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Kidney regeneration in vivo

Extracellular vesicles in renal tissue damage and regeneration

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ABSTRACT

Extracellular vesicles (EVs) appear as important actors in cell-to-cell communication. EV content is characterized by proteins and RNA species that dynamically reflect cell and tissue state. Urinary EVs in particular may act in inter-nephron communication with possible beneficial or detrimental effects. Increasing interest is addressed to the pharmacological properties of EVs as a cell-free therapy, since several of the effects crAQ/tgqcedited to stem cells have been recapitulated by administration of their EVs. Preclinical data in models of renal damage indicate a general regenerative potential of EVs derived from mesenchymal stromal cells of different sources, including bone marrow, fetal tissues, urine and kidney. In this review we will discuss the results on the effect of EVs in repair of acute and chronic renal injury, and the mechanisms involved. In addition, we will analyse the strategies for EV pharmacological applications in renal regenerative medicine and limits and benefits involved.

1. Introduction

Intercellular communication plays a key role in the regulation of tissue organization and functions of multicellular organisms. During the last years, extracellular vesicles (EVs) have been discovered as important actors in cell-to-cell interaction. Indeed, these small vesicles, secreted by cells and present in almost every body fluid, appear as an ancestral mechanism of communication among cells and tissues (Valadi et al., 2007).

EVs act on target cells in different ways, including cell stimulation, transfer of genetic material, mainly messenger RNA (mRNA) and microRNAs (miRNAs), but also of proteins and lipids to target cells, even at distant sites (Nawaz et al., 2016). As a result, EVs may modify target cells and reprogram their biological function with a long lasting outcome (Bruno and Camussi, 2013). Several evidences show the implication of EVs in controlling physiological processes such as stem cell maintenance (Ratajczak et al., 2006), tissue repair (Gatti et al., 2011) and immune surveillance (Lugini et al., 2012). In addition, in pathology, EVs appear as a mechanism to amplify and propagate diseases. Examples were shown in Parkinson and Alzheimer diseases (Bellingham et al., 2012; Emmanouilidou et al., 2010), tumorigenesis (Rak and Guha, 2012) or primary hypertension (Aliotta et al., 2016). On the other side, increasing interest is addressed to the pharmacological properties of EVs as a cell-free therapy, since several of the effects credited to stem cells have been recapitulated by administration of their EVs.

In this review, we will focus on the role of EVs in renal pathology and repair. We will first discuss the data of the literature on the role of urine-derived EVs (uEVs) within the nephron as tools for inter-nephron communication, repair or damage. Moreover, we will describe the preclinical data on the use of stem-cell derived EVs for therapeutic applications in renal pathology, and the mechanisms involved. Finally, we will dissect the strategies for EV pharmacological applications, and limits and benefits involved.

2. General characteristics of EVs

2.1. EV biogenesis

EVs appear to be released from nearly all mammalian cell types. Among the most studied cell types are stem cells and progenitors (Lai et al., 2011; Ratajczak et al., 2006), primary cells of the immune and nervous systems (Chavez-Muñoz et al., 2008; Potolicchio et al., 2005) as well as numerous cancer cell lines (Al-Nedawi et al., 2009; Nakano et al., 2015). In addition, EVs have been isolated from the majority of biological fluids (Keller et al., 2011; Lässer et al., 2011; Wang and Sun, 2014; EL Andaloussi et al., 2013; Turturici et al., 2014).

Although distinct biogenesis pathways lead to different types of extracellular vesicles, EVs could be grouped in three main classes: *exo*somes, microvesicles and apoptotic bodies (Table 1) (Akers et al., 2013; EL Andaloussi et al., 2013; Katsuda et al., 2013). Exosomes are an homogeneous population with a ranging size of 30–

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Table 1
Summary of principal features of EVs.

Vesicles types	Features			
	Source	Size	Markers	Contents
Exosomes	Endosomal origin and storage with subsequent release by fusion of MVB to plasma membrane	30–150 nm	Tetraspanins (CD9, CD63 and CD81), ESCRT components, Alix and Tsg101	Cytoplasmic proteins with the presence of mRNAs, miRNAs and other non-coding RNAs
Microvesicles	Direct budding from plasma membrane	200–1000 nm	Integrins, Selectins, Tetraspanins and membrane related cell markers	Cytoplasmic proteins with the presence of mRNAs, miRNAs and other non-coding RNAs
Apoptotic Bodies	Fragmentation of plasma membrane during apoptosis	1000–5000 nm	Phosphatidyl-serine and membrane related cell markers	Nuclear and organelles fragments, cytoplasmic proteins

EV population can be classified in exosomes, microvesicles and apoptotic bodies by different mechanism of generation. Other features of these EV classes such as dimension, marker expression and content are reported.

