Extracellular Vesicles: Cell-Derived Biomarkers of Glomerular and Tubular Injury

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Abstract
Extracellular vesicles (EVs) are important mediators of intercellular communication. Since EVs are also released during pathological conditions, there has been considerable interest in their potential as sensitive biomarkers of cellular stress and/or injury. In the context of kidney disease, urinary EVs are promising indicators of glomerular and tubular damage. In the present review we discuss the role of urinary EVs in kidney health and disease. Our focus is to explore urinary large EVs (lEVs, often referred to as microparticles or microvesicles) as direct and non-invasive early biomarkers of renal injury. In this regard, studies have been demonstrating altered levels of urinary lEVs, especially podocyte-derived lEVs, preceding the decrease of renal function assessed by classical markers. In addition, we discuss the role of small EVs (sEVs, often referred to as exosomes) and their contents in kidney pathophysiology. Even though results concerning the production of sEVs during diseased conditions are varied, there has been a consensus on the importance of urinary sEV content assessment in kidney disease. These mediators, including EV-released miRNAs and mRNAs, are responsible for EV-mediated signaling in the regulation of renal cellular homeostasis, pathogenesis and regeneration. Finally, steps necessary for the validation of EVs as reliable markers will be discussed.

Introduction

Extracellular vesicles (EVs) are emergent mediators of intercellular communication as well as biomarkers of disease [1–3]. These biologically active membrane-coated vesicles...
are released by cells during physiological conditions in addition to conditions of stress, injury or death. Mechanisms of EV-mediated signaling occur through antigen presentation, receptor-mediated signaling, cell membrane fusion and/or endocytosis [4, 5]. EV content or “cargo” includes functional cytoplasmic proteins, peptides, lipids, nucleic acids (DNA, mRNA, microRNA, lncRNAs) and other signaling molecules that modulate cellular function, promoting autocrine or paracrine responses [6, 7]. On their surface, EVs present characteristic protein markers which can be used to identify their origin [7].

Size and mechanism of biogenesis should be considered when distinguishing different types of EVs: (i) apoptotic bodies are > 1 µm EVs that are formed during the late stages of cellular death by apoptotic pathways; (ii) microparticles (MPs), also termed microvesicles (MVs) are ~100 – 1000 nm fragments released by membrane blebbing and eventual shedding into the extracellular milieu; and, (iii) exosomes are smaller EVs (~20 – 150 nm), formed in a multi-step mechanism, where intracellular vesicles accumulate within multivesicular bodies, which merge with the plasma membrane and release exosomes. Other EV subpopulations (i.e. apoptotic nanovesicles, exomeres, oncosomes, migrasomes) have also been described, often to refer to EV populations in a specific setting (i.e. oncosomes are EVs released by cancer cells; migrasomes are generated during cell migration) [8]. These subpopulations tend to be much less pervasive in literature. A summary of the biogenesis of major EV classes is described in Fig. 1 and readers are directed to Chuo et al. 2018, D’Souza-Schorey & Schorey 2018, Abels & Breakefield, 2016 and Kalra et al. 2016 for more detailed reviews of EV biogenesis [4, 7–9]. Recent guidance from the International Society of Extracellular Vesicles suggests identifying ~100-1000 nm vesicles as “large EVs” (lEVs) and ~40-100 nm vesicles as “small EVs” (sEVs) when size is the primary descriptor and their origin is unclear. This represents a convenient distinction as many separation techniques effectively segregate based on size, however it is important to note that there is significant overlap of sizes between exosomes and MVs such that one cannot simply conclude that large EVs ≠ MV and small EVs ≠ exosomes. Regardless, these terms sEV and lEV will be used as appropriate throughout the present discussion, regardless of the terminology used in the original manuscript. When the size of the EVs is not clear from a manuscript we will employ the term “EV”.

Several studies have reported circulating lEVs as biomarkers of vascular disease, reflecting early vascular damage during prothrombotic and proinflammatory states [6]. In plasma, lEVs derived from endothelial cells, platelets and leukocytes have been associated with the development/progression of kidney diseases, in acute kidney injury (AKI), chronic

**Fig. 1.** Biogenesis of major extracellular vesicles classes. Apoptotic bodies are larger EVs (> 1 µm) formed during cellular death by apoptotic pathways. Microparticles (MPs) are sized ~100 – 1000 nm and released by membrane blebbing and shedding into the extracellular space. Exosomes are smaller (~40 – 100 nm), formed in a multi-step mechanism, with formation of multivesicular bodies (accumulation of intracellular vesicles) and posterior merge with the plasma membrane.
kidney disease (CKD), diabetic nephropathy (DN), lupus nephritis and nephrotic syndrome, among others [2, 3,10, 11]. However, urine is also a rich source of EVs [12]. In the present review we discuss the role of urinary EVs in kidney disease. In this context, circulating EVs are not likely able to cross the glomerular filtration barrier in significant numbers due to their size, thus, EVs in urine are believed to arise mainly from epithelial and parenchymal cells in direct contact with the urine throughout the nephron and bladder [12, 13]. This has led to the assessment of urinary EVs as putative non-invasive markers of renal injury [12–14].

