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Anti-fibrotic and anti-inflammatory effect of mesenchymal stromal cell-derived extracellular vesicles in chronic kidney disease

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Abstract: Renal fibrosis and inflammation are common pathological features of chronic kidney disease (CKD). Since currently available treatments can only delay the progression of CKD, the outcome of patients with CKD is still poor. One therapeutic option for the prevention of CKD-related complications could be the use of mesenchymal stromal cells (MSCs), which have shown beneficial effects in tissue fibrosis and regeneration after damage. However, safety issues, such as cellular rejection and carcinogenicity, limit their clinical application. Among the bioactive factors secreted by MSCs, extracellular vesicles (EVs) have shown the same beneficial effect of MSCs, without any notable side effects. This heterogeneous population of membranous nano-sized particles can deliver genetic material and functional proteins to injured cells, prompting tissue regeneration. Here we describe the anti-fibrotic and anti-inflammatory properties of MSC-derived EVs in CKD preclinical models and summarize the potential molecular mechanisms involved in the regulation of renal fibrosis and inflammation.

List of Abbreviations

α-SMA	Alpha-smooth muscle actin
AT	Adipose tissue
AT-MSC-EV	Extracellular vesicles from adipose tissue-derived mesenchymal stromal cells
BM	Bone marrow
BCL2	B-cell lymphoma 2
BMP7	Bone morphogenetic protein 7
BM-MSC-EV	Extracellular vesicles from bone marrow-derived mesenchymal stromal cells
CCL	Chemokine (C-C motif) ligand
CCR2	C-C chemokine receptor type 2
CKD	Chronic kidney disease
CX3CL1	C-X3-C motif chemokine ligand 1
EMT	Epithelial-to-mesenchymal transition
EV	Extracellular vesicle
HLSC	Human liver stem cell
IL	Interleukin
IRI	Ischemia-reperfusion injury
IFNγ	Interferon gamma
miRNA	MicroRNA
MSC	Mesenchymal stromal cell
MSC-EV	Mesenchymal stromal cell-derived extracellular vesicles
TEC	Tubular epithelial cell
TGF-β	Transforming growth factor-beta
UC-MSC-EV	Extracellular vesicles from umbilical cord-derived mesenchymal stromal cells
UUO	Unilateral ureteral obstruction

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Introduction

Chronic kidney disease (CKD) is a progressive and irreversible loss of kidney function that could ultimately lead to end-stage renal failure, one of the major causes of mortality worldwide (Carney, 2020). CKD affects around 10% of the global population, but its incidence is expected to increase due to the enhanced prevalence of diabetes, hypertension, and obesity, which are considered important risk factors for CKD development (Jha *et al.*, 2013; Djudjaj and Boor, 2019; GBD Chronic Kidney Disease Collaboration, 2020) (Fig. 1).

One of the hallmarks and the final common pathway of CKD is renal fibrosis, characterized by an excessive accumulation of extracellular matrix and responsible for the degeneration of kidney structure and function (Fig. 1). Pro-inflammatory stimuli released by damaged renal cells recruit

