

# Circulating and Urinary microRNAs in Kidney Disease

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## Summary

microRNAs are small, noncoding RNAs that control gene/protein expression through target messengerRNA degradation and/or inhibition of protein synthesis. An array of experimental studies has shown the importance of microRNAs for disease initiation/progression. microRNAs are generally considered to act as intracellular modulators of gene expression. However, first studies in the cancer and cardiovascular field have elucidated that microRNAs are remarkably stable in the extracellular compartment (e.g., in blood or urine). The detection and quantification of circulating microRNAs may, thus, represent a novel noninvasive tool to detect and monitor disease activity. In addition, there might be a potential biologic relevance of circulating microRNAs for cell/cell communication. The aim of the present article is to give an outline of recent work on circulating and urinary microRNAs as well as their potential paracrine activity in kidney disease.

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## Introduction

MicroRNAs (miRNAs) are a class of small, noncoding transcripts with lengths of ~22 nt; they lead to the repression of gene/protein expression through the post-transcriptional degradation of messengerRNA by targeting its 3' untranslated region and/or translational inhibition of protein synthesis. The nomenclature was introduced by the Sanger institute (<http://www.sanger.ac.uk/>). The search for genetic loci responsible for temporal control of postembryonic development in *Caenorhabditis elegans* revealed the first miRNA lin-4 (1,2). miRNAs are formed in a highly regulated process; miRNAs are transcribed as primary miRNA transcripts (pri-miRNAs), which are then processed in the nucleus by Drosha (a ribonuclease) to 70-nt-long precursor miRNAs (pre-miRNAs) (3,4). The pre-miRNA associates with a Ran-guanosine-5'-triphosphate-dependent transporter, exportin 5, which regulates the shuttling into the cytoplasm (5,6). Here, it is processed by a second ribonuclease, Dicer, into a small, double-stranded RNA duplex (miRNA:miRNA\*) that contains an miRNA guide strand and its complementary strand (miRNA\*) consisting of 22 nt (7). The complementary strand is believed to be degraded, whereas the guide strand is incorporated into an RNA-induced silencing complex (Figure 1), in which it specifically targets and degrades messengerRNA (7). Mounting evidence suggests that miRNAs fundamentally impact on the development of various diseases, including kidney and cardiovascular disease (8,9). Intriguingly, a single miRNA may alter the expression of a large number of target genes, thus influencing a specific pathology by regulating whole disease-specific pathways and signaling cascades rather than a single gene. This finding has been elegantly shown for miRNA (miR) -29b, which regulates several collagen genes in Dahl-sensitive hypertension (10). This unique function underlines the immense importance

of these small molecules. miRNAs are generally believed to act as endogenous intracellular modulators of genetic information. However, recent studies have shown that miRNAs are remarkably stable in blood and other body fluids and thus, may be useful as biomarkers of disease activity (11–14). Interestingly, circulating miRNAs may transfer genetic information from a donor to a recipient cell, thereby altering cellular biologic processes by influencing the transcriptional program (15). These early observations have sparked an interest into whether circulating miRNAs may have a use as diagnostic and/or prognostic biomarkers in patients with kidney diseases.

In the present article, we outline the recent work on circulating miRNAs in kidney disease and discuss their potential pathophysiological relevance.