**Urinary EVs as biomarkers of kidney injury**

Podocyte-derived vesicles were first described as membrane-bound vesicles enriched in phospholipids and cholesterol, found in the urine of patients with minimal change disease, membranous nephropathy, focal sclerosis, and DN [15]. These vesicles were approximately 100-200nm in size, consistent with the definition of EVs. In 2010, Hara et al. demonstrated that podocyte-EVs were derived from the tip vesiculation of podocyte’s apical membrane, more specifically by microvilli transformation and shedding into Bowman’s space. These urinary EVs from nephritic and nephrotic patients had a mean size of 200nm and absence of “exosomal” markers, such as CD24 and CD63 [16]. In addition, podocalyxin-associated EVs were isolated from the urine of patients with idiopathic membranous nephropathy and focal segmental glomerulosclerosis and then characterized as released by podocytes in glomerular diseases [17]. Based on these observations, podocyte-EVs gained consideration as direct, non-invasive markers of podocyte injury.

Our group has been investigating the role of podocyte-EVs in diabetic kidney disease. We previously demonstrated that podocytes release EVs in vitro when exposed to high glucose for 24h [18]. In addition, we were amongst the first to use nanoscale flow cytometry for assessment of urinary EVs. Using this approach, we reported that urinary levels of podocyte-EVs (podocalyxin’ or podoplanin’) were increased in diabetic mice (streptozotocin-treated, db/db, OVE26 and Akita mice) [18]. Levels of urinary podocyte EVs were strongly correlated with albuminuria in these mice; however, elevated levels of podocyte EVs could be identified before the development of albuminuria. Recently, we reported that significant increases in podocyte-EVs may be detected in type 1 diabetes patients also in the absence of albuminuria, nephrinuria or glomerular filtration rate (GFR) decline [19]. In the same study, we observed that podocyte-EVs (podoplanin’) were significantly higher during clamped hyperglycemia, suggesting glucose-mediated induction of EVs formation in accordance with previous in vitro experiments [19]. In type 2 diabetes, urinary EVs were reported to be associated with DN progression. De and colleagues (2017) observed a progressive increase in urinary total EVs and ≥130nm EVs (podocalyxin’) in diabetic patients with increases in albuminuria [20]. In addition, Kamińska et al. (2016) identified ~100nm sized urinary EVs inversely correlated with GFR in diabetic patients; however, no differences were observed in patients with advanced kidney disease [21]. Collectively, these findings indicate that in diabetic kidney disease, total and podocyte-derived EVs can be altered when classical markers of kidney function are still unaltered, which suggests that they are reflective of early glomerular damage. Of note, higher levels of urinary EVs during hyperglycemic states suggest that more podocyte damage could occur in diabetic patients with poor glycemic control. However, whether these events are associated with a premature development of kidney disease in type 1 or 2 diabetes, is a matter of further investigation.

Other studies have examined EVs derived from podocytes, in various kidney disorders. For example, Kwon et al. (2017) reported higher levels of podocyte-EVs (podocalyxin’/ nephrin’) in patients with renovascular hypertension when compared with hypertensive patients with similar blood pressure values [22]. They concluded that this reflects podocyte injury associated with the decrease of renal blood flow and perfusion. It is worth noting that the authors did not provide any information regarding the size of the vesicles studied and...
only flow cytometry was used to assess EVs in this study. Accordingly, it may be that only a fraction of urinary EVs were analyzed. In contrast with these observations, podocyte-EVs were not altered in renovascular hypertension in the study of Santelli et al. (2019). Despite that, the authors reported an increase in tubule-derived p16⁺ (marker of cellular senescence) EVs in patients with renovascular hypertension and essential hypertension, which were directly associated with circulant proinflammatory biomarkers and negatively associated with GFR [23]. Recent work by Zhang et al. (2019) reported podocyte-lEVs (podocalyxin⁺/nephrin⁺) to be significantly increased in obese patients as well as in an experimental porcine model of metabolic syndrome. In this study, the authors reported that urinary lEVs were directly associated with podocyte damage (foot process effacement, podocyte size and number, nephrin and podocalyxin tissue expression) and with renal dysfunction [24]. Higher levels of podocyte-derived lEVs were associated with podocyte injury in lupus nephritis and with increased lupus disease activity [25]. In patients with idiopathic membranous nephropathy, the same group reported that the decrease in urinary podocyte-lEVs were associated with disease remission after immunosuppressive therapy [26]. In addition, urinary lEVs of podocyte origin (podocin⁺/nephrin⁺) were significantly correlated with albuminuria in preeclampsia [27]. Taken together, these studies highlight the potential of urinary podocyte EVs as early biomarkers of podocyte damage in a wide range of clinical and metabolic disorders.

