Circulating non-coding RNAs in kidney disease

Zhao Q.

CHAPTER 2

Circulating non-coding RNAs in chronic kidney disease and its complications

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Abstract

Post-transcriptional regulation by non-coding RNAs (ncRNAs) can modulate the expression of genes involved in kidney physiology and disease. A large variety of ncRNA species exist, including microRNAs, long non-coding RNAs, piwi-interacting RNAs, small nucleolar RNAs, circular RNAs and yRNAs. Despite early assumptions that some of these species may exist as by-products of cell or tissue injury, a growing body of literature suggests these ncRNAs are functional and participate in a variety of processes. Although they function intracellularly, ncRNAs are also present in the circulation, where they are carried by extracellular vesicles, ribonucleoprotein complexes or lipoprotein complexes such as HDL. These systemic, circulating ncRNAs are derived from specific cell types and can be directly transferred to a variety of cells, including endothelial cells of the vasculature and virtually any cell type in the kidney, and thereby affecting the function of the host cell and/or its response to injury. Moreover, chronic kidney disease itself, as well as injury states associated with transplantation and allograft dysfunction, is associated with a shift in the distribution of circulating ncRNAs. These findings which may provide opportunities for the identification of biomarkers with which to monitor disease progression and/or the development of therapeutic interventions.

Introduction

Advances in sequencing technology have enabled complete sequencing of the human genome. Alongside these efforts, which have provided important efforts into genome biology and the consequences of variants, the Encyclopedia of DNA Elements (ENCODE) project — a landmark study that was set up to identify the functional elements of the human genome[1] — has vastly increased our understanding of the organizational and regulatory elements that regulate gene expression and function. Findings from genome sequencing studies quickly revealed that it is not merely the number of genes that forms the basis of animal complexity. For instance, the human genome contains an estimated 20,000 protein-coding genes — unexpectedly similar to the total number of genes of nematodes that comprise~1,000 cells[2]. By contrast, these studies have shown that the non-coding part of the DNA extends with increasing organismal complexity[3], suggesting that non-coding RNA is central to the evolution. In humans, up to 98.8 % of the genome is non-coding; over 80% of the total genome is transcribed into a large variety of non-coding RNA species, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), circular RNAs (circRNAs) and yRNAs, that can together be referred to as the 'regulome'[4,5]. The function of many of these

non-coding RNAs remains unknown, but they are generally considered to function as post-transcriptional modulators of regulatory networks that control gene expression under both physiological and pathophysiological conditions. ncRNAs have various regulatory roles in the pathophysiology of kidney diseases. For example, miRNA-193a may induce focal segmental glomerulosclerosis (FSGS) through downregulation of its target *Wt1*[7], whereas lnc-MGC, a lncRNA that that hosts a miRNA megacluster and is induced by endoplasmic reticulum stress, promotes features of diabetic kidney disease (DKD) in mice, including the expansion of extracellular matrix and glomerular hypertrophy[8].

Despite the fact that ncRNAs function intracellularly, ncRNAs also exist in the circulation where they are packaged into extracellular vesicles,[9] lipoprotein complexes (in particular those containing high-density lipoprotein [HDL])[10] or ribonucleoprotein complexes (for example, those containing the RNA-binding protein argonaute 2 (AGO2)[11]. Within these carriers, ncRNAs are protected from degradation by RNases that are abundantly present in the circulation. Although these circulating ncRNA populations were initially thought to exist as shed by-products of tissue damage, a growing body of literature has provided convincing evidence that circulating ncRNA populations — like hormones and neurotransmitters — serve as mediators of cell-to-cell communication, and can be transported via cell-specific uptake mechanisms[12]. These insights also suggest that circulating ncRNA populations may have diagnostic and prognostic potential as biomarkers.

In this Review, we discuss our current understanding of the relevance of circulating and renal extracellular ncRNAs in the development of kidney disease. We describe the carrier types that transport these ncRNAs throughout the body and discuss the potential use of circulating ncRNAs as diagnostic factors and therapeutic targets in kidney disease.

Diversity of ncRNAs in the circulation

Non-coding RNAs can most likely be secreted by all cell types; however, the origin of many circulating, non-coding transcripts is often unclear. Most studies of circulating ncRNAs have focused on relatively well-known classes of ncRNAs, such as miRNAs, lncRNAs and circRNAs. However, transcriptomic studies have shown that tRNAs, yRNAs and ribosomal RNAs (rRNAs), and fragments thereof, are the most abundant classes of ncRNAs in the circulation [13,14]. In addition, piRNAs and snoRNAs are commonly classes of ncRNAs found in the circulation.

Of note, our ability to correctly identify ncRNAs has, until recently, been hindered by traditional sequencing approaches**,** which have often led to different readouts in ncRNA profiling studies, potentially as a consequence of differences in isolation and sequencing methods[14]. Moreover, the presence of RNA-nucleotide modifications, such as m1A base methylations, in ncRNAs interferes with traditional RNA sequencing processes and these modified ncRNAs are therefore not detected. Novel sequencing approaches, such as PANDORA-seq, can overcome these limitations and have led to the identification of many previously undetected short ncRNAs, including tRNA- and rRNA-fragments[15]. Interestingly, some of these fragments seem to be identical to sequences previously annotated as miRNAs, suggesting that some previously identified ncRNAs may have been incorrectly annotated. Hence, it is likely that technical developments in profiling approaches will improve our ability to annotate the vast repertoire of ncRNAs in the circulation and provide insights into their function.

miRNAs

Of all circulating ncRNAs, the function of miRNAs is best understood. miRNAs are negative regulators of gene expression. When bound to AGO2 — a key component of the RNA-induced Silencing Complex (RISC), — miRNAs facilitate RNA degradation or inhibit the translation of mRNAs that contain complementary sequences[16]. Some miRNAs can simultaneously repress multiple genes to directly influence the output of functionally-related biological pathways[17]. An example relevant to kidney injury is miR-182-5p, which targets several genes involved in cell-cycle control in ischaemia–reperfusion injury (IRI). Through these mechanisms, miRNAs can control cell fate via the temporal and spatial regulation of gene expression[18]. The cellular function of microRNAs in kidney injury and disease has been described elsewhere[19].

lncRNAs and circRNAs

LncRNAs are defined as non-protein coding RNA molecules that are longer than 200 nucleotides. LncRNAs have specific, regulated, patterns of expression in cell types, tissues and during development[20]. They can regulate gene expression through a variety of mechanisms, including through the control of gene transcription or by modulating mRNA stability and translation[21]. Although thousands of lncRNAs have been identified in the human and mouse genome[22], only a limited number have been functionally characterized. Some of these seem to have important roles in the development of kidney disease[23,24].