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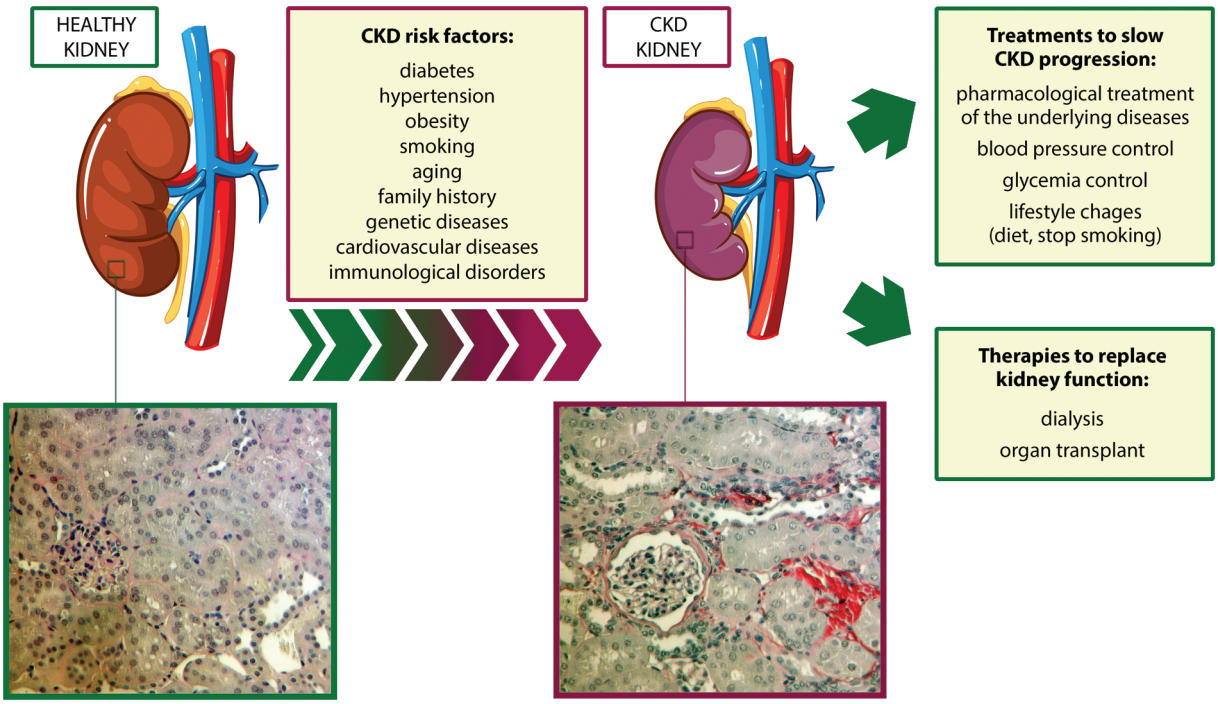

FIGURE 1. Risk factors associated with chronic kidney disease development and currently available treatments. bottom, two representative micrographs of Sirius Red staining of sections of a murine healthy kidney (left) and a murine CKD kidney after two months of ischemia-reperfusion injury (right). The red color highlights the presence of collagen fibrils, typical signs of fibrosis. Magnification 400x. Abbreviation: CKD: chronic kidney disease.

monocytes from the circulation into the interstitial space, leading to the release of inflammatory and fibrogenic cytokines (Cho, 2010). This process triggers epithelial-to-mesenchymal transition (EMT) in fibroblasts and tubular epithelial cells (TECs), which convert into myofibroblasts and increase the production of extracellular matrix components, thus perpetuating tubulointerstitial fibrosis, glomerulosclerosis, and inflammatory cell infiltration (Liu, 2010; Menon and Ross, 2016).

At present, available therapies to slow CKD progression aim to pharmacologically treat the underlying diseases and to control blood pressure and glycemia (Fig. 1), but the outcome of CKD patients is still poor (Turner *et al.*, 2012; Humphreys, 2018). Renal-replacement therapies, such as hemodialysis and kidney transplantation, are limited by high costs and organ availability (Abecassis *et al.*, 2008). For these reasons, new therapeutic strategies for CKD are urgently needed.

Mesenchymal Stromal Cell-Derived Extracellular Vesicles (MSC-EVs)

Stem cell-based therapy is a novel approach that takes advantage of the pro-regenerative properties of stem cells and their bioproducts to induce the repair of damaged or dysfunctional cells and tissues in the human body (Wong, 2021). In the last decade, the therapeutic contribution of mesenchymal stromal cells (MSCs) in kidney repair and regeneration after the injury has been extensively explored (Morigi *et al.*, 2016; Zhuang *et al.*, 2019). Originally described as fibroblast-like cells in the bone marrow (BM) (Friedenstein *et al.*, 1974), this population of multipotent stem cells can be obtained from different adult and fetal tissues, expanded *in vitro*, and differentiate into adipocytes, osteoblasts, and chondrocytes (Pittenger *et al.*, 1999).

The pro-regenerative activity of MSC is carried out in a paracrine manner, mainly through the release of soluble nutritional factors and extracellular vesicles (EVs), nanometer-range particles surrounded by a lipid bilayer. Based on the size and biological origin, this heterogeneous population of vesicles can be classified into exosomes, ectosomes, and apoptotic bodies (Meldolesi, 2018; Bruno *et al.*, 2020). At present, EVs can be purified from biological fluids or the supernatant of cultured cells using the 'gold standard' method of differential ultracentrifugation, or other more specific techniques, such as density gradient centrifugation, filtration, size exclusion chromatography, and immuno-affinity capture (Chiabotto *et al.*, 2020).