Similar results have been obtained when looking at urinary EVs from other cell types. For example, in kidney transplant recipients, urinary CD133⁺ EVs (derived from progenitor cells) were demonstrated to be released by the donor’s glomeruli (CD2AP⁺) and proximal tubule cells (megalin⁺). The presence of CD133⁺ vesicles was associated with graft function, since patients with poor graft function presented lower urinary levels of these progenitor EVs. This suggests a potential protective role of progenitor cell-derived EVs during the reestablishment of kidney function. Consistent with this, CD133⁺ EVs were not detected in the urine of patients with end-stage kidney disease [28]. We recently studied proximal tubule-derived lEVs (megalin⁺) in a mouse model of adenine-CKD treated with PBI-4050. PBI-4050 is an agonist of the G protein-coupled receptor 40 that has been consistently shown to reduce kidney fibrosis and inflammation in preclinical models [29,30]. We observed that PBI-4050 treatment was associated with lower levels of tubular lEVs parallel to improvement in tubular injury in adenine-fed mice [30]. Thus tubular lEVs appeared to be indicative of response to therapy in this animal model. Turco et al. (2016) identified that urinary EV levels decrease in parallel with the decline of kidney function associated with aging. Significant reductions in urinary EVs were also associated with nephron hypertrophy and global glomerulosclerosis in the same study [31]. The authors suggested that a significant decrease in EV production could be correlated with the renal function decline, but at which stage of kidney disease progression the “drop” in EV levels happens and if this is directly associated with extensive cellular death and nephron loss remains to be clarified. Of note in this study is the fact that the authors identified EVs by flow cytometry and that certain antibodies used targeted intracellular antigens (i.e. cytokeratins). Thus it is possible that non-EV cell fragments were enumerated by this approach.

Although there is some evidence suggesting that tubular lEVs are altered in kidney diseases, further studies are necessary to clarify the role of tubule-derived lEVs during acute and chronic renal damage. In this regard, it is interesting to note that De and colleagues have suggested that tubular markers such as megalin are more likely to be associated with sEVs, while podocyte markers are more frequently observed in urinary lEVs [20]. Notably, urinary lEVs from extrarenal cell sources could also provide insights about cellular activation in renal pathophysiology, as recently demonstrated by Burbano et al. (2019), who observed higher levels of urinary lEVs expressing specific markers of monocyte activation in lupus nephritis [32]. The authors speculated that this could reflect on the presence of inflammatory infiltrates in renal parenchyma. Urinary lEVs containing other biomarkers, such as monocyte chemoattractant protein-1 (MCP-1) and neutrophil gelatinase-associated lipocalin (NGAL), were also investigated in kidney stone disease [33]. Therefore, while it is believed that kidney
cells are the major source of lEVs in urine, these findings suggest that the assessment of renal endothelial and inflammatory responses might be possible when investigating urinary lEVs.

In summary, urinary lEVs are promising biomarkers of kidney injury, with significant increases observed in early stages of kidney diseases. Podocyte-lEVs, in particular, have been shown to associate with early glomerular damage in metabolic disease, renovascular hypertension, pre-eclampsia and lupus. An important consideration here is the fact that urinary flow rate can be highly variable between and even within individuals. This can have a profound impact on the concentration of urinary EVs. As such it is advisable to normalize in some fashion, although this has not always been done. We, and others have normalized to urinary creatinine levels in an effort to address this issue [18, 19, 33]. Approaches such as measuring levels over a 24 hour time-period or normalizing to urine osmolality may also be considered however at the moment there is no consensus on the best approach to optimization and we await guidance on this.

Urinary sEVs as biomarkers of kidney diseases

Urinary sEVs can be derived from renal cells in all nephron segments, as well as the bladder and prostate [34]. Conflicting results concerning the production of sEVs by kidney cells in the setting of clinical diseases can be found in the literature. On one hand, some authors reported unaltered urinary levels of sEVs in kidney disease, [28, 35–39] while others have demonstrated significant increases in urinary sEV levels, especially during AKI [40–43]. Evidence supporting the latter hypothesis includes the production 60-80% more sEVs by proximal tubular epithelial cells subjected to inflammatory or hypoxic conditions in vitro [44]. In addition, a recent study reported significant increases in sEV production in IgA nephropathy, which was correlated with tubular injury, histologic activity and proteinuria [41]. Similarly, Yu et al. (2018) observed higher numbers of urinary sEVs in CKD patients [45]. Further studies are needed to confirm whether direct increases in sEV release by kidney cells is reflected in urine as increased urinary sEVs.

