KIAA0825, CSMD2, PITX3, ITGB8, PLAG1, CPEB4, ZNF770, ZNF404, FEM1C, ARID4B, METTL4, ZBTB37, TP53, ZNF701, SPAG9, SLC4A10, ICAM3, EGFLAM, PRKD3, SCARNA15, CDCP1, RASSF8, RSBN1, RPS6KL1, HMGCLL1, ELMOD2, CEP120, RNF183, ESCO1, MAP3K2, CHD1, CEP97, XRN1, RASAL1, SWT1, ZNF33B, ZNF827, FNDC3A, USP34, BICC1, PCMTD1, ZFHX3, NFAT5, MEGF10, FRMD4B, FREM2, NAA16, SNORA55, GALNTL5, SNORA70, PCDHA3, NBEAL1, TRPM7, NKTR, DAZAP1, GABRB1, GPR137B, NIN, BNC1, RNF217, C5orf34, SNORA46, MFI2, LHX9, MDM2, EXOC5, FHAD1, TNFSF10, PHC3, SNX29, NGFR, LRGUK, SF3B1, PNPLA8, SIRT2, ZNF354C, HSPA13, GOLGB1, RYR2, TMEM100, FAM214A, STK17B, CSPP1, DTX1, TPRG1, NTN5, CEP350, THAP5

Table 2

Evidence of microRNAs enriched in MSC-derived EVs.

Study	Source of EVs	microRNAs
He et al [44], Nephrology, 2015	Mouse Bone-marrow MSCs	miRNA-29 (29a-3p, 29b-3p, 29b-1-5p), mi-RNA-30 (30b-3p, 30b-5p, 30d-5p, 30e -3p, 30c-5p), mi-RNA-210-3p
Li et al [92], EBioMedicine, 2016	Human Umbilical cord MSCs	miRNA-181c
Shao et al [41], Biomed Res Int, 2017	Rats bone-marrow MSCs	miRNA-29, miRNA-24
Aliotta et al [93], Cardiovasc Res, 2016	Mouse bone-marrow MSCs	miRNA-34a, miRNA-122, miRNA-124, miRNA-127
Qin et al [94], Sci Rep, 2016	Human bone-marrow MSCs	miRNA-196a, miRNA-27, miRNA-206
Muntion et al [95], PLoS One, 2016	Human bone marrow MSCs	miRNA-10a, miRNA-15a
Zhang et al [53], J Am Heart Assoc, 2016	Rats bone-marrow MSCs	miRNA-147, let-7i-3p, miRNA-503-5p, miRNA-362-3p
Cui et al [96], FEBS Lett, 2016	Mouse bone-marrow MSCs	miRNA-1192, miRNA-680, miRNA-302a
Phinney et al [56], Nat Commun, 2015	Human bone-marrow MSCs	miRNA-451a, miRNA-1202, miRNA-630, miRNA-638
Collino et al [40], J Am Soc Nephrol, 2015	Mouse bone-marrow MSCs	miRNA-483–5p, miRNA-191, miRNA-28–3p, miRNA-423–5p, miRNA-744, miRNA-129–3p, miRNA-24, miRNA-148a
Wang et al [97], Stem Cell Res Ther, 2015	Rat bone-marrow MSCs	miRNA-133b-3p, miRNA-294
Ti et al [52], J Transl Med, 2015	Human Umbilical cord MSCs	Let-7b
Wang et al [98], Sci Rep, 2015	Mouse bone-marrow MSCs	miRNA-223
Eirin et al [39], Gene, 2014	Porcine adipose tissue MSCs	miRNA-148a, miRNA-532-5p, miRNA-378, let-7f
Baglio et al [55], Stem Cell Res Ther, 2015	Human bone-marrow MSCsHuman adipose-tissue MSCs	miRNA-486-5p, miRNA-10a-5p, miRNA-10b-5p, miRNA-191-5p, miRNA-222-3p,miRNA-143-3p, miRNA-10b-5p, miRNA-486-5p, miRNA-22-3p, miRNA-21-5p
Nakamura et al [99], FEBS Lett, 2015	Human bone-marrow MSCs	miRNA-494
Vallabhaneni et al [57], Oncotarget, 2015	Human bone-marrow MSCs	miRNA-21, miRNA-34a
Xu et al [54], PLoS One, 2014	Human bone-marrow MSCs	let-7a, miR-199b, miR-218, miR-148a, miR-135b, miR-203, miR-219, miR-299-5p, miR-302b
Garcia-Contreras et al [49], PLoS One, 2014	Human adipose tissue MSCs	let-7-a-1, miR-21, miR-143, miR145, miR-451a, miR-338-3p, miR-1260b, miR-1908
Ono et al [100], Sci Signal, 2014	Human bone-marrow MSCs	miRNA-23b
Feng et al [101], PLoS One, 2014	Mouse bone-marrow MSCs	miRNA-22
Lee et al [102], PLoS One, 2013	Mouse bone-marrow MSCs	miRNA-16
Xin et al [103], Stem Cells, 2013	Rat bone-marrow MSCs	miRNA-133b

Table 3

List of microRNAs consistently enriched in MSC-derived EVs across studies.

microRNA	Study
miR-29	[41, 44]
miR-24	[40, 41]
miR-34	[57, 93]
let-7	[39, 49, 52-54]
miR-302	[54, 96]
miR-451	[49, 56]
miR-191	[40, 55]
miR-148	[39, 40, 59]
miR-133	[97, 103]
miR-143	[49, 55]
miR-22	[55, 101]
miR-21	[49, 55]

Table 4

Methodological summary of published studies using MSC-derived EVs for renal repair.