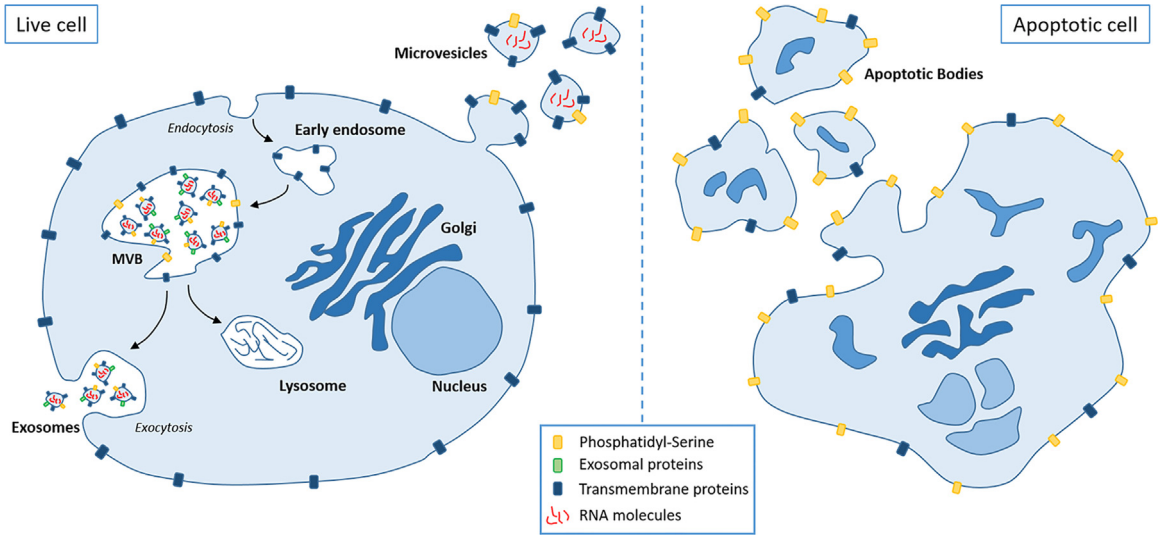

Fig. 1. Schematic representation of biogenesis and release of extracellular vesicles. Extracellular vesicles can be classified into three main classes: exosomes, microvesicles and apoptotic bodies. Exosomes are formed from inward budding of early endosomes and are subsequently release by the fusion of multivesicular bodies (MVB) to the plasma membrane through the exocytosis pathway. Exosomes may contain several proteins and specific nucleic acids (mRNAs and miRNAs) and are highly enriched in glycosphingolipids, sphingomyelin, cholesterol, and phosphatidyl-serine. MVB can also be degraded into lysosomes. On the other hand, microvesicles are formed from the direct outward budding of the plasma membrane and they may transport RNA molecules and both membrane and cytoplasmic proteins. Apoptotic bodies are heterogeneous in size and are formed by random blebbing of plasma membrane of cells undergoing apoptosis. Apoptotic bodies may contain membrane proteins, parts of cytoplasmic organelles and nuclear fragments as well as a large amount of phosphatidyl-serine residues.

150 nm (Greening et al., 2015). They have an endosomal origin and they are stored and released by the fusion of multivesicular bodies with the plasma membrane (Fig. 1) (Mathivanan et al., 2010). While the correct assembly and sorting of exosomes remain not completely clear, several mechanisms have been recently identified in the regulation of exosomes biogenesis. These mechanisms guide protein and RNA cargo sorting thus generating exosomes with a precise biochemical composition (Baietti et al., 2012; Raiborg and Stenmark, 2009). In particular, cargo sorting into exosomes involves the Endosomal Sorting Complex Required for Transport (ESCRT-3) and other associated proteins such as Apoptosis-Linked Gene-2-Interacting Protein X (Alix) and Tumour Susceptibility Gene 101 (Tsg101). Microvesicles, also known as shedding vesicles, are larger particles of 200–1000 nm in size, and represent a relatively heterogeneous population of vesicles (EL Andaloussi et al., 2013; Katsuda et al., 2013). Formation of shedding vesicles takes place from the budding of small cytoplasmic protrusions followed by their detachment from the plasma membrane (Fig. 1). Therefore, it has been suggested that the membrane composition of microvesicles reflects the one of the parent cells in a closer fashion than that in exosomes (EL Andaloussi et al., 2013). Moreover, the microvesicle biogenesis is an energy-dependent process, depending on calcium influx and cytoskeleton reorganization (Cocucci et al., 2009). However, calcium is not the only second messenger involved in inducing vesicle release as the activation by phorbol ester of Protein Kinase C has also been showed to play a role in various cell types (Baj-Krzyworzeka et al., 2006). A third type of vesicles are the apoptotic bodies, large 1000–5000 nm size particles released upon fragmentation of plasma membrane that occurs during apoptosis (Akers et al., 2013) (Fig. 1). When referring to EVs, the majority of studies comprise a heterogeneous population of exosomes and microvesicles, whereas the apoptotic bodies are distinct in activity and content.

2.2. EV isolation and characterization

EVs present in cell supernatants or biological fluids represent a heterogeneous population of vesicles. Common EV isolation protocols used include density gradient centrifugations and ultracentrifugation, polymer-based methods (used by some commercial kits), chromatography, filtration, and immunoaffinity. As these different procedures may impact on EV type, number, integrity and cargo (Taylor and Shah, 2015), a precise analysis of the biochemical and biophysical characteristics of the isolates is required. The International Society for Extracellular Vesicles (ISEV) provided a combination of minimal experimental requirements needed to properly define EV isolates in the different experimental

conditions in use (Lötvall et al., 2014). Analysis of EV dimension within the isolates should combine two different techniques to couple the measurement of the distribution size (using nanoparticle-tracking analysis (Nanosight), dynamic light scattering or resistive pulse sensing) with single vesicle analysis based on microscopical techniques (such as electron microscopy, atomic force microscopy or immunofluorescence).