By acting as mediators of intercellular communication, MSC-EVs can transfer a large variety of bioactive molecules between cells, including functional proteins, lipids, and microRNAs (miRNAs), and reprogram cell fate by modulating different molecular pathways (Quesenberry *et al.*, 2015). The cargo of MSC-EVs can also be genetically modified to deliver specific therapeutic molecules to target cells (Varderidou-Minasian and Lorenowicz, 2020).

Several advantages make MSC-EVs more suitable for therapeutic use than MSCs. MSC-EVs preparations are more stable than cells since they are not subjected to deactivation, even after repeated freezing and thawing (Kou *et al.*, 2022), and can be stored for a longer time at −80 °C (Eirin and Lerman, 2021). Due to their nanometer size and low expression of membrane histocompatibility molecules, MSC-EVs have lower immunogenicity than MSCs (Reis *et al.*, 2016). Furthermore, being acellular products, MSC-EVs

do not self-replicate and lack potentially hazardous properties, such as ectopic differentiation, genetic instability, and tumor formation (Jafarinia *et al.*, 2020).

Renoprotective Effects of Mesenchymal Stromal Cell-Derived Extracellular Vesicles in Animal Models of Chronic Kidney Disease

The renoprotective effect of MSC-EVs has been demonstrated in different *in vivo* models of CKD (Table 1) (Grange *et al.*, 2019a; Eirin and Lerman, 2021; Liao *et al.*, 2022). To date, BM-derived MSC-EVs (BM-MSC-EVs) are the most widely used source of EVs for CKD treatment. However, also MSC-EVs from alternative sources, such as perinatal tissues, adipose tissue (AT), liver, kidney, and urine, have shown to ameliorate CKD conditions (Fig. 2).

An important mechanism underlying the renoprotective effect of MSC-EVs consists in the modulation of markers of fibrosis and inflammation, whose expression is increased in the renal tissue of CKD animals. In different *in vivo* models of CKD, treatment with MSC-EVs from different sources down-regulates the expression of fibrosis-associated genes, such as alpha smooth muscle actin (α-SMA) (He *et al.*, 2015; Choi *et al.*, 2015; Grange *et al.*, 2019b; Kholia *et al.*, 2018, 2020; Wang *et al.*, 2020; Bruno *et al.*, 2022), transforming growth factor-β (TGF-β) (Nagaishi *et al.*, 2016; Grange *et al.*, 2019b; Kholia *et al.*, 2018, 2020; Ishiy *et al.*, 2020; Bruno *et al.*, 2022), and collagen I (Grange *et al.*, 2019b; Kholia *et al.*, 2018, 2020; Ishiy *et al.*, 2020; Bruno *et al.*, 2022). Furthermore, the administration of MSC-EVs can effectively reduce the expression of pro-inflammatory genes, such as interferon gamma (IFNγ) (Grange *et al.*, 2019b; Ramírez-Bajo *et al.*, 2020), interleukin (IL)-6 (Eirin *et al.*, 2017; Bruno *et al.*, 2022), and tumor necrosis factor-α (Eirin *et al.*, 2017; Grange *et al.*, 2019b; Bruno *et al.*, 2022), and increase the expression of anti-inflammatory genes, such as IL-10 (Eirin *et al.*, 2017; Ishiy *et al.*, 2020).

Ischemia-reperfusion injury (IRI)

IRI is considered a major cause of acute kidney injury. It is associated with oxidative stress and inflammation that trigger apoptosis and necrosis of TECs, leading to rapid deterioration of renal function and morphology (Tögel and Westenfelder, 2014). The maladaptive repair following acute kidney injury predisposes the progression to CKD and to the development of fibrosis. In experimental models of IRI-induced CKD, treatment with BM-MSC-EVs improves renal function and morphology, with a significant reduction of fibrosis (Gatti *et al.*, 2011). The ameliorative effect on renal inflammation and fibrosis has also been observed using EVs isolated from umbilical cord-derived MSCs (UC-MSC-EVs) (Zou *et al.*, 2016a, 2016b), Wharton's Jelly-derived MSCs (Zou *et al.*, 2014), and human liver stem cells (HLSCs) (Bruno *et al.*, 2022). In addition to reducing the expression of fibrosis and inflammation markers, HLSC-EVs modulate several genes associated with EMT, upregulating the expression of epithelial marker zonula occludens-1, and down-regulating the expression of mesenchymal markers Twist-related protein 1 and vimentin in renal tissue (Bruno *et al.*, 2022). By transferring vascular endothelial growth factor to TECs, UC-MSC-EVs may also increase renal angiogenesis (Zou *et al.*, 2016a).