There has been considerable focus on urinary sEV content in kidney disease. Of note, sEV content is protected from degradation by proteases and nucleases in urine by the vesicle’s bilayered lipid membrane. This may facilitate analysis of protein expression inside sEVs as a form of “liquid biopsy” to identify molecular determinants of renal pathology. Early studies examining sEVs in human urine focused on proteomic analysis of the sEV urinary fraction after ultracentrifugation [46]. Pisitikun et al. (2004) initially identified ~300 proteins including many proteins well-established to play a critical role in kidney function. More recent studies report over 2000 different proteins expressed within urinary sEVs [47, 48]. Next, we discuss the evaluation of sEV content in kidney disease with a specific focus on transcription factors, sodium (Na+) and water transporters and RNA species.

Ion and water transporters

Emerging evidences suggest that levels of Na+ transporters within sEVs are consistent with their expression in kidney tissue in experimental models [49, 50]. Du Cheyron et al. (2003) reported the presence of Na+/H+ exchanger 3 in “urine membrane fractions” of patients with prerenal azotemia and acute tubular necrosis [51]. Of note, as sEVs were not explicitly demonstrated in these urine samples, the authors used the term “membrane fractions” to describe urine pellets obtained after ultracentrifugation (200,000g for 120 minutes). Further studies demonstrated urinary sEV excretion of other Na+ transporters as sensitive markers of Na+ handling and blood pressure regulation in kidney diseases: Na+Cl-K co-transporter 2 (NKCC2) is significantly elevated in urine samples of patients with pre-eclampsia [52] and DN [53]; increased activation of the epithelial sodium channel (ENaC) was also reported in diabetic patients [54]; alterations in sEV sodium-chloride cotransporter (NCC) were associated with mineralocorticoid administration [55] and calcineurin-induced hypertension in kidney transplant recipients [38]. Significant decreases in sEV-NCC and
NKCC2 has been used to phenotypically differentiate hereditary salt-losing tubulopathies [56]. Moreover, the detection of the B1 subunit of the distal tubule V-ATPase ion exchanger in urinary sEVs during induced metabolic acidosis suggests that these EVs could be involved in the regulation of acid-base homeostasis [57].

Aquaporins (AQPs) were also reported in urinary EVs [58]. Ikeda’s group demonstrated significant reductions in urinary sEV APQ-1 and -2 in early stages of I/R-AKI [35, 40]. In gentamicin-treated rats, they observed significant early increases in urinary sEV excretion of AQP-2 followed by significant reductions six days after, which was accompanied by urinary concentration defects [42]. The dynamics of AQP-1 and -2 release in EVs after cisplatin treatment were also studied; while urinary sEV AQP-1 increases at early stages and significantly decreases in late AKI, dramatic reductions in AQP-2 can be found even at 24h after AKI induction, which suggests that sEV APQ-2 in urine can be used as an early biomarker of cisplatin-induced AKI [59]. Recently, the group observed that reductions of sEV AQP-2 are significantly associated with lower urine osmolality in kidney transplant recipients. This alteration may reflect concentration defects in transplant recipients, however it is difficult to determine whether the reduction in AQP-2 precedes the drop in urine osmolality or vice-versa [60]. In addition, the role of sEV APQs has been also investigated in DN. In fact, one of the first studies that demonstrated urinary EVs, focusing on the increase of AQP-2 expression in response to vasopressin, was performed in diabetic patients [61]. More recently, Rossi et al. (2017) reported significant increases in urinary excretion of AQP-1, -2 and -5 in diabetic patients with albuminuria, and AQP-2 and -5 were significantly associated with the progression of DN [53]. These results can elucidate mechanisms of urinary concentration defects in some disorders.

Transcription factors

The presence of transcription factors in urine was specifically demonstrated in sEV fractions [62, 63]. The presence of various transcription factors in urinary sEVs has been reported in both AKI and CKD [62]. Significant increases in urinary sEV activating transcription factor 3 (ATF3) was shown in early sepsis-induced and ischemic AKI [63, 64]. ATF3 may, in fact, play a causal role in AKI as Chen et al. (2014) have shown that ATF3 inhibits MCP1-expression in vitro and ATF3-knockout mice showed a higher I/R-induced inflammation [63].