Study	Isolation procedure	Qualitative and quantitative assays	EV Dose	Route of administration	Mechanism of action
Bruno et al [37], 2009	Ultracentrifugation of human bone-marrow MSCs medium	RT-qPCR, RT-PCR, Micro-array data analysis	15μg	Intravenous injection into tail vein of mice	mRNA transfer
Tomasoni et al [38], 2013	Ultracentrifugation of human bone-marrow MSCs medium	Electron microscopy, RT-qPCR, RT-PCR, IF	In vitro study	No systemic administration	mRNA transfer
Collino et al [40], 2015	Ultracentrifugation of human bone-marrow MSCs medium	RNA-seq, RT-qPCR, Micro-array data analysis, microRNA target sites enrichment analysis	2.2×10^8 EVs/mouse	Intravenous injection into tail vein of mice	microRNA transfer
He et al [44], 2015	Ultracentrifugation of mice bone-marrow MSCs medium	Western blot, RT-qPCR, RT-PCR, RNA assays, biochemical and histopathological analysis	30mg	Intravenous injection into causal vein of mice	microRNA transfer
Bruno et al [62], 2012	Ultracentrifugation of human bone-marrow MSCs medium	Morphological studies, Apoptosis TUNEL assay, IF, RT-PCR, RT-qPCR	100μg	Intravenous injection	Not studied
Choi et al [63], 2015	Ultracentrifugation of mouse kidney MSCs medium	EMT differentiation, western blot, IHC, IF	2×10^7 EVs/mouse	Intravenous injection into tail vein	mRNA transfer
Reis et al [64], 2012	Ultracentrifugation of rat bone-marrow MSCs medium	Flow cytometry, differentiation assays, biochemical and histological analysis, IF, RT-PCR, RT-qPCR, Electron microscopy	Unknown	Intravenous injection	RNA-dependent, but protein independent
Gatti et al [65], 2011	Ultracentrifugation of human bone-marrow MSCs medium	Biochemical and histological analysis, Apoptosis TUNEL assay, RNA micro-assay	30μg	Intravenous injection	RNA-dependent
Lindoso et al [66], 2014	Ultracentrifugation of human bone-marrow MSCs medium	Apoptosis TUNEL assay, RT-qPCR, RT-PCR, microRNA target prediction	In vitro study	No systemic administration	microRNA transfer
Zhang et al [69], 2014	Ultracentrifugation of human Warton Jelly MSCs medium	Electron microscopy, Surface marker analysis, biochemical analysis, histological analysis, ROS level analysis, IHC, IF, apoptosis TUNEL assay, western blot, RT-qPCR,	100μg	Intravenous injection into rat causal vein	Not studied
Zou et al [70], 2014	Ultracentrifugation of human Warton Jelly MSCs medium	Electron microscopy, Surface marker analysis, biochemical analysis, histological analysis, ELISA, IHC, apoptosis TUNEL assay, western blot, RT-qPCR,	100μg	Intravenous injection into rat causal vein	microRNA transfer
Zhou et al [71], 2013	Filtration of human umbilical cord MSCs medium	Histological study, Apoptosis TUNEL assay, IHC, mitochondrial membrane potential assay, western blot	200μg	Rat renal capsule injection	Not studied
Ju et al [73], 2015	Ultracentrifugation of human umbilical cord MSCs medium	Biochemical analysis, Histological analysis, IHC, RT-PCR, Western blot	30μg	Intravenous injection into rat causal vein	RNA transfer
Wang et al [74], 2014	Ultracentrifugation of rat bone-marrow MSCs medium	Biochemical analysis, Histological analysis, Apoptosis TUNEL assay, RT-PCR, RT-qPCR, Western blot	100μg	Unknown	Not studied

Study	Isolation procedure	Qualitative and quantitative assays	EV Dose	Route of administration	Mechanism of action
He et al [75], 2012	Ultracentrifugation of mice bone-marrow MSCs medium	Biochemical analysis, Histological analysis	30μg	Intravenous injection into mouse causal vein	Not studied
Eirin et al [76], 2017	Ultracentrifugation of porcine adipose-tissue MSCs medium	Flow cytometry, IF, surface marker analysis, Histological analysis, ELISA, RT-qPCR	1×10^10 EVs/pig	Injection into renal artery of pigs	mRNA and protein transfer
Lin et al [77], 2016	The method of harvest unknown, rat adipose-tissue MSCs medium	Biochemical analysis, Histological analysis, western blot, IHC, IF	100μg	Intravenous administration	Not studied
Nassar et al [79], 2016	Ultracentrifugation of umbilical cord MSCs medium	Flow cytometry, electron microscopy, histologic analysis, IHC, ELISA	100μg/Kg	Intravenous and intra-arterial injection	Not studied

Abbreviations: MSC: Mesenchymal stem cell; EV: Extracellular vesicle; RT-qPCR: Real time quantitative polymerase chain reaction; RT-PCR: Reverse transcriptase polymerase chain reaction; IF: Immunofluorescence; RNA-seq: RNA sequencing; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; IHC: Immunohistochemistry; ELISA: Enzyme-linked immunosorbent assay