Unilateral ureteral obstruction (UUO)

UUO is another *in vivo* model of tubulointerstitial fibrosis. It is characterized by alterations in the renal cell phenotype and accumulation of excessive extracellular matrix (Chevalier *et al.*, 2009). Another histologic alteration is renal microvasculature injury, which results in chronic tissue hypoxia, thus contributing to the progression of renal disease (Ohashi *et al.*, 2002). In experimental models of UUO-induced CKD, treatment with BM-MSC-EVs preserved renal function and morphology and reduced fibrosis, as shown by the reduction in α-SMA expression and the increase in E-cadherin expression (He *et al.*, 2015; Wang *et al.*, 2020). In addition, kidney-derived MSC-EVs reduce inflammatory cell infiltration and improve peritubular rarefaction by inhibiting the endothelial-to-mesenchymal transition of peritubular capillaries (Choi *et al.*, 2015).

The protective role of the miRNA content of BM-MSC-EVs in preventing UUO-induced CKD has been demonstrated by two different research groups (He *et al.*, 2015; Wang *et al.*, 2020). He and colleagues have found 81 miRNAs upregulated BM-MSC-EVs compared to BM-MSCs, including miRNAs belonging to the miR-29 and to the miR-30 family (He *et al.*, 2015), whose anti-fibrotic effect is well-described in CKD (Lv *et al.*, 2018). Wang and colleagues have observed a correlation between the downregulation of miR-294 and miR-133 in BM-MSC-EVs of aged rats and a reduced capability of BM-MSC-EVs to inhibit renal fibrosis (Wang *et al.*, 2020).

Drug-induced nephrotoxicity

CKD is frequently associated with nephrotoxicity induced by drugs, in particular immunosuppressors and antineoplastics. In general, treatment with MSC-EVs induced morphological and functional recovery of the kidney, also reducing fibrosis, EMT, and TEC apoptosis (Bruno *et al.*, 2012; Nagaishi *et al.*, 2016; Jiang *et al.*, 2016; Kholia *et al.*, 2018, 2020; Zhong *et al.*, 2018; Grange *et al.*, 2019b; Ramírez-Bajo *et al.*, 2020). In cyclosporine-induced CKD, both the preventive and the curative treatment with BM-MSC-EVs attenuate renal fibrosis by down-regulating the expression of plasminogen activator inhibitor-1, TIMP metallopeptidase inhibitor 1, IFN-γ (Ramírez-Bajo *et al.*, 2020). A protective effect on renal function and morphology was also observed in the aristolochic acid-induced CKD model, treated with BM-MSC-EVs (Kholia *et al.*, 2020) and with HLSC-EVs (Kholia *et al.*, 2018). In both cases, EV treatment attenuated the expression of fibrosis markers, such as α-SMA, collagen I, TGF-β, and latent transforming growth factor-beta binding protein, and restored baseline levels of several fibrosis-related miRNAs, whose expression was altered in the intoxicated kidney (Kholia *et al.*, 2018, 2020).

Interestingly, a considerable number of fibrosis-related genes were also modulated in an experimental model of streptozotocin-induced diabetic nephropathy, treated with BM-MSC-EVs and with HLSC-EVs (Grange *et al.*, 2019b).