## Cellular miRNA Release and Extracellular Stabilization

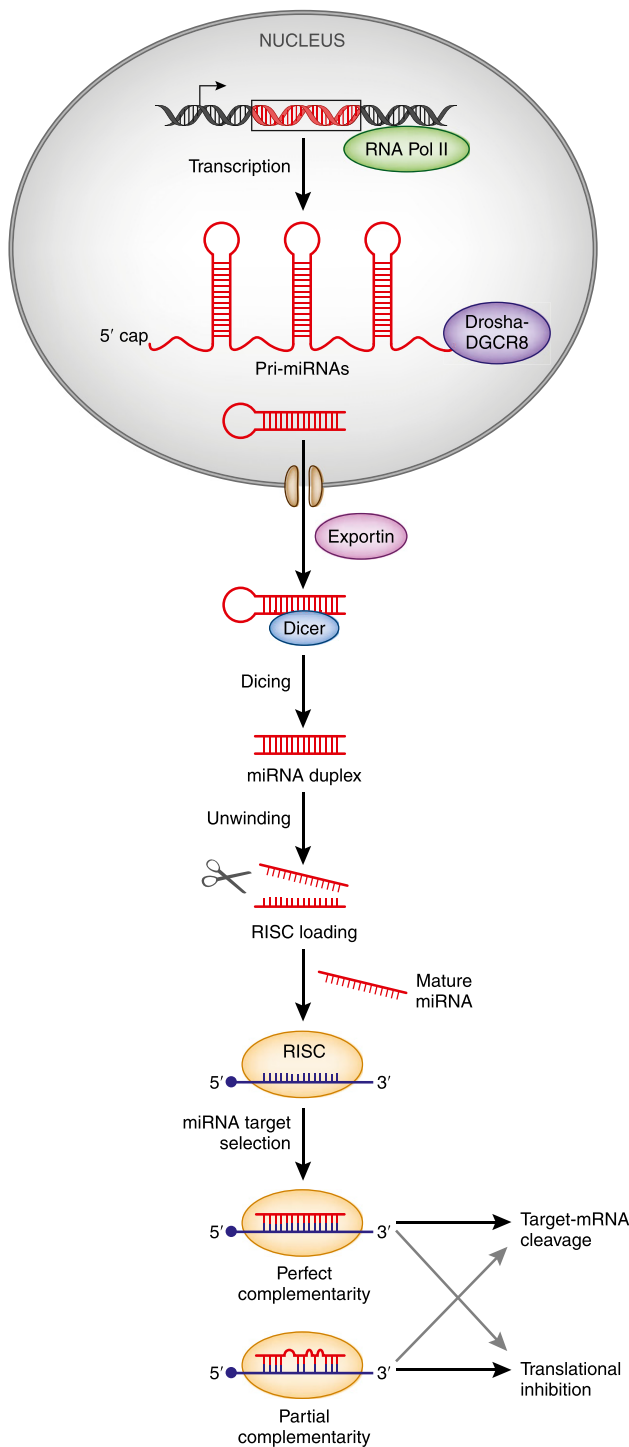
Recent work suggests that circulating miRNAs do not only function as disease biomarkers, which are released from the cellular compartment as a result of cell death on injury, but also are actively secreted and carry genetic information from one cell to another, thereby functioning as critical regulators of cellular crosstalk. To function as intercellular communicators, circulating miRNAs must be protected against degradation and because of their relatively low concentration in circulating blood, must be actively taken up by recipient cells.

miRNAs are present in the blood in a remarkably stable form that even withstands repetitive freezing/thawing cycles and are protected against RNases (11,12,16). Several distinct mechanisms of miRNA transport have been reported thus far.

Circulating miRNAs are included into lipid or lipoprotein complexes such as microvesicles/microparticles or exosomes. Although larger microvesicles/microparticles (100 nm to 1  $\mu$ m) are shed from the cell membrane of a

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**Figure 1. | Cellular biogenesis of microRNAs.** Primary microRNA transcripts are processed in the nucleus by Drosha to 70-nt-long precursor microRNAs. Then, a Ran-guanosine-5'-triphosphate-dependent transporter, exportin 5, transports pre-microRNAs into the cytosol, where the ribonuclease Dicer forms the mature, double-stranded RNA duplex that contains a microRNA strand and its complementary strand. The guide strand is incorporated into the RNA-induced silencing complex (RISC) complex to target messengerRNA transcripts. In mammals and humans, target silencing mainly occurs through partial complementarity, whereas perfect complementarity occurs in plants. However, both mechanisms may function in humans. Main mechanisms are depicted by black arrows, and subordinate mechanisms are depicted by gray arrows.

variety of different cell types under stress-dependent and physiologic conditions (17), smaller exosomes (30–100 nm) are derived from endolysosomal compartments called multivesicular bodies (18). The association of the RNA-induced silencing complex with multivesicular bodies is a prerequisite for miRNA loading into exosomes (18). The release of exosomes has been shown to be regulated, at least in part, by an enzyme implicated in ceramide biosynthesis called neutral sphingomyelinase 2 (19).