Once specific subtype of lncRNAs that have gained a lot of attention are circRNAs. These are single-stranded, circular ncRNA molecules, which are protected from exonucleases owing to their circular form[25]. Various functions have been ascribed to circRNAs, including the sponging (that is, suppression) of miRNAs and proteins, an ability to act as a scaffolding protein for enzyme–substrate complexes and roles in the recruitment of intracellular proteins. In addition, circRNAs have potential coding ability and can translate into (poly)peptides[27]. Available evidence suggests that circRNAs have important roles in kidney disease as described elsewhere[26].

yRNAs

yRNAs are ncRNAs of 83–110 nucleotides in size. The human genome contains four yRNAs (hY1, hY3, hY4, and hY5), whereas two yRNAs (mY1 and mY3) exist in rodents [28]. yRNAs were originally discovered as RNA components of the circulating Ro60 ribonucleoprotein complex — a complex that is targeted by autoimmune antibodies in patients with systemic lupus erythematosus (SLE) and Sjögren's syndrome[29]. Available data suggests that yRNAs have roles in DNA replication and in the regulation of misfolded RNA degradation (through interaction with Ro60)[30]. yRNAs can be cleaved into 5′ and 3′ fragments of specific lengths[31]; interestingly, one study has shown these yRNA fragments to comprise up to 67% of RNA sequence reads in plasma[13]. Although a miRNA-like role was proposed for those yRNA fragments, it was demonstrated that yRNA fragments bound to AGO2 do not repress the expression of reporter mRNAs, suggesting an alternative function[32].

tRNA-fragments

tRNAs are highly structured ncRNAs that are 76–90 nucleotides long and known for their essential role in protein translation. However, studies from the past few years have demonstrated the presence of tRNA halves and tRNA-derived fragments (tRFs) that are 31-40 nucleotides and 14-30 nucleotides long, respectively[33]. These tRNA-derived transcripts have been functionally linked to several biological processes, including cell proliferation[34], the inheritance of acquired metabolic disorders[37] and adaptations to stress[38]. Transcriptomic analyses have indicated that reads that map to tRNAs or their derived transcripts also represent a major fraction of circulating ncRNAs[38]. Furthermore, available evidence suggests that tRNAs, as well as yRNAs, are released into the circulation as full-length RNAs, after which they are processed by RNase 1 into their fragments[39]. Interestingly, differential expression of tRFs has been reported in ischemia-reperfusion injury (IRI)[40], hypertension-associated kidney

injury[41] and focal segmental glomerulosclerosis (FSGS)[42], although their function in these diseases remain to be determined.

piRNAs, snoRNAs, vtRNAs and snRNAs

Other types of less well-studied ncRNAs that are present in the circulation include piRNAs, snoRNAs, vault RNAs (vtRNAs) and small nuclear RNAs (snRNAs). piRNAs are small RNAs (26–31 nucleotides in length) that are important for the silencing of genes and genetic elements, in particular transposons, in the germline[43]. SnoRNAs are around 60–300 nucleotides in length and function to stabilize RNA through 2'O-ribose-methylation (that is, the addition of a methyl group to the 2' hydroxyl of the ribose moiety of a nucleoside) or through pseudouridylation (involving conversion of a uridine to a pseudouridine within an RNA chain)[44]. snoRNAs can also be processed into 21-nucleotide fragments that can function as miRNAs in RISC complexes[45]. piRNAs and snoRNAs are both abundant in the circulation[46].

vtRNAs are RNAs that are 88–98 nucleotides in length. Although their function is not fully understood, they form part of the vault ribonucleoprotein complex, and vtRNA-derived small RNA fragments have been shown to associate with argonaute proteins to guide the sequence-specific cleavage of specific fragments that can regulate gene expression similar to the actions of miRNAs[47].

snRNAs are nuclear RNAs that are typically ~150 nucleotides in length. They form components of small nuclear ribonucleoprotein complexes, which are involved in mRNA splicing[48]. Finally, non-host ncRNA species, such as virus-encoded, bacterial RNAs and food-derived RNAs, are also present in the circulation[49,50].

Types of non-coding RNA carriers

Extracellular ncRNAs are carried in the circulation either by extracellular vesicles (EVs), ribonucleoprotein complexes or lipoprotein complexes (**Figure 1).**

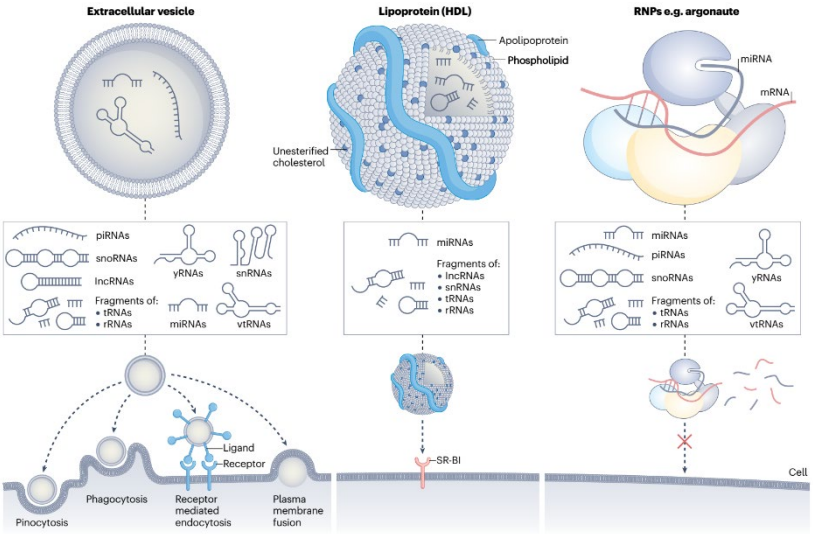

Fig. 1 | Types of circulating non-coding RNAs and their carriers. Cells can release non-coding (nc)RNAs or fragments thereof that can be specifically associated with either extracellular vesicles, or lipoproteins (in particular HDL) or ribonucleoprotein complexes (RNPs, and associated proteins such as argonaute-2). ncRNAs can then be selectively transferred to recipient cells and exert their function, thereby facilitating cell–cell communication. In patients with kidney disease, different pathophysiologies may contribute to the selective release of ncRNAs and their association with specific carriers. For example, cellular injury, endothelial activation or mesenchymal stem cell therapy may result in the shedding of extracellular vesicles that contain specific ncRNAs. These extracellular vesicles may be taken up by recipient cells via direct plasma membrane fusion or endocytotic mechanisms. During systemic inflammation, monocytes and/or macrophages may induce ncRNA loading of HDL that is taken up by recipient cells via a SR-BI-dependent mechanism. RNP–ncRNA complexes are often considered the result of passive release by dead or apoptotic cells that remain in the extracellular space owing to the stability of the RNPs, but may not be involved in functional ncRNA transfer. miRNAs, microRNAs; lncRNAs, long non-coding RNAs; snRNAs, small nuclear RNAs; tRNAs, transfer RNAs; rRNAs, ribosomal RNAs; piRNAs, piwi-interacting RNAs; snoRNAs, small nucleolar RNAs; vtRNAs, vault RNAs. Image courtesy of Manon Zuurmond.