In addition, criteria for EV identification include the assessment for the presence of EV-associated proteins as well as absence of contaminant-associated proteins (Lötvall et al., 2014). Proteomic studies identified various proteins expressed on EV membranes, which can be used as EV markers (Table 1). They include tetraspanins (CD9, CD63 and CD81), Alix, Major Histocompatibility Complex (MHC) molecules and cytosolic stress-related proteins (Heat Shock Proteins; HSPs), Tsg101 and ESCRT-3 (Witwer et al., 2013). Although some of these molecules were initially proposed as specific markers for exosomes, such as CD9, CD63 and CD81, they were subsequently shown to be present in apoptotic bodies and microvesicles as well (Crescitelli et al., 2013). Therefore, at present, a specific marker for the distinction of EV subtypes is lacking.

This notion was recently underlined by an extensive quantitative proteomic analysis performed on human dendritic cell-derived EVs (Kowal et al., 2016). In this study, it appeared that several classically used exosome markers (MHC class I, Flotillin, HSPs 70-kDa) were similarly present in all EV types. In addition, more selective markers such as Syntenin-1, Metalloproteinase Domain-Containing Protein 10 (ADAM-10) and Tsg101 were shown to be associated with Tetraspanin-enriched small EVs, representing new markers for exosomes (Kowal et al., 2016). This work provides guidelines to define subtypes of EVs for future functional studies.

2.3. General content and mechanism of action

EVs are composed of membrane fragments, which encloses a broad variety of bioactive lipids, cytoplasmic proteins and nucleic acids (mRNAs, non-coding RNAs, miRNAs and other small RNAs) (Witwer et al., 2013). In general, the content of EVs reflects that of the cell of origin (Quesenberry et al., 2014). However, selected molecules appear specifically enriched in EVs in respect to the originating cell. The lipid content of EVs, for instance, appears highly enriched in glycosphingolipids, sphingomyelin, cholesterol, and phosphatidyl-serine (Llorente et al., 2013). Of interest, EV-associated RNA is predominantly smaller in size than within the cell fraction (Eirin et al., 2014). Indeed, RNA sequencing of urine or serum-derived EVs indicated a predominance of small non coding RNAs (Bellingham et al., 2012; Miranda et al., 2014). In addition, specific miRNAs have been enriched in EVs of MSCs (Collino et al., 2015). Possible mechanisms involving RNA packaging within EVs have also been identified (Zaborowski et al., 2015). Among them, it has been proposed that 3′ end post-transcriptional modifications may drive miRNA sorting into EVs (Koppers-Lalic et al., 2014).

Extensive catalogues of proteins, lipids and RNAs in different types of EVs are available in online databases: ExoCarta, EVpedia, and Visiclepedia (Kalra et al., 2012; Keerthikumar et al., 2015; Kim et al., 2015). In addition, EVs content may be influenced by physical or chemical stresses, including oxidative stress, hypoxia and inflammation (Robbins and Morelli, 2014). Growth factor stimulation of the originating cells may also influence the content and functional properties of EVs. When adipose tissue derived stem cells were treated with Platelet Derived Growth Factor (PDGF), an increased pro-angiogenic activity of deriving EVs was observed (Lopatina et al., 2014). This concept is relevant both for the possible use of EVs as marker of pathological conditions and as possible therapeutic option to ameliorate EV content.

EVs have the capacity to modify the activity of target cells using different mechanisms, such as extracellular cell stimulation through surface receptor interactions and receptor transfer to the target cell membrane (Cocucci et al., 2009). Alternatively, EVs can modify target cells due to their membrane fusion or their endocytosis/phagocytosis, with subsequent release and transfer of proteins and nucleic acids to target cell (Mathivanan et al., 2010). In particular, mRNAs present in EVs can be transferred to recipient cells and translated into functional proteins. Furthermore, also miRNAs can be shuttled between cells and EVs leading to the repression of specific mRNAs present in recipient cells (Collino et al., 2015). Finally, non-coding RNA present inside EVs may have a role in cell regulation. Therefore, among the possible mechanisms of action, EV-mediated RNA horizontal transfer may be instrumental for the reprogramming effect of target cells, as suggested in pioneering studies of the field (Deregibus et al., 2007; Ratajczak and Ratajczak, 2016).