Urinary sEV Wilm’s tumor protein 1 (WT-1) was demonstrated to be significantly increased in patients with type 1 diabetes and associated with the decline of kidney function in diabetic kidney disease [65]. Recently, the work of Abe et al. (2018) reported that hyperglycemia can stimulate the mobilization of WT-1 from the podocyte cytoplasm to be released within sEVs. This may explain the association between sEV WT-1 and DN progression, since they also observed that higher basal levels of urinary WT-1 were significantly associated with rapid decline of kidney function in type 2 diabetes [66]. In summary, these studies demonstrated that the alterations in urinary sEV levels of transcription factors could reflect the regulation of gene expression during kidney injury.

mRNAs and miRNAs

Nucleic acid content in urine sEV has also emerged as a possible avenue for biomarker discovery. As sEVs can be released by kidney cells during stress conditions (i.e. hypoxia, hyperglycemia, oxidative stress, inflammation, acidosis), [67] several studies have examined the role of mRNAs and miRNAs delivered by sEVs during kidney injury and their effects on the recipient cell. In this regard, the transference/exchange of mRNAs and miRNAs between cells from different segments of the nephron (as discussed in the next topic) is emerging as a novel regulator of kidney function [14]. Independent of this, sEVs secreted by renal cells in contact with the urinary space may be assessed for their RNA content. In fact, sEV RNAs are more stable in urine samples than “free” RNA [68, 69]. In Table 1, studies investigating urinary sEV RNAs in kidney disease are summarized.
Collectively, these findings confirm that sEV RNA and miRNA may be useful biomarkers. Should such markers be altered during renal cellular stress and/or injury it is possible that they can be assessed non-invasively in urinary EVs to evaluate the molecular status of a given patient for the purpose of individualized medicine. For example, kidney fibrosis is associated with increased excretion of specific exosomal miRNAs and mRNAs in the urine, [39, 41, 45, 70–73] as described in Table 1. This may provide evidence of fibrosis without a need of a biopsy. The evaluation of specific molecules involved in gene regulation and protein expression, such as exosomal transcription factors, miRNAs and mRNAs, could directly reflect possible mechanisms of altered cellular function in renal pathology.

Table 1. Summary of studies demonstrating urinary small extracellular vesicles-derived RNA species as biomarkers of kidney disease. Patterns of exosomal RNAs expression are demonstrated as increased (↑) or decreased (↓).

<table>
<thead>
<tr>
<th>Kidney disease</th>
<th>sEV-derived RNA</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute kidney injury</td>
<td>↑ ATF3 mRNA</td>
<td>Mice/Human</td>
<td>[62]</td>
</tr>
<tr>
<td>Autoimmune GN</td>
<td>↑ miR-26a</td>
<td>Mice</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>↓ miR-29c</td>
<td>Human</td>
<td>[72]</td>
</tr>
<tr>
<td></td>
<td>↓ miR-181a</td>
<td>Human</td>
<td>[131]</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>↓ miR-29, ↓ miR-200</td>
<td>Human</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td>↓ miR-29c, ↓ miR-141, ↓ miR-200b, ↓ miR-429</td>
<td>Human</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>↑ CD2AP mRNA</td>
<td>Human</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>↑ IL-18 mRNA</td>
<td>Human</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-145</td>
<td>Mice</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-451-5p, ↑ miR16</td>
<td>Rat</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-192</td>
<td>Human*</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-877-3p</td>
<td>Human*</td>
<td>[134]</td>
</tr>
<tr>
<td>Diabetic Nephropathy</td>
<td>↑ miR320c, ↑ miR6068</td>
<td>Human*</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-192, ↑ miR-194, ↑ miR-215</td>
<td>Human*</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-15b, ↑ miR-34a, ↑ miR-636</td>
<td>Human*</td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-133b, ↑ miR-342, ↑ miR-30a</td>
<td>Human*</td>
<td>[138]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-130a, ↑ miR-145, ↑ miR-155, ↑ miR-424</td>
<td>Human*</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>↑ WT-1 mRNA</td>
<td>Human*</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>↑ UMOD, ↑ SLC12A1, ↑ NDUFB2, ↑ OAZ1 mRNA</td>
<td>Human*</td>
<td>[139]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-193a</td>
<td>Human</td>
<td>[140]</td>
</tr>
<tr>
<td>Minimal change disease</td>
<td>↑ miR-155, ↓ miR-1915, ↓ miR-663</td>
<td>Human</td>
<td>[141]</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
<td>↑ miR-1225-5p, ↑ miR-1915</td>
<td>Human</td>
<td>[141]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-149-5p, ↑ miR-146b-5p, ↑ miR-378a-3p, ↑ miR-23b-3p, ↑ miR-30a-5p</td>
<td>Human</td>
<td>[142]</td>
</tr>
<tr>
<td></td>
<td>↑ CysC mRNA</td>
<td>Mice</td>
<td>[143]</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>↑ miR-215-5p, ↑ miR-378i, ↑ miR-365b-3p, ↑ miR-135b-5p</td>
<td>Human</td>
<td>[144]</td>
</tr>
<tr>
<td></td>
<td>↑ CCL2 mRNA</td>
<td>Human</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-26a</td>
<td>Human</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>↓ miR-29c</td>
<td>Human</td>
<td>[72]</td>
</tr>
<tr>
<td>Lupus nephritis</td>
<td>↓ miR-21, ↓ miR-let-7a</td>
<td>Human</td>
<td>[145]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-146a-5p, ↑miR-3135b, ↑ miR-654-5p</td>
<td>Human</td>
<td>[146]</td>
</tr>
<tr>
<td></td>
<td>↑ miR-146a, ↑ miR-200c, ↑ miR-302d, ↑ miR-335</td>
<td>Human</td>
<td>[37]</td>
</tr>
<tr>
<td>Renal fibrosis</td>
<td>↓ miR-21, ↓ miR-29b, ↓ miR-29c</td>
<td>Human</td>
<td>[39,45,69,70]</td>
</tr>
<tr>
<td></td>
<td>↑ CCL2 mRNA</td>
<td>Human</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>↓ E-cadherin mRNA, ↑ Vimentin mRNA</td>
<td>Human</td>
<td>[39]</td>
</tr>
</tbody>
</table>
EV signaling across the nephron