Extracellular vesicles

Most research on ncRNAs in a specific carrier have focused on extracellular vesicles. These structures can be secreted by almost all mammalian cells and are divided into several categories according to their size and origin: exosomes (50-150nm), which are derived from endosomes; microvesicles, which are derived from cell membranes (~100-1000nm); and apoptotic bodies, which are derived from apoptotic cells (~1000-4000nm)[51]. Of note, definitions of EVs and their subtypes are often inconsistent. This inconsistency, combined with between-laboratory differences in methods used to isolate EVs, complicates comparisons of studies, and should be considered when evaluating and interpreting EV-relevant literature.

Encapsulation of ncRNAs by EVs might reflect a mechanism of RNA disposal, but may also enable the effective transport of ncRNAs to recipient cells and thereby facilitate their downstream function[52,53]. Adipose tissue has been identified as a major source of circulating exosomal miRNAs, which can subsequently regulate gene expression in distant tissues[54]. The relevance of this cross-tissue signalling was demonstrated in experimental work; for example, inhibition of exosomal miRNA secretion from adipose led to an increase in hepatic, circulating and renal FGF21 levels owing to the decreased expression of miR-99a, miR−99b, and miR−100.

Of note, the levels of circulating EVs are altered in the context of disease, and may affect the extent to which ncRNA cargo is transferred to target cells. For instance, patients with type 2 diabetes have higher absolute numbers of circulating EVs (as well as EVs derived from specific cell types, such as platelets, monocytes and endothelial cells) than do healthy controls[55]. RNA profiling studies have demonstrated that EVs contain many if not all ncRNA subtypes, including miRNAs, lncRNAs, circRNAs, yRNAS, tRNAs, piRNAs, snoRNA, vtRNAs, snRNAs and their fragments[56]. Interestingly, many studies have observed that ncRNAs are relatively enriched in EVs compared to those in various cells, suggesting that a process exists for the specific and selective sorting of these RNAs into these EVs[51]. For example, a remarkable enrichment of yRNA fragments (>75-fold) was found in endothelial cell-secreted exosomes compared with the intracellular yRNA fraction of endothelial cells. Moreover, circRNAs with small lengths are more likely than longer circRNAs to be released by platelets through EVs[57]. However, this enrichment of ncRNAs in EVs is not a universal finding, suggesting that ncRNAs may also be passively secreted into EVs[58]. Nevertheless, several mechanisms have been suggested to mediate the sorting of specific ncRNAs into EVs, including ncRNA sequence motifs, the actions of RNA-binding proteins, lipid membrane interactions and RNA modifications[51]. For example, the presence of specific sequence motifs may determine the whether miRNAs are secreted

into EVs or retained in the cell; recognition of these motifs by different cell types, such as adipocytes and endothelial cells, may enable those cells to selectively realize a cell-type specific EV-miRNA signature[59].

Following their packaging in EVs, ncRNAs must be delivered to recipient cells. The above-mentioned ncRNA sequence motifs may — in addition to facilitating EV packaging — facilitate delivery of the miRNA to recipient cells. The delivery of ncRNAs to target cells requires either direct fusion of the EVs with the plasma membrane of the target cell or uptake of the EVs via endocytic pathways. Uptake of EVs is the more common process and can involve clathrin-dependent endocytosis or clathrin-independent pathways, such as caveolin-dependent uptake, phagocytosis, macropinocytosis or lipid raft–mediated internalization[60]. The uptake of EVs by recipient cells may also depend on the presence of proteins and glycoproteins on the vesicle and cell surface, which might contribute to cell-selective and specific ncRNA uptake. For example, the presence of specific integrins on tumor exosomes contribute to organotropic metastasis through their organ and cell-selective uptake, which primes the pre-metastatic niche[61]. In the context of kidney disease, presence of C-X-C Motif chemokine receptor 4 (CXCR4) on exosomes, which can bind to SDF-1α on endothelial cells, is critical to the transport of miRNAs to endothelial cells[62]. Various other studies have also demonstrated evidence of cross-organ communication through EV-derived ncRNA transfer. For example, the transfer of miRNAs and lncRNAs from liver-derived EVs to the kidney may regulate kidney fibrotic[63] and nephrotoxic[64,65] responses. Conversely, in mice, myocardial infarction induced the secretion of miR-1956-containing EVs from the kidney, which were subsequently transported to adipose tissue. There, they activated adipose-derived mesenchymal stem cells through the stimulation of pro-angiogenic VEGF-signaling[66].

The fact that EVs that are generated by different tissues demonstrate capacity for homing to specific recipient organs may have important implications for the development and progression of kidney disease and its complications. Therapies that target such organ crosstalk may be of interest in multimorbid diseases, such as chronic kidney disease (CKD).

Lipoproteins

Beyond EVs, circulating ncRNAs can also be transported by lipoproteins. Although binding of ncRNAs to LDL has been observed, the majority of ncRNAs that are transported by lipoproteins are bound to HDL[11,49]. The ncRNAs bound to HDL are 10–60 nucleotides in size, with miRNAs being the most thoroughly studied to date. In plasma, HDL transports extracellular miRNAs

that are distinct from those transported by EVs[11]. Of note, HDL has a similar density as EVs, and thus, HDL and EVs are collected into the same fraction when they are isolated from plasma using density gradient ultracentrifugation[67]. Thus, in studies in which no further separation was performed, subsequent RNA isolation will reflect a combination of EV- and HDL-bound RNAs.

Myeloid cells, in particular, are thought to export ncRNAs to HDL through a mechanism that is independent of scavenger receptor class B member 1 (SR-BI). However, pancreatic beta cells and neurons can also load HDL with miRNAs[68]. The subsequent transfer of HDL-bound ncRNAs to recipient cells is dependent on SR-BI[11]. Although the transfer of HDL–miRNA to target cells was initially thought to inefficient and insufficient to have functional implications[69], several studies have now shown this transfer process to impact gene expression in the target cell[68]. Interestingly, patients with CKD demonstrate impaired reverse cholesterol transport and functionality and composition of HDL. Moreover, low levels of HDL-C are associated with CKD progression[70,71]. These alterations in cholesterol metabolism might also affect ncRNA binding and transport by HDL and therefore affect target cell function s. Differences in HDL-bound ncRNAs might also contribute to sex differences in disease, since women have higher HDL levels than men[72].