miRNAs have been shown to be actively secreted by different cell types in either exosomes (19,20) or microvesicles (20,21). Uptake of miRNA-enriched microvesicles or exosomes by recipient cells induces an altered transcriptional program (22). For instance, miRNA-enriched microvesicles derived from mouse embryonic stem cells transfer a subset of miRNAs to mouse embryonic fibroblasts influencing the genetic program (23). Dendritic cells have been shown to release miRNA-enriched exosomes, and release is followed by uptake and repression of target messengerRNAs in recipient dendritic cells (24). Recently, it was shown that vesicles secreted by endothelial cells after transduction with Krüppel-like factor 2 or stimulation by shear stress are enriched in miR-143/145 and that this miRNA cluster controls target gene expression in cocultured smooth muscle cells (15). In addition, atherosclerotic lesion formation in the aorta of ApoE(-/-) mice was reduced by uptake of these vesicles (15). This study highlights a pivotal function of circulating miRNAs packaged in vesicles, underlining that circulating miRNAs are capable of affecting the genetic program in recipient cells.

Transport in RNA binding proteins represents another mechanism of miRNA stabilization in plasma. In plasma, the majority of circulating miRNAs were shown to be associated with the RNA binding protein Argonaute 2, which is part of the RNA-induced silencing complex, whereas a smaller fraction of miRNAs was transported in microvesicles (25). Certain miRNAs were mainly detected in microvesicles (e.g., let-7a), whereas others were associated with Ago2 (e.g., miR-16 and miR-92a) (25). miRNA selection into either microvesicles or RNA binding proteins may, thus, represent cell type-specific miRNA expression and/or release mechanisms. Nucleophosmin 1 is another RNA binding protein shown to transport miRNAs (26).

Cell damage culminating in apoptosis induction may also be a source of circulating miRNAs. Endothelial apoptotic bodies were shown to carry miR-126, thereby providing alarm signals to recipient adjacent endothelial cells in atherosclerotic lesions and attracting progenitor cells to repair damaged endothelium (27).

Recently, HDL was found to carry miRNAs (28). Patients with familial hypercholesterolemia displayed a distinct HDL-miRNA profile compared with healthy controls. *In vitro*, native HDL was incorporated with exogenous miRNAs and introduced to cultured hepatocytes. The cellular uptake of miR-223-enriched HDL resulted in increased intracellular miRNA levels and a significant loss of miR-223 targets. Interestingly, HDL-dependent miRNA delivery to cells is dependent on scavenger receptor class B type I. Potential transport mechanisms of circulating miRNAs in kidney disease are summarized in Figure 2.

Circulating RNase levels have been shown to be influenced by renal function (29). One might speculate that