TABLE 1

Renoprotective effect of mesenchymal stromal cell-derived extracellular vesicles in chronic kidney disease. EV sources, animal models, EV cargo and effect are listed, with particular reference to fibrosis and inflammation-related markers modulated in CKD

MSC origin	*In vivo* model	EV content	EV effect	Down-regulated markers	Up-regulated markers	References
Bone marrow	IRI (mouse)	RNAs?	Improve renal function and morphology, reduce renal fibrosis			Gatti *et al.* (2011)
	Cisplatin (mouse)	RNAs?	Improve renal function and morphology, reduce tubular apoptosis			Bruno *et al.* (2012)
	5/6 subtotal nephrectomy (mouse)		Preserve the remnant renal function and morphology, ameliorate renal injury and prevent renal fibrosis			He *et al.* (2012)
	UUO (mouse)	miR-29 (29a-3p, 29b-3p, 29b-1-5p), miR-30 (30b-3p, 30b-5p, 30d-5p, 30e-3p, 30c-5p), and miR-210-3p	Preserve renal function and morphology and reduce renal fibrosis	α-SMA	E-cadherin	He *et al.* (2015)
	Streptozotocin-induced diabetic nephropathy (mouse)		Preserve renal function and morphology and reduce tubular apoptosis, renal fibrosis and inflammation	TGF-β	ZO-1	Nagaishi *et al.* (2016)
	Streptozotocin-induced diabetic nephropathy (mouse)	*(top 15 expressed)* hsa-miR-222-3p, hsa-miR-24, hsa-miR-302c-3p, hsa-miR-99a-5p, hsa-let-7b-5p, hsa-miR-1243, hsa-miR-100-5p, hsa-let-7e-5p, hsa-miR-191-5p, hsa-miR-125b-5p, hsa-miR-21-5p, hsa-miR-193b-3p, hsa-miR-30a-5p, hsa-miR-574-3p	Ameliorate renal function and glomerular alterations, reduce tubular damage and renal fibrosis	Pro-fibrotic markers (TGF-β, α-SMA, Collagen I), FASL, SERPINA1a, MMP3, ITGA2, IFNγ, BMP7, IL5, COL1A2, CCL3, INHBE, TNF, SNAI1	IL1a, MMP1a, CCR2, BCL2, TGFB3	Grange *et al.* (2019)
	Cadmium-exposed (medaka)	RNAs ?	Improve renal morphology and animal survival			Matsukura *et al.* (2019)
	Aristolochic acid (mouse)	hsa-miR-194-5p, hsa-miR-192-5p, mmu-miR-378a-3p	Improve renal function and morphology, reduce renal injury and fibrosis	pro-fibrotic markers (α-SMA, Collagen I, TGF-β, LTBP1), hsa-miR-21-5p, hsa-miR-34a-5p, hsa-miR-34c-5p, hsa-miR-132-3p, hsa-miR-342-3p, mmu-miR-212-3p, hsa-miR-214-3p	hsa-miR-194-5p, hsa-miR-192-5p, mmu-miR-378a-3p	Kholia *et al.* (2020)
	Cyclosporine A (mouse)		Improve renal function and morphology, reduce	PAI1, TIMP-1, IFN-γ		Ramírez-Bajo *et al.* (2020)

(Continued)

Table 1 (continued)