EVs may also alter signaling to target cells by delivering different functional molecules including miRNAs and mRNAs. Studies have shown that kidney cells can deliver EVs and promote intra- and intercellular communication within the nephron in order to modulate cellular homeostasis [12, 74]. Gracia et al. (2017) demonstrated that sEV-derived miRNAs could influence downstream signaling responses in cultured renal epithelial cells, altering the expression of potassium channel ROMK1 and calcium-transporter PMCA1 in collecting duct cells, as well as regulating the expression of amino acid-transporter SNAT2 in proximal tubular cells [75]. It is worth noting that the down-regulation of proteins was measured by Western blot and RNA interference was not validated directly (i.e. luciferase reporter assay). Accordingly, one cannot conclude that the down regulation of protein was definitively a result of miRNA transfer. Co-culture experiments have also demonstrated that sEVs can transfer functional AQP-2 in response to vasopressin, regulating water reabsorption by collecting duct cells [76, 77]. Fig. 2 highlights intercellular communication between kidney cells and other cell types promoted by EVs and their effects in renal homeostasis.

Importantly, the health status of the donor cells seems to determine the effect (beneficial or deleterious) on recipient cells. For example, EVs derived from healthy tubular cells can promote mesenchymal-to-epithelial transition and contribute to tissue repair [78] whereas tubular cells exposed to hypoxia or inflammatory mediators can promote macrophage infiltration and tubulointerstitial inflammation via EV signaling [79, 80]. Thus, the effects promoted by EVs in kidney cells may also contribute to renal pathogenesis. In this regard we recently demonstrated that podocyte-derived lEVs can induce a pro-fibrotic signaling response in the proximal tubule. This process involved a p38 MAPK/Smad3-associated

Fig. 2. Extracellular vesicles-promoted signaling in kidney homeostasis and pathogenesis. EVs can participate in kidney physiological processes, such as transference of aquaporins. EVs can also regulate apoptosis and reactive oxygen species (ROS) production in tubular and collecting duct cells under pathological conditions, induce epithelial-to-mesenchymal transition (EMT) and contribute to tubulointerstitial fibrosis. In addition, the cross-talk between renal cells and other cell sources (immune cells, fibroblasts, endothelial cells) can also contribute to kidney injury.
TGF-β activation and extracellular matrix production [81]. Consistent with this, sEVs enriched with TGF-β released by cells exposed to hypoxia or high glucose are implicated in the development of renal fibrosis [82–84]. In addition, other studies have also reported EV signaling contributing to other pathological processes such as ROS production [85] and epithelial-to-mesenchymal transition [70, 84]. Lastly, the cross-talk between kidney and immune cells promoted by EV signaling has also been recognized as an important factor associated with renal tubulointerstitial inflammation. In vitro studies demonstrated that lEVs and sEVs released by immune cells stimulate the production of inflammatory mediators by podocytes and epithelial tubule cells, respectively [86, 87]. The work of Lv et al. (2018) demonstrated that tubule-derived sEVs are packed with cytokine associated mRNAs in AKI and CKD experimental models. In addition, the group observed an increase in CCL2 expression within sEVs from cells exposed to albumin-induced inflammation, which then induced an up-regulation of inflammatory makers by macrophages [80]. Consistent with this, urinary exosomal CCL2 mRNA is significantly associated with proteinuria in IgA nephropathy patients [41].

EVs and kidney repair

It is important to emphasize that intercellular communication promoted by EVs are not always deleterious. In this regard, pioneering studies investigating the therapeutic potential of stem cells in kidney disease consistently observed that treatment with different types of progenitor cells (or their conditioned media) could ameliorate kidney function, however, the mechanisms involved in the recovery were not well understood [88–90]. As EVs became increasingly recognized as bioactive mediators of horizontal communication between cells, the role of progenitor cell-derived EVs in kidney regeneration was investigated. Several studies have reported that EVs released by different types of progenitor cells (e.g. mesenchymal stromal cells, endothelial cells, liver and kidney stem cells, adipose cells) were involved in tissue repair, cellular recovery and reduction of apoptosis [12, 91–94]. In Fig. 3, the protective role of EVs delivered by progenitor cells during kidney injury is illustrated.