Ribonucleoprotein complexes

Despite interest in HDL and EV-bound ncRNAs, most ncRNAs, including tRNA fragments and miRNAs, are in fact found in a non-vesicular fraction associated with ribonucleoprotein complexes[10],[38],[73]. Specifically, most miRNAs are bound to the AGO2–ribonucleoprotein complex, whereas only a proportion of miRNAs are predominantly found in EVs[10]. The presence of circulating AGO2–miRNA complexes in blood suggests that cells may release a functional miRNA-induced silencing complex that might be directly transported to recipient cells to repress gene expression. Interestingly, AGO2–miRNA complexes are also found within EVs[74] and the possibility exists that the presence of non-vesicular, extracellular AGO2–ncRNAs may reflect a by-product of dead or apoptotic cells that may not contribute to cell-cell communication. AGO1 has also been shown to carry a distinct subset of miRNAs in the circulation[75]. Furthermore, ribosome-bound ncRNAs have also been detected in blood, suggesting that ribosomes may also act as a carrier for specific ncRNA types[73].

Exomeres and supermeres

Small (<50 nm), non-membranous extracellular nanoparticles termed 'exomeres' are also able to carry ncRNAs.[76] Exomeres are weakly negatively-charged, have a greater stiffness than EVs, distinct protein, DNA and RNA content and different organ biodistribution patterns. Finally, 'supermeres' are also carriers of ncRNA. These EVs, which are morphologically and structurally distinct from exomeres, exhibited a markedly greater uptake *in vivo* by all tissues compared to sEVs and exomeres[76,77], have distinct ncRNA and protein content, and are enriched for argonaute proteins[78]. It has been suggested that the majority of circulating RNA may actually be associated with these supermeres[76], and studies to assess the relevance of these particles in ncRNA-mediated cell–cell communication and their relevance to kidney disease are warranted.

Circulating ncRNAs in CKD

Despite long-standing interest in ncRNA transcripts as possible biomarkers of disease onset and progression, circulating ncRNAs have not received the same attention, given concerns that they were random degradation products of RNA turnover and dying cells. However, an increasing body of evidence has demonstrated that circulating ncRNAs can function as regulatory molecules and may contribute causally to a variety of kidney diseases (**Figure 2 and Supplementary Table 1)**.

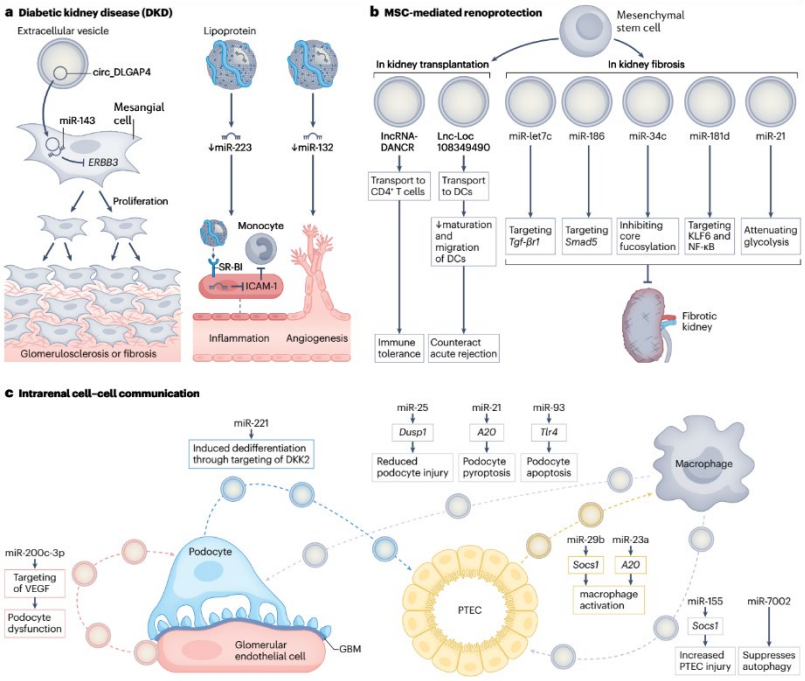

Fig. 2 | Functional role of carrier-associated non-coding RNAs in kidney disease. Many carrier-associated non-coding (nc)RNAs are dysregulated in patients with kidney diseases and involved in disease pathogenesis. **a**, In diabetic kidney disease — a condition that is associated with microvascular injury and ultimately leads to kidney fibrosis — extracellular vesicle and HDL-bound ncRNAs have been linked to mesangial cell proliferation, fibrosis, inflammation and angiogenesis. **b**, Mesenchymal stem cell (MSC)-derived extracellular vesicles are considered to provide protection against kidney injury in the context of transplantation, acute kidney injury and chronic kidney disease. For example, MSC-derived extracellular vesicle-associated long non-coding (lnc)RNAs can attenuate inflammatory processes following kidney transplantation. Several MSC-derived extracellular vesicle-micro (mi)RNAs can also inhibit pro-fibrotic pathways and reduce kidney fibrosis following injury. **c**, In the kidney, different cell types can communicate with each other via the release of extracellular vesicles that transfer ncRNAs to recipient cells. Such communication can occur, for example, between macrophages and proximal tubular epithelial cells (PTECs), macrophages and podocytes, and podocytes and glomerular endothelial cells. Image courtesy of Manon Zuurmond. GBM, glomerular basement membrane.

EV-associated ncRNAs in CKD and associated complications

Vascular injury. One of the earliest indications that EV-associated ncRNAs may have a functional role in CKD and its associated complications came from the observation in a rat model that endothelial progenitor cells (EPCs) release miRNA-containing EVs following the induction of IRI, which can activate an angiogenic program. Intravenous injection of these microvesicles following IRI induced protection from both acute and chronic kidney injury, as demonstrated by enhanced tubular cell proliferation, reduced apoptosis and leukocyte infiltration, and attenuation of capillary rarefaction, glomerulosclerosis and tubulointerstitial fibrosis. This effect was shown to be mediated by miR-126 and miR-296[79,80]. Interestingly, EV transfer of miR-126 may underlie the vascular protective effects observed following hematopoietic overexpression of miR-126 in mice following IRI[81]. Moreover, EPC-derived EVs were also shown to confer protection against sepsis-induced acute kidney injury (AKI), again via the actions of miR-126[82,83]. Specifically, miR-126-5p and miR-126-3p attenuate the lipopolysaccharide (LPS) induced upregulation of *HMGB1* and *VCAM1* in endothelial cells, respectively[83], whereas miR-93-5p targets *KDM6B* and thereby inhibits TNF signalling[82]. A further study also found that sepsis-induced AKI was attenuated via EV-derived miR-21 through targeting of the downstream PDCD4–NF-kB and PTEN–AKT pathways, thereby yielding anti-inflammatory and anti-apoptotic effects[84]. In addition to EPC-derived EVs, endothelial colony forming cells also secrete EVs that selectively target the kidney after ischemic injury and home to endothelial cells[62]. The subsequent transfer of miR-486-5p targets *PTEN* and reduces ischemic injury[85].