MSC origin	*In vivo* model	EV content	EV effect	Down-regulated markers	Up-regulated markers	References
			tubular apoptosis and renal fibrosis			
	UUO (rat)	miR-294, miR-133	Reduce EMT and renal fibrosis	α-SMA, phosphorylation of SMAD 2/3 and ERK1/2	miR-294/miR-133, E-cadherin	Wang *et al.* (2020)
Umbilical cord	IRI (rat)	VEGF, inflammation-related miRNAs	Reduce renal injury and NK cell infiltration, increase angiogenesis	CX3CL1/TLR-2	VEGF	Zou *et al.*, 2016a, 2016b
	Streptozotocin-induced diabetic nephropathy with hyperuricemia (mouse)	miR-451a	Reduce EMT and fibrosis	cell cycle inhibitors (P15/P19), α-SMA	miR-451a, E-cadherin	Zhong *et al.* (2018)
Wharton's Jelly	IRI (rat)	miR-16, miR-15a, miR-15b	Reduce macrophages infiltration, renal inflammation and fibrosis	CX3CL1		Zou *et al.* (2014)
	Ischemia-partial nephrectomy (rat)	HGF	Increase of M2 macrophages polarization and reduce renal fibrosis		HGF	Du *et al.* (2021)
Adipose tissue	Metabolic syndrome and renal artery stenosis (pig)	IL-4, IL-10	Reduce renal inflammation and fibrosis, and improve renal function and medullary oxygenation	inflammatory cytokines (MCP-1, IL-1β, IL-6, TNF-α)	IL-4, IL-10	Eirin *et al.* (2017)
	Metabolic syndrome and renal artery stenosis (pig)	pro-angiogenic (e.g., VEGF), anti-apoptotic and anti-oxidant genes and proteins	Improve renal function and perfusion, ameliorate renal injury	Oxidative stress markers dihydroethidium and nitrotyrosine on endothelial cells	VEGF, Notch1, DLL4	Eirin *et al.* (2018)
	Renal artery stenosis (rat)		Improve renal function and perfusion, reduce renal fibrosis and inflammation	Collagen I, TGF-β, IL-1β, HIF-1α	IL-10, SDF1-α	Ishiy *et al.* (2020)
Liver	Aristolochic acid (mouse)		Improve renal function and morphology, reduce renal fibrosis and inflammation	Pro-fibrotic markers (α-SMA, Collagen I, TGF-β, LTBP1, CCL12, CCR2, SERPINA1a, SNAI1), mmu-miR-448-3p, mmu-miR-377-3p, mmu-miR-329-3p, mmu-miR-294-3p, mmu-miR-880-3p, mmu-miR-9-3p, mmu-miR-297c-3p, mmu-miR-376c-5p	mmu-miR-466f, mmu-miR-469b, mmu-miR-331-5p, mmu-miR-327, mmu-miR-291a-5p, mmu-miR-495-3p, mmu-miR-490-3p, miR-469, mmu-miR-208a-3p, mmu-miR-212-3p, mmu-miR-363-3p, mmu-miR-220, mmu-miR-2183, mmu-miR-1942, mmu-miR-1983, mmu-miR-300-5p,	Kholia *et al.* (2018)

(Continued)

Table 1 (continued)

MSC origin	*In vivo* model	EV content	EV effect	Down-regulated markers	Up-regulated markers	References
					mmu-miR-708-3p, mmu-miR-743a-3p, mmu-miR-127-5p, mmu-miR-689	
	Streptozotocin-induced diabetic nephropathy (mouse)	*(top 15 expressed)* hsa-miR-24, hsa-miR-191-5p, hsa-miR-146a-5p, hsa-miR-222-3p, hsa-miR-31-5p, hsa-miR-574-3p, hsa-miR-484, hsa-miR-16-5p, hsa-miR-29a-3p, hsa-17-5p, hsa-miR-106a-5p, hsa-miR-19b-3p, hsa-miR-409-3p, hsa-miR-155-5p, hsa-miR-99a-5p	Ameliorate renal function and glomerular alterations, reduce tubular damage and renal fibrosis	Pro-fibrotic markers (TGF-β, α-SMA, Collagen I), MMP3, CCL3, FASL, IL13, TIMP1, IFNγ, SERPINA1a, DCN, COL1A2	TGFB3	Grange *et al.* (2019)
	IRI (mouse)		Reduce EMT, renal fibrosis and inflammation	Pro-fibrotic markers (α-SMA, Collagen I, TGF-β), inflammatory genes (IL-6, TNF-α), EMT markers (TWIST1, VIM)	epithelial gene ZO-1	Bruno *et al.* (2022)
Renal	UUO (mouse)		Ameliorate rarefaction and endothelial-to-mesenchymal transition of peritubular capillaries, reduce tubulointerstitial fibrosis, tubular apoptosis, and inflammatory cell infiltration	α-SMA, F4/80		Choi *et al.* (2015)
Urine	Streptozotocin-induced diabetic nephropathy (rat)	BMP-7, VEGF, TGF-β1 and angiogenin	Reduce urine volume and urinary microalbumin excretion, prevent podocyte apoptosis, increase glomerular endothelial cell proliferation	caspase-3		Jiang *et al.* (2016)