The beneficial effects promoted by EVs in kidney disease have been reported in experimental models of AKI, CKD and DN as summarized in Table 2. Biodistribution experiments from two studies demonstrated that mesenchymal stem cell (MSC)- and endothelial progenitor cell-derived EVs are significantly increased in kidney tissue during AKI, accumulating within endothelial and injured tubular cells at 2h after I/R [95, 96]. In control animals, MSC-derived EVs did not traffic specifically to the kidney [95, 96]. Similarly, Viñas et al. (2018) recently reported that sEVs can target to kidney cells at 30min and 4h after I/R and transfer miR-486-5p to glomerular, tubular and endothelial cells. CXCR4/SDF-1α was observed to be involved in sEV uptake, microRNA transfer and improvement of kidney function during ischemic conditions [97]. Despite increasing evidence, additional in vivo studies are needed to clarify mechanisms of action and relative efficacy of sEVs from various origins in promoting kidney repair.

In summary, EVs are not only promising biomarkers of kidney disease, but active contributors to both pathogenesis and recovery. Importantly, as reviewed in this section, the health status of the donor cell directly impacts EV signaling. The cellular effects can be either beneficial, or deleterious, depending on whether the donor cells are injured or not. However, how and when the switch of the EV content during disease state happens, remains unclear. Moreover, progenitor cell-derived sEVs are associated with cellular recovery but lEVs have been reported to promote beneficial [98, 99] and deleterious effects [93, 100] during disease conditions. Thus, this it may not simply be a case of “small EV= good” and “large EV = bad”? Further study will be necessary to gain a greater appreciation for the role of EVs in intercellular communication in the kidney and the impact of disease on this. Finally, EV size categorization studies have shown that different molecular signatures can be observed according to sEV size [101, 102]. Even EVs within the same “category” (i.e. sEVs or lEVs) can be sub-categorized according to size, and this can directly impact the effect promoted by EVs in recipient cells [101, 102]. This challenges the current classification of major EV subtypes.
Fig. 3. Extracellular vesicles delivered by progenitor cells contribute to recovery of kidney injury. Previous studies have demonstrated that progenitor cell-derived EVs can promote beneficial effects and attenuate kidney injury, which includes inhibition of apoptosis, mitochondrial stress, oxidative stress, inflammation, endothelial dysfunction and fibrosis. Furthermore, these EVs can stimulate cellular proliferation and angiogenesis and contribute to kidney repair.

Applications of EVs in kidney health and disease

Methodological aspects

Studies on the EV field substantially increased in the last decade, and different approaches to isolate EVs have been described in the literature. EVs can be isolated and characterized from a host of sources. These include, but are not limited to, urine, serum, dialysate, cerebrospinal fluid, synovial fluid, bronchoalveolar lavage fluid, breast milk, bile, and saliva [18, 103–113]. Moreover, new technologies are constantly emerging that claim to increase the efficacy for EV detection and its applicability to clinical practice [114, 115]. In this regard, appropriate use of methods for EV isolation and measurement (and adequate reporting of the methods used) is critical to our understanding of EVs in kidney physiology and pathology. Recently, the EV scientific community has advocated for improved reporting practices [116]. Further, the International Society for Extracellular Vesicles (ISEV) recently published a comprehensive guideline (MISEV2018) which highlights prerequisites to validate EVs as biomarkers in different scenarios [117]. Each isolation method has advantages and disadvantages in terms of purity and enrichment of EVs. The decision of which technique to use is left to the investigator who must consider the focus of research, type of sample, disease and, ultimately, the requirements for downstream analysis (single EVs analysis, functional analysis or content analysis). For a complete review of methodologies for EV isolation and characterization readers are directed to Islam et al. (2019), Coumans et al. (2017), Giebel & Helmbrecht (2017), and Momen-Heravi et al. (2017) [118–121].
Table 2. Summary of studies investigating protective effects of extracellular vesicles in renal cellular recovery and kidney disease. Arrows indicate paracrine signaling promoted by EVs. AA = arachidonic acid; ADSCs = adipose-derived stem cells; ADMSCs = adipose-derived mesenchymal stem cells; AKI = acute kidney injury; Ang II = angiotensin II; BUN = blood urea nitrogen; CDGs = cardiosphere-derived cells; CKD = chronic kidney disease; Cr = creatinine (plasma or serum); CrCl = creatinine clearance; DN = diabetic nephropathy; ECs = endothelial cells; EPCs = endothelial progenitor cells; GCs = glomerular cells; GFR = glomerular filtration rate; GN = glomerulonephritis; HSLCs = human liver stem cells; I/R = ischemia-reperfusion; KMSCs = kidney mesenchymal cells; MetS = metabolic syndrome; MSCs = mesenchymal stromal cells; NGAL = neutrophil gelatinase-associated lipocalin; POD = podocytes; PTEN = phosphatase and tensin homolog; RAS = renal artery stenosis; RBF = renal blood flow; STCs = scattered-like cells; TCs = tubular cells; TGFβ = transforming growth factor β; USCs = urine-derived stem cells; UUO = unilateral ureter obstruction; VEGF = vascular endothelial growth factor.
Clinical perspectives