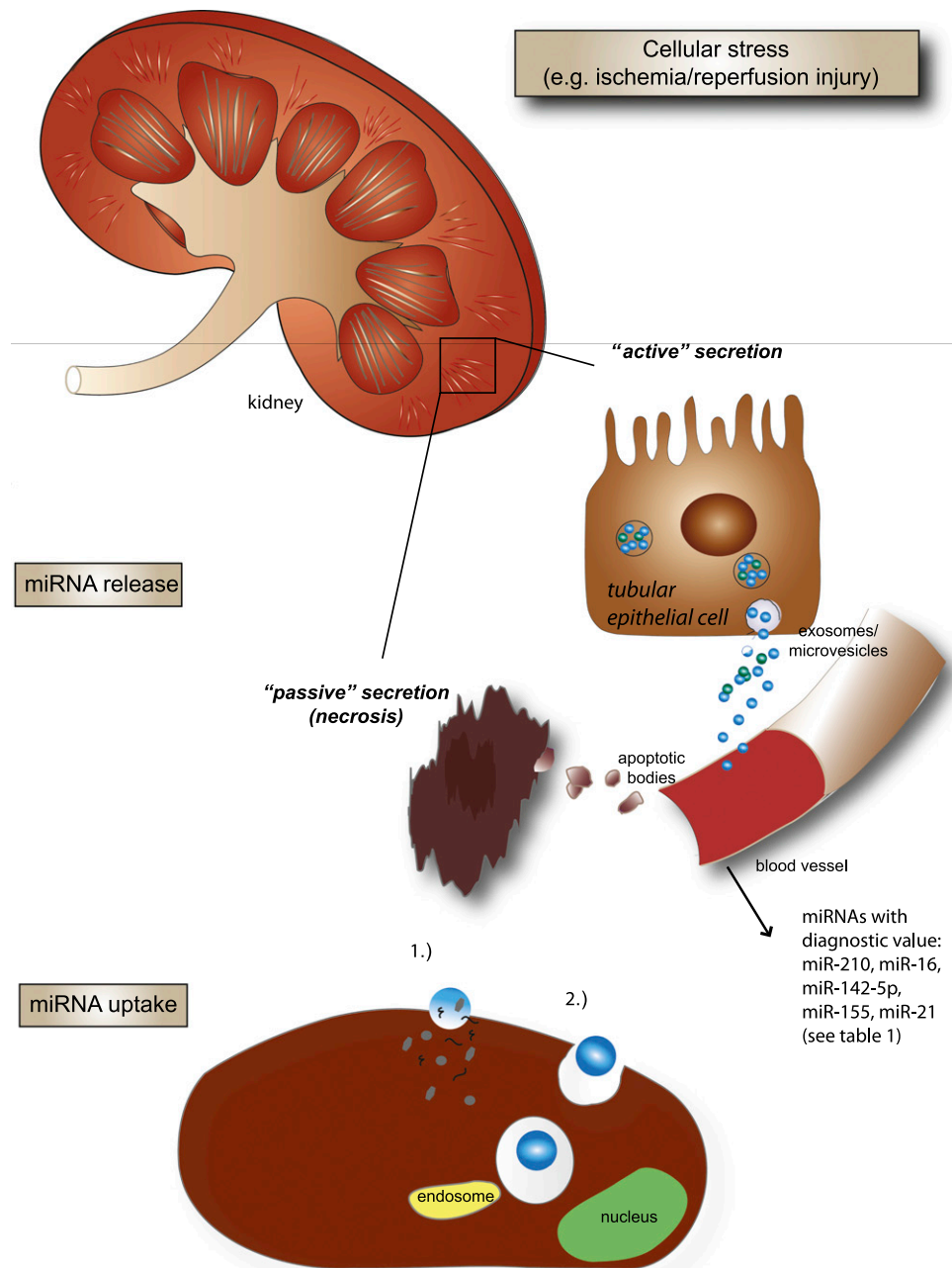
alteration in plasma RNase activity depending on kidney function might lead to the degradation of circulating miRNAs. Because miRNAs are bound to RNA binding proteins or packaged in microvesicles/exosomes in the circulation, they are protected against degradation by RNases.

### Circulating and Urinary miRNAs in Kidney Transplant Patients

The work by Anglicheau et al. (30) investigated the miRNA profile in PBMCs as well as kidney biopsies in patients with acute renal allograft rejection. These patients

were shown to display a unique miRNA expression profile in PBMCs and paired renal biopsy specimens. miRNAs overexpressed in renal biopsies (miR-142-5p, miR-155, and miR-223) followed the same pattern of expression in PBMCs, indicating that miRNA expression analysis might serve as a diagnostic test in this patient cohort.

To further assess the diagnostic potential of miRNAs in this patient population, our group analyzed the miRNA expression profile in urine samples (quantitative reverse transcription PCR-based miRNA microarray) of patients with acute T cell-mediated renal allograft rejection (31). This approach was chosen to identify miRNAs deregulated



**Figure 2. | Potential transport mechanisms of circulating microRNAs.** Cellular microRNAs are released from kidney cells (e.g., tubular epithelial cells) to the circulation through exosomes/microvesicles or in apoptotic bodies after cellular injury. Microvesicles (1) then fuse with the cell membrane of recipient cells, whereas exosomes are processed through the endosomal compartment (2).