3. EVs in the nephron inter-cellular communication

3.1. Urinary EVs

Urine is a rich reservoir of EVs released by cells lining the nephron. The contribution of plasma circulating EVs to the urinary pool appears quite limited, as confirmed by uEVs proteomic analysis showing that uEVs are largely made up of exosomes derived from the tubular cell apical plasma membrane (Pisitkun et al., 2004). In particular, the majority of EVs released into the pre-urine appeared to derive from the first part of the nephron's cells, with a limited contribution of the lower urinary collecting system (Pisitkun et al., 2004). However, a minor contribution of sera EVs could be speculated and possibly increased in condition of renal pathology. Indeed, a recent publication reported that when labelled EVs were administered i.v., around 2.5% of injected EVs were found in the urine (Oosthuyzen et al., 2016). In general, uEVs are characterized by high expression of CD24 (Dimuccio et al., 2014; Keller et al., 2007), a membrane glycoprotein able to interact with various ligands, including adhesion molecules such as P-selectin and L1 cell adhesion molecule (Ayre et al., 2016). The expression of specific podocyte markers, such as Podocin and Podocalyxin, or tubular segment specific markers may sign the origin of the uEVs from the different cells along the nephron (Salih et al., 2014). In addition, normal urine contains EVs expressing CD133, a marker of scattered $CD133^+$ cells along the nephron. Alterations in the levels of $CD133^+$ EVs were reported in patients with end stage kidney disease or transplanted patients with slow graft function suggesting that CD133 may represent a marker of renal function and possibly reflect the activity of $CD133^+$ cells along the nephron (Dimuccio et al., 2014).

The number and content of uEVs may also vary during disease (Zhou et al., 2006). In these terms, uEVs may reflect the physiopathological state of kidneys (Record et al., 2011). A number of molecules (including mRNA, proteins and miRNAs) have been identified as candidate markers for kidney injury (Ranghino et al., 2015). Among the most promising markers, the podocyte marker Wilms' Tumour 1 (WT1) was successfully correlated to glomerular damage and chronic injury whereas the Activating Transcription Factor 3 and the Neutrophil Gelatinase-Associated Lipocalin appeared to be early markers for detection of acute kidney injury (Ranghino et al., 2015). Therefore, uEVs are highly studied as markers of renal damage and repair. However, the detailed analysis of this aspect is beyond the scope of the review.

3.2. Functional effect of nephron cell-derived EVs

The unidirectional flux of urine along the nephron likely suggests that EVs from upper nephron segments could be uptaken by distal cells. Indeed, both distal tubule and collecting duct cells have the capacity to taken up EVs released by proximal tubule cells

(Gildea et al., 2014). It has been demonstrated that EVs derived from the upper collecting duct were able to transfer Aquaporin-2 to the lower collecting duct cells, thereby increasing the water flow of recipient cells (Street et al., 2011). In contrast, in polycystic kidney disease, EVs derived from proximal tubular cells mainly interact with primary cilia of recipient cells increasing intracellular calcium level (Hogan et al., 2009; Pocsfalvi et al., 2015). The hormonal mechanisms regulating the uptake of EVs in renal cells has been highlighted in a recent work. In this paper, Desmopressin, a Vasopressin analogue, selectively stimulated EV uptake selectively in tubular cells *in vitro* and in parallel a Vasopressin antagonist reduced the uptake of injected EVs within renal tissue *in vivo* (Oosthuyzen et al., 2016).

In addition, preliminary data indicate a possible role of uEVs in communication along the nephron. EVs from proximal tubular cells cultured in presence of dopamine receptor agonist were able to decrease radical production in distal tubular cells, indicating the transfer of an anti-inflammatory message (Gildea et al., 2014). Nephron cell-released EVs also appeared as a mechanism involved in progression toward renal tissue maladaptive repair and fibrosis after damage. Tubular epithelial cell-derived EVs subjected to hypoxic damage *in vitro* studies were shown to induce fibroblast activation and proliferation, by the transfer of TGF-β1 mRNA (Borges et al., 2013). This mechanism underlines the role of the EVs in modulation of the microenvironment and in amplification of damage.

All together, these studies indicate the role of EVs in cell-to-cell communication along the nephron during both physiological and pathological conditions, even though the mechanisms are not completely clear.

4. Therapeutic effect of EVs derived from different sources of stem/progenitor cells on kidney regeneration

4.1. Therapeutic effect of mesenchymal stromal cell-derived EVs

From a therapeutic point of view, EVs have been exploited for their ability to act on different cell types and modulate relevant cellular processes, such as proliferation (Zhan et al., 2015), angiogenesis (Gai et al., 2016; Merino-González et al., 2016) and immune tolerance (Robbins and Morelli, 2014). The resulting effect, summarized as a general regenerative potential, has been investigated in different experimental animal models of renal injury mainly using mesenchymal stromal cells of different origin (Table 2).

EVs derived from bone marrow mesenchymal stromal cells (EV-MSCs) were the first to be tested *in vivo* in models of acute kidney injury (AKI). A single intravenous injection of EV-MSCs during the peak of renal damage resulted in acceleration of morphological and functional recovery of glycerol-induced AKI in SCID mice (Bruno et al., 2009). The effect of EV-MSCs treatment was comparable to that obtained with the cells of origin, indicating that EVs might mimic the beneficial effects of MSCs. In a different model of ischemia and reperfusion injury (IRI), a single administration of EV-MSCs immediately after damage protected rats from AKI and prevented chronic kidney disease (CKD) development (Gatti et al., 2011). EV-MSCs were also able to minimize gentamicin-induced AKI in rats (Reis et al., 2012). In analogy, in the lethal model of AKI induced by cisplatin, a single dose of EV-MSCs improved the survival of SCID mice, but chronic tubular injury developed in surviving mice. In this experimental model, multiple doses of EV-MSCs, at different time points after cisplatin administration, were required to improve the survival of SCID mice and to abolish the development of chronic tubular injury (Bruno et al., 2009).