Given that EVs are measurable, specific and becoming well-studied, it is certainly feasible that they will ultimately be developed as new biomarkers of renal disease in clinical practice. Furthermore, their potential use as biological delivery systems are also an area of great interest. As mentioned above, in order for EVs to be reliable markers in clinical practice, they need to be easily obtained, quantified and characterized. This also needs to occur in a reasonably fast and affordable manner. As such, there are several groups studying the use of EVs in pre-clinical and clinical studies as they pertain to medical conditions including cancer, [122, 123] rheumatologic disease, [124] cardiovascular disease [125] and renal disease [2, 13].

As biologically active biomarkers, EVs present a new and exciting opportunity. Regarding urinary EVs in particular, it is thought they can function as both endogenous communication links between renal cells and perhaps exogenous tools to protect against injury or promote recovery [2, 14, 91, 100]. Because it is easy and non-invasive to collect urine, using urinary EVs as markers is very clinically appealing. Fresh urine is ideal; [126] however, samples can be centrifuged and stored frozen, making batched analysis a viable option that would be clinically feasible. Nevertheless, numerous challenges remain and further information is needed with respect to normalization, optimal collection parameters, and an appreciation of the influence of protein aggregates, urine viscosity, pH and osmolality. As such one must be careful not to over-conclude based on our current knowledge. While the development of individual patient urinary EV proteomes or transcriptomes to diagnose renal conditions, or monitor disease progression is appealing, much is still not known about possible confounding variables. In this context, further "omic"-based studies should continue to elucidate specific changes in the expression EV-derived factors in kidney disease (i.e. exosomal derived-mRNAs and miRNAs) but also remain conscious of the need to ensure reproducibility.

Using EVs as clinical tools for delivering "cargo" was recently discussed in an ISEV-led white paper [127]. An example of this would be the ability of EVs isolated from human MSCs to protect against I/R injury following both acute and chronic kidney injury [91, 128]. As a singular example, one can see the importance of translating this finding to clinical practice. AKI remains a leading cause of morbidity and mortality among adult and pediatric patients, alike [129, 130]. This paper highlights several additional examples of clinically applicable uses for EVs in diagnosis of disease ranging from genetic disorders (i.e. exosomal NKCC2), water homeostasis defects (i.e. urinary EV-AQP2), and glomerular injury secondary to podocytopathies (i.e. podocyte-derived lEVs). Importantly, these conditions normally require serum testing and long wait times. Could urinary EVs facilitate a faster diagnosis and be employed as markers of disease severity or remission? At present, it is impossible to answer this, but it would appear this is a clinically important question to ask.

Our approach for this review was to highlight the potential of EVs as mediators of intercellular communication and biomarkers of kidney disease. The number of studies on the EV field is increasing substantially in the past few years, however there are still several unanswered questions. For example, to identify lEV’s cellular origin, a range of surface markers have been studied individually or at most in combination of two; however, it is unclear if the EV phenotype (density of specific markers) can change according the diseased condition. Therefore, a panel of markers for the same cellular origin in different scenarios should be performed. In the context of glomerulopathies, for example, the expression of podocyte markers in lEVs such as podocin, podocalyxin, nephrin and podoplanin; could vary according to the etiology and phenotype of glomerular disease.

We have also discussed EV-mediated interactions involved in both kidney pathophysiology and repair; however, the possible routes for EV signaling and how donor cells target different cells across the nephron require further study. Recent studies suggest that cells can communicate by EV delivery throughout urinary space (e.g. podocytes → proximal tubular cells), but we speculate that it may also be possible for mesangial and endothelial cells to transfer EVs in the vasa recta and promote "interstitial signaling". Future
experimental studies performing renal punctures and isolation of mesangial-derived EVs could test this hypothesis.

**Conclusion**

In conclusion, this review summarizes some of the many applications for urinary EVs in clinical medicine. While assessment of EV levels and their content shows promise it is clear that we are only scratching the surface of our what is possible. Future studies should focus on standardization of methodology to facilitate adoption to routine clinical practice and further expanding our knowledge of EVs in kidney health and disease.

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