Note: α-SMA: alpha-smooth muscle actin; BCL2: B-cell lymphoma 2; BMP7: bone morphogenetic protein 7; CCL: chemokine (C-C motif) ligand; CCR2: C-C chemokine receptor type 2; COL1A2: collagen type I alpha 2 chain; CX3CL1: C-X3-C motif chemokine ligand 1; DCN: decorin; DLL4: delta like canonical notch ligand 4; ERK1: extracellular signal-regulated kinase 1; EMT: epithelial-to-mesenchymal transition; FASL: Fas ligand; HGF: hepatocyte growth factor; HIF-1α: hypoxia-inducible factor-1 alpha; IL: interleukin; INHBE: inhibin beta E chain; IRI: ischemia-reperfusion injury; IFNγ: interferon gamma; ITGA2: integrin subunit alpha 2; LTBP1: Latent transforming growth factor-beta binding protein; MCP-1: monocyte chemoattractant protein-1; miRNA: microRNA; MMP: matrix metalloproteinase; PAI1: plasminogen activator inhibitor-1; SDF1-α: stromal cell-derived factor 1-alpha; SMAD 2/3: mothers against decapentaplegic homolog 2/3; TEC: tubular epithelial cell; TGF-β: transforming growth factor-beta; TIMP-1: TIMP metallopeptidase inhibitor 1; TLR-2: toll-like receptor 2; TNF: tumor necrosis factor; TWIST1: Twist-related protein 1; UUO: unilateral ureteral obstruction; VEGF: vascular endothelial growth factor; VIM: vimentin; ZO-1: zonula occludens-1.

For these two populations of MSC-EVs, Grange and colleagues have identified distinct miRNA signatures, which include common anti-fibrotic miRNAs, such as miR-21, miR-24, miR-29a, miR-30a, and let-7 family. Moreover, Zhong and colleagues found UC-MSC-EVs enriched in miR-451a, which can restart TEC cycle by inhibiting P15 and P19 and reverse EMT in a streptozotocin-induced diabetic nephropathy model with hyperuricemia (Zhong *et al.*, 2018). Jiang and colleagues also observed that urinary-derived MSC-EVs contain growth factors with reno-protective activity, such as TGF-β1, angiogenin, and bone morphogenetic protein-7, which can reduce podocyte

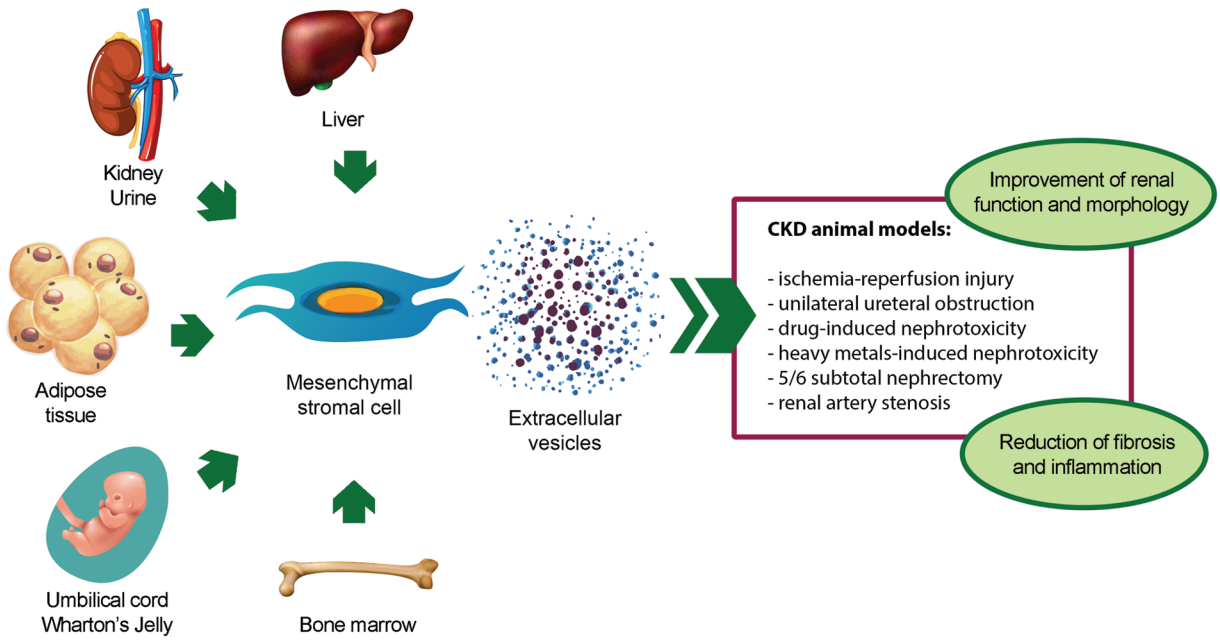

FIGURE 2. Pro-regenerative effects of extracellular vesicles derived from mesenchymal stromal cells isolated from different sources in animal models of chronic kidney disease. Abbreviation: CKD: chronic kidney disease.

apoptosis and promote vascular regeneration and cell survival in streptozotocin-induced diabetic nephropathy (Jiang *et al.*, 2016).