The need for multiple EV injections was not confirmed in the remnant kidney CKD mouse model (He et al., 2012). In this model of 5/6 subtotal nephrectomy, a single EV-MSCs administration was sufficient to preserve the function of the remnant kidney, to prevent tubular atrophy and interstitial lymphocyte infiltration, and to limit renal fibrosis.

The detailed effect of MSC-EVs was explored in *in vitro* experiments on murine tubular renal cells. MSC-EVs prevented apoptosis and promoted proliferation (Bruno et al., 2012, 2009). The inhibition of *in vitro* apoptosis was associated with the down-regulation of genes involved in the execution phase of cell apoptosis (Caspase 1 and 8) and with the up-regulation of anti-apoptotic genes (Bcl-xL and Bcl2) (Bruno et al., 2012). In addition, in an *in vitro* model of IRI (Lindoso et al., 2014), the survival effect of

Table 2
Effects of EVs of different origin in animal models of renal injury.

Cell sources of EVs	*In vivo* models of renal injury	Doses	Therapeutic effect	Ref.
BM-MSCs	Glycerol-induced AKI	15 μg (by 7.5×10^5 cells)	Yes	(Bruno et al., 2009)
		2.2×10^8 EVs		(Collino et al., 2015)
	IRI	30 μg		(Gatti et al., 2011)
	Cisplatin-induced AKI	100 μg	No	(Bruno et al., 2012)
		100 μg +50 μg every 4 days	Yes	
	Remnant kidney (CKD)	30 μg	Yes	(He et al., 2012)
	Gentamicin-induced AKI	100 μg	Yes	(Reis et al., 2012)
CB-MSCs	Cisplatin-induced AKI	200 μg	Yes	(Zhou et al., 2013)
	IRI	30 μg	Yes	(Ju et al., 2015)
WJ-MSCs	IRI	100 μg	Yes	(Zou et al., 2014)
Kidney-MSCs	IRI	2×10^7 EVs	Yes	(Choi et al., 2014)
HLSC	Glycerol-induced AKI	1.9×10^9 Evs (by 3.5×10^5 cells)	Yes	(Herrera Sanchez et al., 2014)
		5.5×10^9 Evs (by 10×10^5 cells)		
EPC	IRI	30 μg	Yes	(Cantaluppi et al., 2012)
	Thy1.1 glomerulonephritis	30 μg/100 g body weight	Yes	(Cantaluppi et al., 2015)
Urine derived- MSCs	Diabetic nephropathy	100 μg weekly	Yes	(Jiang et al., 2016)
Embryonic-MSCs	Remnant kidney and diet (CKD)	7 μg twice daily for 4 consecutive days	No	(van Koppen et al., 2012)
Fibroblast-EVs	Glycerol-induced AKI	15 μg (by 7.5×10^5 cells)	No	(Bruno et al., 2009)
	Thy1.1 glomerulonephritis	30 μg/100 g body weight	No	(Cantaluppi et al., 2015)

EVs from different sources and different administration modality have been tested in models of AKI and CKD. EV origin, experimental model and resulting effect in terms of renal repair are reported. **Abbreviations:** AKI: acute kidney injury, CKD: chronic kidney disease, BM: bone marrow, CB: cord blood; WJ: Wharton's Jelly, HLSC: human liver stem cells, IRI: ischemia reperfusion injury.

MSC-EVs on renal proximal tubular cells was associated with miRNA transfer. In parallel, a transcriptional modulation of endogenous miRNAs was observed within injured cells. Prediction of miRNA targets showed that miRNAs modulated were involved in downregulation of coding-mRNAs associated with apoptosis, cytoskeleton reorganization, and hypoxia.

Similar results showing induction of renal regeneration were also obtained using MSCs from fetal tissues. EVs produced by human Wharton's Jelly MSCs (WJ-MSCs) (Zou et al., 2014) and by umbilical cord MSCs (UC-MSCs) (Ju et al., 2015) were able to reverse the morphological and functional alteration in IRI rats, in both the acute and chronic stage. Moreover, *exo*somes produced by human UC-MSCs were able to repair cisplatin induced AKI in rats by ameliorating oxidative stress, reducing cell apoptosis and promoting cell proliferation (Zhou et al., 2013). At variance, no therapeutic effect was obtained using EV-MSCs derived from human embryonic stem cells in a CKD model (van Koppen et al., 2012). In this study, EV-MSCs were administered twice daily for 4 consecutive days in rats with established CKD induced by 5/6 nephrectomy combined with L-N^G–nitroarginine and 6% NaCl diet, using therefore a curative protocol (van Koppen et al., 2012). This is the only paper, to our knowledge, where EVs were used in an established model of CKD. At variance, in the other papers in the field, EVs were injected soon after injury to prevent development of CKD in a preventive approach (Gatti et al., 2011; Ju et al., 2015; Zou et al., 2014).