in easily accessible urine to circumvent the potential risks associated with an invasive procedure such as a kidney biopsy. In the first step, the expression of a highly abundant miRNA (miR-21) was shown to be unaffected by incubation of urine samples at room temperature for up to 24 hours as well as a number of freeze/thaw cycles, indicating the stability of miRNAs in urine. Urine samples of patients were obtained at the time of biopsy. Patients with an acute rejection as diagnosed by a kidney biopsy showed reduced urinary levels of miR-210. In samples taken before and after the rejection period, miR-210 increased to levels of controls. In addition, a disease control group of transplant patients with urinary tract infection did not display altered miR-210 levels. These results underline a potential specificity of urinary miR-210 for the diagnosis of acute T cell-mediated rejection. Moreover, urinary miR-210 at the time of rejection predicted GFR decline at 1 year after transplantation. Hypoxia has been assigned a major role in miR-210 regulation. In endothelial cells, miR-210 was shown to be induced by hypoxia-inducible factor (32). In renal carcinoma, miR-210 was shown to be regulated by tumor hypoxia (33). It is, thus, conceivable that miR-210 might be regulated by hypoxia during renal allograft rejection, leading to an enrichment in kidney tissue and a subsequent reduction in urine.

The work by Scian *et al.* (34) investigated the miRNA expression profile in transplant patients with chronic allograft dysfunction characterized by interstitial fibrosis and tubular atrophy in kidney biopsies and paired urine samples. Differential expression was detected for miR-142-3p, miR-204, miR-107, miR-211, and miR-32 in kidney biopsies. The expression profile could be confirmed for miR-142-3p, miR-204, and miR-211 in paired urine samples. In addition, a gene expression profile was generated. Interestingly, the work by Scian *et al.* (34) found a high degree of correlation between different miRNA/messengerRNA pairs in kidney biopsies. Pathway analysis indicated that regulation of lymphocyte proliferation, B cell and T cell activation/differentiation, natural killer cell differentiation, and positive regulation of apoptosis as well as lipid oxidation/modification and protein dephosphorylation were most prominent in the biopsies.

Circulating or urinary miRNAs might, thus, represent an attractive, noninvasive tool for the early detection of acute or chronic rejection. This finding is the basis for the timely initiation of measures to prevent graft loss.

### Circulating miRNAs in AKI and CKD

Our group recently analyzed the miRNA expression profile in plasma samples of critically ill patients with dialysis-dependent AKI before the inception of renal replacement therapy (35). A total of 13 miRNAs were found to be significantly deregulated in the initial miRNA microarray (5 upregulated and 8 downregulated miRNA). In the additional analyses involving the whole cohort of patients ( $n=77$ ), the expression levels of miR-210, which were found to be upregulated, as well as miR-16 and miR-320, which were downregulated, were investigated. The level of deregulation could be confirmed for these miRNAs in the entire cohort. In uni- and multivariate Cox regression as well as Kaplan–Meier curve analysis, miR-210 was

found to predict the survival of patients at the study endpoint (day 28 after induction of renal replacement therapy). In addition, levels of miR-210 were significantly elevated in nonsurvivors. This study represents an initial report that circulating miRNAs are stably detectable in plasma of patients with kidney disease. Moreover, miR-210 was identified as a powerful predictor of mortality. The results for miR-210 in AKI are in contrast to those results reported in urine of patients with acute T cell-mediated renal allograft rejection. The reduction in urine might be caused by an enrichment in kidney tissue during acute rejection. Levels of plasma-derived miR-210 were shown to be upregulated in other acute ischemic disease states such as myocardial infarction (36), suggesting differences in miRNA transport and/or secretion dependent on the disease condition.

The work by Neal *et al.* (37) investigated the miRNA deregulation in patients with CKD. miR-16, miR-21, miR-155, miR-210, and miR-638 were analyzed in CKD patients and controls on the basis of detectability in plasma as previously shown by the group. All five miRNAs displayed a significant inverse correlation with kidney function (estimated GFR). Levels of miR-210 and miR-16, in particular, showed a higher degree of deregulation with progress of kidney disease (*e.g.*, healthy controls/CKD stage 3 versus CKD stage 4/5). The work by Neal *et al.* (37) also showed significant correlations between individual levels of miRNAs and clinical variables (*e.g.*, miR-16, miR-155, miR-210, miR-638, and hemoglobin; miR-21, miR-155, miR-638, and parathyroid hormone; miR-16, miR-155, miR-210, miR-638, and phosphate; miR-638 and albumin). With the exception of miR-638, none of the investigated miRNAs showed a differential excretion in urine of CKD patients compared with controls.