Compared to hemodialysis, hemodiafiltration is associated with reduced inflammation and potentially lower cardiovascular mortality. One study that examined endothelial cell-derived EVs in patients on hemodialysis and hemodiafiltration, showed that those from patients on hemodiafiltration had lower levels of miR-223. Follow-up in vitro studies in cultured endothelial cells showed that EVs derived from patients on hemodiafiltration induced stronger angiogenic responses and reduced apoptosis and calcification than those from patients on hemodialysis[86]. Of note, patients on hemodialysis had higher plasma levels of endothelial cell-derived EVs than healthy individuals, suggesting a relationship between EV levels and endothelial cell activation. Higher levels of endothelial cell-derived EVs were also observed in patients with diabetic kidney disease [87] (in addition to increased levels of EVs derived from platelets, leukocytes and erythrocytes), and in patients with CKD and coronary artery disease (CAD)[88]; thus higher levels of EVs seems to reflect EC dysfunction. Moreover, EVs from patients with CKD and CAD contained lower levels of the vasculo-protective miRNAs miR-130a-3p and

miR-126-3p than those from patients with CAD without CKD. The different miRNA contents of these EVs may lead to differences in endothelial cell in CKD and CAD and may contribute to the development of CAD or other vascular disorders in patients with CKD[88].

Fibrosis and muscle wasting. Muscle wasting a common complication among patients with CKD[89]. Two studies have found that miR-26a transfer after administration of EVs engineered to overexpress miR-26a, limits muscle wasting in mouse models of CKD[90,91]. MiR-26a directly targets the transcription factor *FOXO1* and in addition to its effects on muscle wasting, it has been shown to reduce cardiac and kidney fibrosis in 5/6 nephrectomized and unilateral ureteral obstruction mouse models, respectively. Inhibition of the transcription factor *YY1* and *TGF-β3* by EV-associated miR-29 also reduces muscle atrophy and kidney fibrosis[92]. Conversely, EV transfer of miR-150 and miR-21 from injured tubule epithelial cells to fibroblasts leads to fibroblast activation and exacerbated kidney fibrosis in mice, possibly via targeting of *Socs*1 and *Pten*, respectively[93-95]. In addition, the transfer of circulating miR-103a-3p to glomerular endothelial cells mediates angiotensin II-induced kidney fibrosis by inhibiting expression of the serine/threonine-protein kinase *SNRK* and inducing activationof NF-kB–p65 signaling. The carrier that transports miR-103a-3p to glomerular endothelial cells is not known; however, the identification of this miRNA in EVs by previous studies suggests that EV-mediated transfer is likely[96].

Epithelial cells, macrophages and glomerular injury. In a mouse model of streptozotocin-induced diabetes, inhibition of miR-221 reversed the abnormal dedifferentiation of proximal tubule epithelial cells. The researchers further showed that injured podocytes release miR-221-containing EVs, which are transferred to proximal tubule epithelial cells and induce their dedifferentiation through targeting of the WNT pathway inhibitor, *DKK2* [97]. Podocytes can also receive miRNAs via EV transfer. For instance, glomerular endothelial cells secrete miR-200c-3p-containing EVs in response to endothelial cell activation. This miRNA cargo are subsequently transferred to podocytes where they decrease VEGF expression and increase mitochondrial stress, ultimately leading to podocyte dysfunction[98]. Several studies have also found that macrophages to transfer miRNA cargo to podocytes via EVs. MiR-25-3p, from 'reparative' type 2 macrophages (M2)-derived EVs, improved high glucose-induced podocyte injury via targeting of *Dusp1* and activation of autophagy[99]. Another study showed that miR-25-3p and miR-21-5p released from macrophage-derived EVs mediate the anti-inflammatory and protective effects of γ-aminobutyric acid (GABA) on glucose-induced podocyte injury[100]. High glucose-induced macrophage-derived EV-miR-21-5p also demonstrated to increase podocyte pyroptosis by inhibiting the ubiquitin editor *A20*[101]. In addition, targeting of Toll-like

receptor 4 by M2-macrophage derived miR-93-5p attenuates LPS-induced podocyte apoptosis [102]. Although these studies provide initial evidence of macrophage–podocyte communication in the kidney via EV-derived ncRNAs, these analyses have mostly used in vitro systems and thus *in vivo* confirmation studies and independent verification by other groups is needed to provide greater insight into the contributions of these factors to kidney injury and repair.

Macrophages also deliver EV-derived miRNAs to tubular epithelial cells. For instance, macrophage-derived EVs were found to transfer miR-155 and promote tubular injury in a model of IRI[103], whereas macrophage-derived miR-7002-5p induces tubule cell injury and inflammation by targeting Atg9b and suppressing autophagy in tubular epithelial cells[104]. Conversely, studies in mice have shown that injured tubule epithelial cells can secrete miR-23a-containing EVs, which activate macrophages and induce a pro-inflammatory state via suppression of *A20*[105]. MiR-29b is also transferred to macrophages in LPS-induced AKI, inducing their activation via targeting of *Socs1*[106]. Finally, EVs derived from cystic renal epithelial cells promoted cyst growth in a model of autosomal dominant polycystic kidney disease (ADPKD), at least in part via the upregulation of miR-200b/c and miR-21, leading to the activation of ADPKD-associated signaling pathways[107].

Kidney transplantation. EV-derived miRNAs may also have a functional role in the setting of allograft rejection in kidney transplantation recipients. For example, plasma EVs derived from patients with antibody-mediated rejection induced senescence in cultured tubule epithelial cells as well as endothelial-to-mesenchymal transition in human umbilical vein endothelial cells. These EVs were enriched for miR-604, miR-515-3p, miR-let-7d-5p and miR-590-3p, whereas levels of miR-24-3p and miR-29a-3p were decreased, as compared to levels in EVs from transplant recipients without antibody-mediated rejection[108]. In kidney transplant recipients with delayed graft function (DGF), kidney perfusate-derived EVs contained higher levels of miR-218-5p, that strongly associated with a T-cell response, namely an increase in the ratio of T-helper 17 (T_H17) to T-regulatory (T_{REG}) cells[109], suggesting that donor EVs may be able to modulate immune responses in transplant recipients. In a mouse model of kidney transplantation, miR-682 was enriched in immature dendritic cells-derived exosomes (imDECs); this miRNA targeted *Rock2* to promoted T_{REG} cell differentiation *2*, thereby inducing immunotolerance[110].