Finally, MSCs resident in adult tissue different from bone marrow, such as kidney and liver were also investigated as possible sources of EVs to obtain acceleration of renal repair. Administration of EVs derived from kidney MSCs into mice with AKI induced by IRI, significant improved renal function and morphology, also inducing an amelioration of microvascular rarefaction by pro-proliferative and anti-apoptotic effects on tubular and endothelial cells (Choi et al., 2014). Similarly, purified EVs derived from human liver stem cells ameliorated renal function and morphology in a manner comparable to the HLSCs themselves, in an experimental AKI model induced by glycerol administration (Herrera Sanchez et al., 2014).

4.2. Other sources of EVs for kidney repair

Human endothelial progenitors cells (EPCs), a source of potent pro-angiogenic circulating progenitors obtained from peripheral blood of healthy donors, have been explored as possible EV source of for kidney regeneration. In a rat model of IRI, i.v. administration of EPCs-derived EVs (EV-EPCs) immediately after IRI prevented the renal functional damage (Cantaluppi et al., 2012). In particular, EV-EPCs conferred functional and morphologic protection from AKI by enhancing tubular cell proliferation and reducing apoptosis and leukocyte infiltration (Cantaluppi et al., 2012). In the same model, EV-EPCs also protected against progression of CKD after IRI by inhibiting capillary rarefaction, glomerulosclerosis, and tubulointerstitial fibrosis (Cantaluppi et al., 2012). Moreover, EV-EPCs were tested in the experimental anti-Thy1.1 glomerulonephritis in rats induced by complement-mediated mesangial injury. After i.v. injection in Thy1.1-treated rats, EV-EPCs inhibited mesangial cell activation, leukocyte infiltration and apoptosis, decreased proteinuria, increased serum complement haemolytic activity and ameliorated renal function (Cantaluppi et al., 2015).

Recently, EVs obtained by multipotent human urine derived MSCs were tested in a rat model of streptozotocin-induced diabetic nephropathy. Weekly repeated intravenous injections of this type of EVs could potentially reduce the urine volume and urinary microalbumin excretion, prevent podocyte and tubular epithelial cell apoptosis, suppress the Caspase-3 overexpression and increase glomerular endothelial cell proliferation in diabetic rats (Jiang et al., 2016).

Although different sources of EVs depicted above seem to display a similar protecting effect on acute kidney damage and to protect from its progression toward fibrosis, the effect cannot be ascribed to a general mechanisms. Indeed, fibroblasts-derived EVs, used as control cell source for EV generation did not ameliorate renal function (Bruno et al., 2012, 2009; Cantaluppi et al., 2015), indicating a specific action of EVs derived from stem cell types. At variance, the protective effect of stem cell-derived EVs in CKD models appears to be confirmed, on the light of negative results obtained using a single (Bruno et al., 2012) or even multiple administrations (van Koppen et al., 2012). Doses, number and timing of administration still require further investigation.

5. Mechanisms of action of EVs in kidney regeneration

5.1. EV uptake and bio-distribution

As depicted in the paragraphs above, EVs from different stem cell sources triggered *in vitro* and *in vivo* pro-proliferative and anti-apoptotic events. Among the possible mechanisms of action, it appears that the entrance of EV-MSCs into target cells is the first requirement for the biological effects *in vitro*. EV-MSCs expressed several adhesion molecules typically expressed by the originating MSCs (CD44, α-1, α-4 and α-5 integrins). CD44 and α-1 integrin were involved in EV internalization into renal tubular epithelial and treatment with specific blocking antibodies prevented EV-MSCs incorporation into target cells (Bruno et al., 2009; Lindoso et al., 2014). In addition, Annexin V was instrumental in EV-MSCs incorporation, since pre-treatment of EVs with Annexin V abrogated their uptake and effects on target cells (Iglesias et al., 2012).

In vivo evidences confirm the requirement of mechanisms of organ targeting and cell entrance for EV regenerative effects. In fact, pre-treatment of EVs with trypsin, that damages the proteins on the surface necessary for EV homing and uptake into target cells, abolished their *in vivo* regenerative capacity. Immunofluorescence experiments indicated that, after injection in mice with glycerol- or IRI-induced AKI, EV-MSCs and EV-EPCs localized within peritubular capillaries and tubules, as soon as 1 h after injection, with a peak of accumulation at 6 h after administration (Bruno et al., 2009; Cantaluppi et al., 2012; Gatti et al., 2011). In analogy, labelled EV-MSCs localized within the renal tissue in an AKI model were rapidly detected using optical imaging technique (Grange et al., 2014). Of interest, normal control mice without AKI showed absence of EV localization within kidneys. Minimal renal localization of i.v. injected EVs was confirmed by a study performing a detailed organ bio-distribution in a healthy animal using fluorescence intravital imaging (Wiklander et al., 2015). These data suggest that increased vascular permeability and tissue damage, or possibly expression of specific cell receptors for EV entrance, are required for EV renal localization in renal pathology. At variance, 60 and 120 min after i.v. administration, EVs were detected within the renal tissue using a sensible bioluminescent EV membrane reporter, suggesting that a small amount of EVs are cleared in physiological condition *via* the renal route (Lai et al., 2014).