Other chronic kidney disease models

Treatment with BM-MSC-EVs has been shown to improve kidney damage also in cadmium-induced nephrotoxicity (Matsukura *et al.*, 2019) and in 5/6 subtotal nephrectomy (He *et al.*, 2012), where it can prevent renal fibrosis by reducing tubulointerstitial collagen deposition. Similarly, Wharton's Jelly-derived MSC-EVs ameliorated renal inflammation and fibrosis by transferring hepatocyte growth factor into the ischemic kidney subjected to partial nephrectomy (Du *et al.*, 2021).

Finally, the anti-inflammatory and anti-fibrotic effect of EVs isolated from AT-derived MSCs (AT-MSC-EVs) was demonstrated in a rat model of renal artery stenosis (Ishiy *et al.*, 2020) and in a porcine model of metabolic syndrome and renal artery stenosis (Eirin *et al.*, 2017, 2018). Eirin and colleagues demonstrated that AT-MSC-EVs contain pro-angiogenic, anti-apoptotic, and anti-oxidant genes and proteins that can restore renal perfusion and ameliorate renal injury (Eirin *et al.*, 2018). In particular, IL-10 was detected among the anti-inflammatory molecules enriched in AT-MSC-EVs, and its knockdown abolished the protective effect of MSC-EVs on the stenotic kidney (Eirin *et al.*, 2017).

Evidence of Renoprotective Effects of Mesenchymal Stromal Cell-Derived Extracellular Vesicles in Human Patients

Several clinical trials have tested the regenerative effect of MSC-EVs in the human body. The first clinical application of MSC-EVs has been described in a patient with steroid-resistant graft *vs.* host disease (Kordelas *et al.*, 2014). In this study, EV administration was shown to be safe and effective in improving cutaneous and mucosal graft *vs.* host disease.

The efficacy of UC-MSC-EVs has also been demonstrated in CKD by Nassar *et al.* (2016). Forty patients with CKD stage III or IV were enrolled in a single-center, randomized, placebo-controlled phase II/III clinical study and followed for 12 months after two administrations of UC-MSC-EVs. Compared with patients administered with placebo, treated patients showed a significant improvement in kidney function and a reduction of inflammation. As no adverse effects were observed throughout the study, EV administration has been proven safe and effective in patients with CKD.

Conclusions

In CKD *in vivo* experimental models, MSC-EVs isolated from different sources exert pro-regenerative, anti-fibrotic, and anti-inflammatory activities, showing the same therapeutic effects as MSCs. By delivering proteins, mRNAs, and non-coding RNAs into the injured kidney, MSC-EVs modulate several molecular pathways related to inflammation, fibrosis, oxidative stress, apoptosis, and angiogenesis, thus impeding the progression of CKD. Based on the promising results obtained in different *in vivo* experimental models, together with the results from the first-in-human study in CKD patients, MSC-EVs may represent supportive care to slow the development of CKD, postpone renal replacement therapies, and improve the quality of life of patients.

Compared with cell therapy, MSC-EVs are safer; as nano-sized particles, they possess both low immunogenicity and biocompatibility. However, there are still challenges that need to be overcome before their clinical application. First, the molecular mechanisms underlying the therapeutic effect of MSC-EVs in the kidney are not fully understood, and an in-depth analysis of the MSC-EV cargo could help to

elucidate how they carry out their *in vivo* anti-fibrotic and anti-inflammatory effect. Second, it would be important to determine the duration of the beneficial effects of MSC-EVs and their adequate dose regimen for the clinical application. Luckily, no long-term detrimental effects of MSC-EVs have been described so far in CKD *in vivo* models. However, possible side effects must be taken into consideration and closely monitored before moving into clinical trials. Finally, the standardization of MSC-EV isolation and characterization techniques, based on the minimum criteria proposed by the International Society of Extracellular Vesicles, could help to increase batch reproducibility and facilitate their clinical application.

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