Mesenchymal stem cell-mediated renoprotection. The application of mesenchymal stem cells (MSCs) for the treatment of kidney disease has been extensively studied with some promising results. The mechanism of action is largely considered to be via paracrine actions, including

the EV-mediated transfer of functional ncRNAs. Several studies demonstrated a protective function of MSC-derived EV-miRs in the development of kidney fibrosis. For example, MSC EV-derived miR-let-7c attenuated kidney fibrosis in mice by reducing the expression of the pro-fibrotic factor, *Tgfβ-r1* [111], whereas miR-186-5p attenuated fibrosis by directly targeting *Smad5*[112]. Furthermore, MSC-derived EV-miR-21 alleviated kidney fibrosis in mice by attenuating glycolysis[113] whereas the protective effects of miR-181d involved inhibition of the zinc finger transcription factor *KLF6* and NF-kB signaling[114]. MSC-derived MiR-34c-5p also ameliorates kidney fibrosis by inhibiting an important post-translational modification, called core fucosylation. The modification of proteins by core fucosylation is important for the activation of pericytes[115] — a cell type that gives rise to kidney myofibroblasts, which are responsible for the excessive deposition of extracellular matrix in the context of fibrosis[116]. In an IRI model, MSC-derived EVs transferred miR-125b-5p predominantly to proximal tubule epithelial cells, thereby promoting tubular repair via repression of p53 and preventing the G2/M cell cycle arrest[117] that contributes to kidney fibrosis[118]. MSC-derived EV-miR-199a-3p also protects from IRI injury by downregulating the expression of *SEMA3A* and subsequently activating AKT–ERK signaling[119]. Beyond MSCs, human urine-derived stem cells have been shown to protect against IRI via vesicle-mediated transfer of miR-146a-5p, resulting in the inhibition of NF-kB signaling through targeting of interleukin-1 receptor-associated kinase 1 (*IRAK1*)[120].

Several studies have also demonstrated roles ncRNAs beyond miRNAs in the response to kidney disease. For example, MSC-derived EVs also contain the lncRNA DANCR, which contributes to the induction of immune tolerance after kidney transplantation. In a mouse model of kidney transplantation, DANCR was transported from MSCs to CD4+ T cells where it promoted the ubiquitination and subsequent degradation of SIRT1. As a consequence, Treg cell differentiation was increased, inducing immune tolerance to kidney transplants[121]. Another MSC-derived EV-lncRNA, Loc108349490, alleviated acute rejection in a rat model of kidney transplantation through TLR4 ubiquitination, and inhibition of dendritic cell maturation and migration[122]. Moreover, The EV-circRNA mmu_circ_0001295 derived from adipose-derived MSCs was shown to inhibit sepsis-induced AKI by suppressing renal vascular leakage[123], whereas EV-circ_DLGAP4 found to be increased in serum EVs from patients with DKD as well s in EVs derived from high glucose-treated mesangial cells, was observed to induce aspects of diabetic kidney disease, mesangial cell proliferation and fibrosis in a rat model for DKD, by sponging miR-143 leading to de-repression of *Erbb3*[124].

HDL-associated ncRNAs in CKD and associated complications

Little is known about the role of circulating HDL-associated ncRNAs in patients with kidney diseases; however, HDL can transport a variety of small ncRNAs, including miRNAs and fragments derived from tRNAs, snRNAs, lncRNAs and rRNAs[49]. Within cells, lncRNAs and miRNAs control several genes involved in HDL metabolism, including *ABCA1* and *SR-BI*[125,126]. A number of studies suggest that circulating HDL–ncRNAs may be useful as biomarkers of disease activity and/or therapeutic targets. For instance, HDL-mediated transfer of circulating miR-223 to endothelial cells can suppress endothelial cell expression of the intercellular adhesion molecule *ICAM-1*, and is therefore considered to confer an anti-inflammatory response[127]. In line with this proposal, patients with diabetic kidney disease have lower levels of both HDL and circulating miR-223 compared to those in healthy controls[128], suggesting that loss of the protective effects of HDL–miR-223 may contribute to the development of vascular complications in patients with diabetes. In support of these findings, HDL–miR-223 was associated with the suppression of angiogenesis in the context of diabetes mellitus[129].

In a study of Australian Aboriginal people, individuals with diabetes and peripheral artery disease had elevated circulating levels of HDL-bound miR-181c-5p levels, which exhibited an anti-angiogenic potential[132]. In our study of patients with diabetic kidney disease in the Netherlands, levels of HDL-miR-132 levels decreased compared to those in healthy individuals; by contrast, total plasma miR-132 levels were increased[133], potentially indicating that HDL-miRNAs more strongly reflect kidney disease progression than whole plasma miRNAs. Interestingly, and in contrast to EV-associated miR-132, which did not demonstrate any angiogenic potential, HDL-miR-132 increased the angiogenic capacity of cultured endothelial cells. This finding suggests that the lower HDL-miR-132 levels of patients with diabetic kidney disease may reflect a reduced capacity to maintain vascular integrity and could be causally involved in the development of disease.

Thus, available evidence suggests that the HDL-facilitated transfer of ncRNAs to endothelial cells might provide a novel mechanistic explanation for the observation that dysfunctional HDL in patients with CKD can promote endothelial dysfunction and an abnormal vascular phenotype[134]. Moreover, we can speculate that differential HDL ncRNA cargos may explain the observation that medications used to increase HDL paradoxically do not improve health, despite the correlation between HDL levels are correlated and cardiovascular health[135].

Ribonucleoprotein-associated ncRNA complexes in CKD and associated complications

The majority of circulating ncRNAs are bound to ribonucleoprotein complexes containing proteins such as AGO2[58]. It is therefore surprising that little is known about ribonucleoprotein-associated -ncRNAs in kidney disease, or in general. Although some studies have investigated the biomarker potential, it remains of interest to assess whether circulating ncRNAs bound to RNPs can also mediate cell–cell communication One study demonstrated that the transfer of platelet microvesicle-derived AGO-2–miR-223 complexes to endothelial cells mediates the expression of specific genes and proteins[137], suggesting that the function of circulating AGO2-miRs is dependent on their physical association with EVs. Whether RNP-bound circulating ncRNAs have function in cell–cell communication or reflect a process of ncRNA degradation and disposal requires further investigation.

Biomarker potential of circulating ncRNAs

As described above, a number of papers have studied the functional role of circulating ncRNAs in relation to kidney function. Many, if not most of the studies that have focused on the potential of ncRNAs as biomarkers of disease have, however, predominantly measured total plasma or serum levels and not considering their carriers. This approach has led to the identification of many ncRNA species that could potentially serve as biomarkers, for example, the development of CKD [23,138,139], the risk of progression to kidney failure in patients with DKD [140,141], or survival or recovery from AKI.[142,143] However, an increasing body of evidence suggests that the evaluation of ncRNA levels in specific carriers may have improved biomarker potential over evaluation of total circulating levels. The below discussion focuses on studies that have investigated ncRNAs in their carriers or that identified novel types of ncRNAs as potential biomarkers in kidney disease (**Table 1 and Figure 3)**.