5.2. Molecular mechanisms of EV activity

As reported above, EVs are enriched in small non-coding RNA species. The mRNA and miRNA content of therapeutic MSC-EVs has been characterized by microarray and RNA sequencing approaches by several investigations (Bruno et al., 2009; Collino et al., 2015, 2010). In particular, MSC-EVs were reported to contain mRNAs of genes involved in the control of transcription (Transcription Factor CP2, Clock Homolog, *etc.*), cell proliferation (RBL1,

SUMO-1, *etc.*) and immune-regulation (Bruno et al., 2009). Sequencing of miRNAs in MSCs and EPCs-derived EVs similarly showed the presence of miRNAs involved in the control of a number of cellular processes (*e.g.* proliferation, angiogenesis, apoptosis, cell adhesion, inflammation) (Cantaluppi et al., 2012; Collino et al., 2010). Moreover, the miRNAs present in the EV-MSCs are functionally active and able to induce the down-regulation of specific targeted proteins (Bcl-2, cyclin D1 and PTEN) in the recipient cells (Collino et al., 2015).

Several studies identified the transfer of extracellular RNA as the main mechanism for the EV therapeutic effects (György et al., 2011). When EVs of different origin were incubated with RNase, the *in vitro* and *in vivo* effects were blunted, indicating that regenerative properties were mediated by RNA species carried by EVs (Bruno et al., 2012, 2009; Cantaluppi et al., 2012; Ju et al., 2015; Reis et al., 2012). The transfer of specific mRNA and its subsequent translation into proteins in renal tubular cells has been shown both *in vitro* and *in vivo* (Bruno et al., 2009; Ju et al., 2015). The mechanisms of endogenous renal tissue repair after AKI include dedifferentiation of surviving cells followed by proliferation to repopulate the damaged tubules (Bonventre, 2003). In this context, *in vivo* and *in vitro* experiments indicated that mRNA for human Hepatocyte Growth Factor (HGF), a potent factor for tubular repair and regeneration, was contained in UC-MSCs-derived EVs and delivered into damaged tubular cells to be translated into protein. In addition, the induction of endogenous rat HGF mRNA and protein synthesis was also observed (Ju et al., 2015). Moreover, *in vitro* studies indicated that transfer of the Insulin Growth Factor 1 (IGF-1) receptor mRNA to renal tubular cells through MSC-EVs potentiated tubular sensitivity to locally produced IGF-1, increasing cell proliferation (Tomasoni et al., 2013). Therefore, trafficking mRNAs from specific growth factors and their receptors could have an important role in the regenerative effect exerted by EVs in different experimental renal injury models. In addition, stem cell derived EVs may contain mRNAs coding for anti-apoptotic molecules and for complement inhibitors such as Factor H, CD55 and CD59, and the related proteins. *In vitro* experiments indicated that EV-EPCs transferred to mesangial cell mRNAs coding for Factor H, CD55 and CD59 and inhibited anti-Thy1.1 antibody/complement-induced apoptosis and C5b-9/C3 mesangial cell deposition (Cantaluppi et al., 2012).

The pivotal role of miRNAs in the renoprotective effect of EVs was clearly proved by its abrogation after nonspecific miRNA depletion by Dicer or Drosha knock-down in the progenitor/stem cells (Cantaluppi et al., 2012; Collino et al., 2015). In addition, depletion of specific miRNAs in EPCs-derived EVs showed the role of the pro-angiogenic miRNA-126 and miRNA-296 (Cantaluppi et al., 2012). These results indicate that EVs derived from EPCs and BM-MSCs improve kidney injuries by delivering their miRNA cargo that contributes to a reprogramming of damaged resident renal cells to a regenerative program.

Extensive proteomic analysis has been performed on EV-MSCs (Kim et al., 2012). EV-MSCs contain proteins that are characteristic of MSCs (*e.g.* CD29, CD73, CD44 and CD105), proteins associated with intra-cellular EV biogenesis and trafficking (RAB protein family) and proteins associated with self-renewal and differentiation processes (TGF-β, MAPK, PPAR, *etc.*). Gene ontology analyses of such proteins indicated that several biological processes are represented, including vesicle-mediated transport, cell cycle and proliferation, cell migration, morphogenesis and developmental processes. In addition, EVs derived from urine MSCs contained specific growth factors, such as Vascular Endothelial Growth Factor, Transforming Growth Factor α1, Angiogenin and Bone Morphogenetic Protein 4, that are known to be podocyte survival factors and could be implicated in renal protection in diabetes (Jiang et al., 2016).

A different possible activity of EVs is the transfer of wildtype proteins to reprogram mutant cells. This activity has been demonstrated for EVs derived from MSCs and amniotic fluid-derived stem cells, that were able to shuttle a cysteine-selective transport channel (cystinosin) that restored function in mutant tubular target cells (Iglesias et al., 2012). This opens new perspectives for possible therapeutic applications of EVs in genetic-based diseases.