These two studies indicate that miR-210 and miR-16 might play distinct pathophysiological roles in patients with kidney disease. Future studies have to be conducted to further analyze and potentially confirm their importance in these patient cohorts. Circulating and urinary microRNAs in patients with kidney disease are summarized in Table 1.

### Current Challenges in Using miRNAs as Biomarkers

Data on circulating miRNAs in kidney disease are still limited. One might question the biologic relevance of circulating miRNAs concerning altered gene expression in recipient cells. In other words, are circulating miRNAs merely biomarkers, or may they be considered as true mediators of disease propagation/containment? Studies in the cardiovascular (27,38) and cancer field (22) have clearly shown the biologic role of circulating miRNAs. It has to be noted, however, that, in patients with renal disease, current studies have merely shown an association with disease activity. Thus, future studies are needed to prove a causative role for circulating miRNAs, which has been done in the cardiovascular field.

A challenge to the analysis of circulating miRNAs is the proper method of normalization. Owing to the low concentration of miRNAs in circulating blood, normalization is of utmost importance. Currently, a stable circulating miRNA is not available. Thus, researchers have to rely on other

**Table 1. Specific circulating microRNAs in patients with kidney disease**

microRNA	Body Fluid	Disease	Organism	Reference
miR-142-5p, miR-155, and miR-223	PBMCs	Acute allograft rejection	Human	30
miR-10a, miR-10b, and miR-210	Urine	Acute T cell-mediated allograft rejection	Human	31
miR-142-3p, miR-204, and miR-211	Urine	Chronic allograft dysfunction	Human	34
miR-16, miR-210, and miR-320	Plasma	AKI	Human	35
miR-16, miR-21, miR-155, miR-210, and miR-638	Plasma	CKD	Human	37
miR, microRNA.				

techniques of normalization. Several groups have supplemented spiked-in control miRNAs to normalize for potential differences in RNA isolation. Synthetic *C. elegans* miRNAs (e.g., miR-39, miR-54, and miR-238) were added after denaturation of plasma/serum during RNA isolation. Other groups have used miR-17-5p (36) and miR-1249 (39) as normalizing controls. Doubts remain about the stability of these miRNAs and other circulating miRNAs under different disease conditions. In fact, levels of miR-17 have been shown to be reduced in patients with acute coronary syndrome and miR-1249 undetectable in some samples (40).

The time-consuming analysis of circulating miRNAs represents an obstacle to their clinical use. Depending on the pathology investigated (e.g., acute myocardial infarction of acute renal allograft rejection), the clinical situation requires an immediate therapeutic decision. A bedside test instead of the complex RNA isolation procedure and the subsequent reverse transcription and quantitative PCR analysis would pave the way for the introduction of circulating miRNAs to broad clinical application.

Several animal studies have shown that the *in vivo* use of specific miRNA antagonists is an effective treatment option in various diseases (41,42). In the future, the detection of deregulated miRNAs in the circulation might lead to the therapeutic decision to use miRNA antagonists in humans in the treatment of kidney disease or a function as a biomarker of treatment efficacy.

In conclusion, because of their non- or minimally invasive nature, determination of blood- and urine-circulating miRNAs represents a fascinating tool to detect or monitor various human diseases. Studies published so far involved a rather low number of patients. In addition, prognostic data in patients with kidney disease concerning miRNAs are still limited. Future studies involving larger cohorts of patients and different etiologies of kidney disease have to further explore their usefulness. An analysis of miRNA transport in circulating blood of kidney disease patients is highly warranted. Moreover, studies exploring the impact of circulating miRNAs on altered transcriptional programs in recipient cells would be desirable. Last, in most studies pertaining to circulating miRNAs in kidney disease, the donor/recipient cells of these circulating molecules are unknown. Thus, an elucidation of the origin and target cells of circulating miRNAs would increase insight into their potential pathophysiological relevance.

### Disclosures

J.M.L. and T.T. have filed patent applications for the use of circulating miRNAs as diagnostic markers in patients with kidney disease.

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