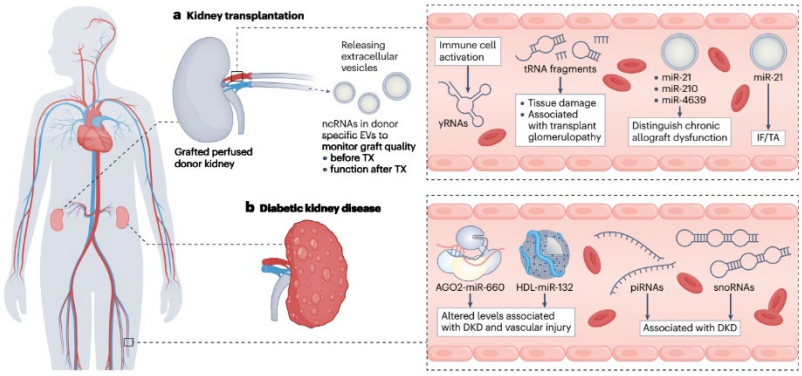

Fig. 3 | Biomarker potential of carrier-associated non-coding RNAs in patients with kidney disease. The selective release of non-coding (ncRNAs) in specific carriers suggests that levels of carrier-specific ncRNA levels may serve as novel biomarkers for kidney diseases. **a**, In the context of kidney transplantation, extracellular vesicle-associated ncRNA complexes derived from the donor graft may serve as a strategy through which to monitor graft quality and function. Similarly, circulating levels of yRNA, tRNA-fragment and miRNA may predict unfavourable outcomes, such as delayed graft function. **b**, In patients with diabetes, different carrier–ncRNA complexes are associated with the development of diabetic kidney disease, including AGO2-miR-660, HDLmiR-132 and sets of snoRNAs and piRNAs. Importantly, although HDL-miR-132 is strongly associated with diabetic kidney disease (DKD) and vascular injury, total plasma miR-132 levels do not, indicating that the measurement of ncRNAs in specific carriers may have increased biomarker potential over total levels. IF/TA, interstitial fibrosis and tubular atrophy; TX, transplantation.

Transplantation

Several studies have investigated whether EV-associated ncRNAs can be used to monitor graft function in the context of kidney transplantation. Levels of miR-21, miR-210 and miR-4639 in circulating EVs discriminated between kidney transplant recipients with chronic allograft dysfunction (estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 m^2) and those with normal graft function (eGFR > 90 ml/min/1.73 m^2)[144]. A study of circulating small ncRNA levels in kidney transplant recipients with biopsy-proven transplant glomerulopathy detected rRNA- and tRNA-derived fragments (mainly from two mature tRNAs: tRNA-Gly and tRNA-Glu), and identified specific signature of tRNA fragments associated with transplant glomerulopathy[146]. These circulating tRNA-fragments have been shown to rapidly increase in several experimental models of kidney injury, suggesting that this change reflects a specific response mechanism to injury. In support of this proposal, levels of these circulating tRNA-fragments increased in humans experiencing acute renal ischemia before other known markers of tissue damage increased[147]. These data provide compelling evidence that tRNA-fragments may serve as an early biomarker of kidney injury[148].

A proof-of-principle study using human-into-mouse xenogeneic transplantation of islets further demonstrated that donor-derived EVs and their miRNA content could be used monitor the allograft[149]. Further work demonstrated the ability to identify and characterize donor exosomes in the plasma of islet and kidney transplant recipients over follow-up periods of up to 5 years. Small ncRNAs in donor-derived EVs isolated from preservation fluid has also been used to monitor organ quality; moreover, the miRNA profile of these donor-derived EVs was associated with post-transplant graft function in the first week after transplantation[150]. EV-derived ncRNAs may therefore potentially provide a means by which to monitor donor organ quality and predict the outcome of transplantation. The ratios of ncRNA subtypes in EVs may also provide important information. One study found that yRNA subtype ratios were indicative of the number and type of immune cells in inflammatory disease[151]. Although speculative, this finding suggests that yRNA subtype ratios might also facilitate diagnosis of inflammatory responses associated with procedures such as kidney transplantation. Finally, levels of plasma EV-derived miR-21, but not from total plasma levels of miR-21, have been associated with interstitial fibrosis and tubular atrophy after kidney transplantation, indicating that ncRNA levels in a specific carrier may serve a better biomarker of disease processes than total ncRNA levels[152].

Diabetic kidney disease and CKD-related vascular complications

An association between carrier-specific ncRNAs and cellular dysfunction has also been demonstrated in patients with diabetic kidney disease. As described earlier, we have reported that although total plasma levels of miR-132 levels are increased in patients with diabetic kidney disease compared to those in healthy individuals, HDL-bound miR-132 levels are decreased; only HDL-bound miR-132 levels were associated with the vascular injury marker angiopoietin-2 and parameters of endothelial cell dysfunction[133]. Similarly, we have also shown that only AGO2-bound miR-660 — and not total plasma miR-660 levels — is associated with elevated levels of angiopoietin-2 and increased microvascular tortuosity, reflecting vascular injury in patients with diabetic kidney disease[133]. A preprint study has also identified small ncRNAs other than miRNAs, including piRNAs, a snRNA and snoRNAs, in association with DKD[153]. Although preliminary, this work highlights a need for further studies to investigate the relationship between these types of ncRNAs and DKD.

As mentioned earlier, patients with CKD are at increased risk of CAD and heart failure[154]. It is therefore of interest that circulating levels of HDL-miR-486 and HDL-miR-92a discriminated between patients with CAD who are stable and those who are vulnerable (that is, with unstable atherosclerotic plaques and therefore at risk of acute coronary syndromes)[155]. Moreover, AGO1-bound miR-222-3p, miR-497-5p and miR-21-5p levels were increased, whereas AGO1-bound miR-let-7a-5p was decreased, in patients with heart failure compared to levels in healthy controls[156]. These findings raise the question of whether these HDL- and AGO1-bound ncRNAs may also identify patients with CKD who are at risk of CAD or heart failure. Finally, studies in patients with hypertension — another common complication of CKD — have identified a signature of differentially expressed lncRNAs, miRNAs and piRNAs in plasma that are associated with urinary albumin excretion[157,158]. Of note, lncRNAs are usually expressed at much lower levels than other ncRNA transcripts[159], which makes their quantification difficult and potentially poses a challenge for the use of lncRNAs as biomarker of disease risk or progression.

However, together, these studies indicate that ncRNAs in specific carriers show promise as potential novel biomarkers for the development of kidney disease and its (cardiovascular) complications. Follow-up studies will be needed to determine whether they exhibit true biomarker potential and have clinical applicability.

Circulating ncRNAs as therapeutics

The growing body of evidence suggesting that circulating ncRNAs have functional roles in CKD and its associated complications suggests it may be of interest to pursue these transcripts or their fragments in therapeutic applications. In fact, the field of RNA-based therapeutics is rapidly growing and many RNA-based drugs — for a variety of diseases — are expected to enter the market in the coming years. Lumasiran is one of the first FDA-approved, RNA-therapeutic drugs of relevance to nephrology. Lumasiran is a short interfering RNA (siRNA) — an exogenous double-stranded small RNA that functions in RNA interference, similar to the actions of endogenous miRNAs — that causes sequence-specific inhibition of gene expression. Lumasiran specifically targets *HAO1* mRNA to inhibit the production of glycolate oxidase and is approved for the treatment of primary hyperoxaluria type 1.[160]

In terms of non-coding ncRNA-based therapeutics, one leading candidate that is currently in phase 1 trials is a second-generation antisense oligonucleotide against miR-17, called 'RGLS8429', that could serve as a potential therapy for ADPKD[161]. Unfortunately, a phase 2 trial of an inhibitor of miR-21 in Alport syndrome was terminated early as no meaningful improvement in kidney function was found compared to placebo[162].