6. Pharmacological application of EVs

6.1. Limits and benefits of EV therapy

The therapeutic effect of EVs in renal regenerative medicine is promising and supported by a number of preclinical studies. However, comparison of results from these studies is limited by a number of reasons. First, different techniques of EV isolation have been used as well as different cell culture conditions of the originating cells that may account for different EV nature or content. Moreover, standardization of the dose of EV administered is still lacking. Several studies quantified EV protein content, that in turn may also be influenced by contaminants such as serum or bovine albumin in the culture medium. Others studies used EVs released by a defined cell number, and most recently the count of the EV number to be administered appears as the most reliable method (Table 2). However, even in this last case, the use of different instruments of quantification, based on different technology, may lead to incomparable results.

In addition, different problems may limit the clinical use of EVs. EVs can be classified as biological medicinal products, and their production needs to follow the guidelines on biological active substances of the different countries. First of all, criteria for scalable and reliable EV isolation methods and the best storage conditions for long term preservation of EV functions, compatible with current good manufacturing practice are required (Taylor and Shah, 2015). In addition, the mechanism of action of EVs including the characterization of the active substances is fundamental for a clinical application. Indeed, the pharmaceutical classification and approval by the National Agencies for Drug Regulation strictly depends on the identification of the active and ancillary components of EVs. A detailed analysis of the criteria for the use of EVs in clinical trials is provided in a statement paper of the ISEV society (Lener et al., 2015). As EV preparations represent a homogenous EV collection, information on possible differential effect of specific EV subtypes could provide a better standardization of EVs. Finally, although stem cell derived EVs such as MSC-EVs may possess immune-modulating activity, (Bruno et al., 2015) immune related problems of EV administration may be present, especially associated with multiple administrations.

On the other side, a number of benefits associate with EV-based therapy and support an active research in the field to improve the knowledge on EV content and mechanisms of action. The first benefit of EV therapy obviously derives from the comparison with the use of the cell source from which they originate. Having a similar therapeutic efficacy, EV therapy is devoid of issues related to ectopic engraftment or tissue formation, maldifferentiation or occlusion of the pulmonary artery that certainly concern the use of cell therapies. Second, characterization of EV cargo in terms of RNAs and proteins appears feasible and more applicable than that of cell therapy. In particular, EVs offer the possibility to use criteria based not only on phenotypic characterization but also on functional assays. The definition of a “therapeutic profile” based on the expression of genes required for the therapeutic effect of EVs would ameliorate the safety and possibly the result of EV administration.

6.2. EVs and clinical trials

Preliminary application of EV therapy has been proposed to exploit the EV immune-modulating and anti-tumour activity. EV-MSCs have been first administered for compassion use in a patient with steroid refractory graft *versus* host disease (Kordelas et al., 2014). Multiple injections (every 2–3 days) of EVs obtained from MSCs of unrelated bone marrow donors showed anti-inflammatory effects and ameliorated the clinical symptoms of the patient without adverse effects, indicating the safeness of EV administration. Three phase 1 clinical immunotherapy trials reporting feasibility and safety of autologous EV therapeutics have also been published (Escudier et al., 2005). Nevertheless, the first trial on MSC-derived Microvesicles and Exosomes on β-cell Mass in Type I Diabetes Mellitus is now enrolling (NCT02138331, www.clinicaltrial.gov). A possible advance in the use of EV based therapies can be envisaged by the use of vegetable EVs which appear as an edible, safe source of nanovectors (Quesenberry et al., 2015). In particular, two trials are currently being conducted using grapefruit-derived exosome-like nanoparticles. In these trials, plant-derived EVs, loaded with drugs, are orally administered for cancer treatment (Wang et al., 2013).

6.3. EV engineering

EVs may also be envisaged as drug carriers, as their surface composition leading to cell specific mechanisms of internalization may be coupled with addition of desired drugs. As discussed above, integrin- or tetraspanin-adhesion molecule complexes are involved in specific cellular uptake of EVs (Lindoso et al., 2014). Indeed, in comparison with liposomes, EVs are considered to display an increased half life and reduced uptake by the reticulo-endothelial system due to presence of an autologous surface molecules system (Johnsen et al., 2014). EVs may be modified for loading of drugs, as shown for the lipophilic drugs Doxorubicine or Curcumine which, being able to spontaneously enter the EVs, concentrate in their inside (Sun et al., 2010; Tian et al., 2014). In these studies, drug-loaded EVs showed an increased efficacy in term of anti-tumour or anti-inflammatory activity as compared to the drug alone. Another possible approach is the EV enrichment with therapeutic miRNAs (Johnsen et al., 2014). Finally, surface peptides might also be added to increase the specificity of the delivery. Although none of these approaches has been applied in renal preclinical models, it appears an interesting possibility.

7. Conclusions

In conclusion, EVs appear an interesting pharmacological tool for renal regenerative medicine. In particular, EVs derived from MSCs of a number of different sources has been proven to ameliorate renal function in AKI models. The application of EV based therapy for CKD is at variance less established. In particular, the definition of the therapeutic doses of EVs, standardization of EV quantification, modalities of administration and the best timing to start the treatment are still required. From all these studies, it can be prospected that the identification of the mechanisms of action of EVs and of the renoprotective molecules involved may allow the generation of modified, enriched EVs or engineered EVs for regenerative renal applications.

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