Targeting and modulating circulating ncRNAs may potentially have an advantage in that the endogenous carriers that mediate their delivery could be hijacked. Moreover, improved understanding of how circulating ncRNAs are transferred through cell–cell communications may also provide opportunities for the design of effective delivery systems, as delivery to specific sites currently remains a challenge. For example, EVs have been proposed as drug delivery tools for RNA-based therapies[163,164], although our limited understanding of the mechanisms underlying their tropism *in vivo* complicates their therapeutic application for treating kidney disease. Hence, their optimal delivery requires a better understanding of their selective uptake and delivery mechanisms. Nonetheless, several approaches have been undertaken to engineer EVs or EV-like particles, such as lipid nanoparticles as delivery vehicles[165]. Since the uptake of EVs by recipient cells may depend on the presence of proteins and glycoproteins on the surface of the vesicle as well as the target cell, it may be necessary to make use of those (glyco)proteins for selective targeting. Relevant examples are the development of KIM-1 targeted EVs as a platform for RNA-inhibitor delivery to treat AKI[166], or engineering of the glycocalyx to achieve cell-specific targeting of EVs[167]. Polysaccharide-based nanoparticles called chitosan-nanoparticles also show potential for the specific delivery of cargo, including ncRNA into cells[168]. For example, these nanoparticles have been used to

deliver plasmid DNA expressing *Bmp7* for gene therapy in mouse models of CKD, which reversed the progression of fibrosis and promoted tubule regeneration[169]. Microbubbles in combination with ultrasound could provide an opportunity for local delivery. For instance, microbubbles could be loaded with the 'drug' of choice and released following the application of local, high-intensity ultrasound[170]. HDL mimetics may also serve as vehicle for the delivery of ncRNAs, possibly with different functional effects compared to those imparted by EVs given their different cellular uptake mechanisms[11,171]. Once successful targeting of local sites or cell types is possible, ncRNAs could be manipulated by delivery of ncRNA mimics or antisense oligonucleotides to enable targeting strategies for cell types and ncRNA functions.

Conclusions and future perspectives

A rapidly growing number of studies demonstrate that circulating ncRNAs are active mediators of cell–cell communication in patients with kidney diseases[171]. Although early studies typically focused on total levels of circulating ncRNAs, it is now clear that ncRNAs can be transported in the circulation in a diverse array of carriers that can demonstrate carrier-specific targeting mechanisms. To fully understand the mechanisms through which circulating ncRNAs exert their function, it is important for future studies to take these different carriers into account. Also, for therapeutic purposes, carrier-selective properties and their function may dictate how specific circulating ncRNAs may be stimulated or inhibited to treat kidney disease.

An interesting observation is that many subtypes of ncRNAs are found to yield specific cleavage products; tRNAs, yRNAs, snoRNAs, vault RNAs all are cleaved with some evidence that the fragments exhibit miRNA-like functions. These fragments are also often enriched in the circulation, suggesting a role for these ncRNAs fragments in providing an acute reservoir of miRNA-like transcripts that may function in conditions of cellular stress, as has been described for tRNAs[172].

Together, current insights into the role of circulating ncRNAs in kidney disease, coupled with developments in advanced 'drug-delivery' systems and RNA-based therapeutics, suggests that the selective modulation of circulating ncRNA levels or activity may provide novel opportunities for the targeting of specific molecular pathways that are involved in the pathophysiology of kidney disease.

Key points

- Circulating non-coding (nc)RNAs are mediators of cell–cell communication; current evidence suggests they have important roles in kidney disease.

- Circulating ncRNAs are selectively loaded into their carriers; selective uptake by recipient cells suggests that transported ncRNAs may have very specific functions in specific cell types.

- NcRNAs in specific carriers may serve as better biomarkers than total levels of circulating ncRNAs.

- A variety of circulating ncRNA species beyond microRNAs, including long non-coding RNAs (lncRNAs), piwi-interacting RNAs, small nucleolar RNAs (snoRNAs), circular RNAs (circRNAs) and yRNAs, as well as tRNA fragments, may have important functions, but their role in kidney physiology and disease remains to be explored

- Selective modulation of carrier–ncRNA complexes may provide novel therapeutic targeting strategies for various kidney diseases.

Table 1 | Circulating non-coding RNAs as potential biomarkers in kidney disease

Context	*Noncoding RNA*	*Disease or condition*	*Relation*	*refs*
Kidney transplantation	EV-miR-21; miR-210; miR-4639	Chronic allograft dysfunction	*Lower EV–miRNA levels inversely correlate with eGFR*	144
	rRNA- and tRNA-fragments	Transplant glomerulopathy	*tRNA-fragment signature (mainly from tRNA-Gly and tRNA-Glu) associates with transplant glomerulopathy*	146
	tRNA-fragments	Renal ischemia	*Circulating tRNA-fragments increased before other known markers of tissue damage increased*	147
	EV-miRNAs	Transplant status	*Donor specific EVs can be characterized to monitor graft status*	149
	EV-miRNAS	Transplant status	*EV-miRNAs in donor organ preservation fluid associated with graft function during the first 7 days post transplantation*	150
	EV-yRNA ratios	Inflammation	*Specific yRNA subtype ratios reflect the number and type of immune cells during inflammation*	151
	EV-miR-21	IF/TA	*Higher miR-21 levels associate with IF/TA*	152
DKD and CKD-related vascular complications	HDL-miR-132	DKD	*HDL-miR-132 levels are decreased in DKD and inversely correlate with angiopoietin-2 levels.*	133
	AGO2-miR-660	DKD	*AGO2-miR-660 levels are increased in DKD and correlate with microvascular tortuosity*	133
	Set of piRNAs snRNA snoRNAs	DKD	*Novel associations: piRNAs, a snRNA and in particular snoRNAs associate with DKD*	153
	HDL-miR-92a/miR-486	CAD	*Higher HDL-miR-486 and HDL-miR-92a discriminates between stable and vulnerable CAD*	155
	AGO1-miR-222-3p/miR-497-5p/miR-21-5p/miR-let-7a-5p	HF	*AGO1-bound miR-222-3p, miR-497-5p and miR-21-5p levels are increased, whereas AGO1-bound miR-let-7a-5p is decreased, in HF*	156
	Set of lncRNAs miRNAs piRNAs	Hypertension	*Signature of differentially expressed lncRNAs, miRNAs and piRNAs in plasma associates with urinary albumin excretion*	157,158

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