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MicroRNAs as novel therapeutic targets to treat kidney injury and fibrosis

Ivan G. Gomez, 1,2 Naoki Nakagawa, 2,3 and Jeremy S. Duffield1,2

1Research and Development, Biogen, Cambridge, Massachusetts; 2Division of Nephrology, Departments of Medicine and Pathology, University of Washington, Seattle, Washington; and 3Division of Nephrology, Asahikawa Medical University, Asahikawa, Hokkaido, Japan

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Gomez IG, Nakagawa N, Duffield JS. MicroRNAs as novel therapeutic targets to treat kidney injury and fibrosis. Am J Physiol Renal Physiol 310: F931–F944, 2016. First published February 24, 2016; doi:10.1152/ajprenal.00523.2015.—MicroRNAs (miRs), a class of small noncoding RNAs that act as post-transcriptional regulators of gene expression, have attracted increasing attention as critical regulators of organogenesis, cancer, and disease. Interest has been spurred by development of a novel class of synthetic RNA oligonucleotides with excellent drug-like properties that hybridize to a specific miR, preventing its action. In kidney disease, a small number of miRs are dysregulated. These overlap with regulated miRs in nephrogenesis and kidney cancers. Several dysregulated miRs have been identified in fibrotic diseases of other organs, representing a “fibrotic signature,” and some of these fibrotic miRs contribute remarkably to the pathogenesis of kidney disease. Chronic kidney disease, affecting ~10% of the population, leads to kidney failure, with few treatment options. Here, we will explore the pathological mechanism of miR-21, whose pre- eminent role in amplifying kidney disease and fibrosis by suppressing mitochondrial biogenesis and function is established. Evolving roles for miR-214, -199, -200, -155, -29, -223, and -126 in kidney disease will be discussed, and we will demonstrate how studying functions of distinct miRs has led to new mechanistic insights for kidney disease progression. Finally, the utility of anti-miR oligonucleotides as potential novel therapeutics to treat chronic disease will be highlighted.

microRNAs; Dicer; chronic kidney disease; fatty acid oxidation; mitochondria; angiogenesis; macrophage activation

MICRORNAS (MIRS) ARE SMALL, 21- OR 22-nucleotide, highly conserved RNAs first identified in 2001 in worms (52, 56, 103). They were found to be temporally expressed but not code for proteins and are therefore “non-coding.” However, their temporal expression and phylogenetic conservation suggested they play regulatory roles in cellular fate and function. In 2002, they were shown to be complementary to the untranslated sequence of genes whose expression they regulated, indicating that they mediate posttranscriptional suppression of gene expression and therefore are a collection of RNA molecules that mediate posttranscriptional regulation of gene expression, which in many settings has equivalent or greater impact on phenotype than transcription factors (5, 53, 105). Since the identification of miRs only 14 years ago, there have been nearly 36,000 peer-reviewed publications about miRs, emphasizing the biological importance of these small molecules.

Over the following years, the complex function of miRs in controlling the expression of genes by preventing mRNA transcripts from being translated to proteins was unraveled (Fig. 1A). miRs are transcribed as precursor transcripts (pri-miR) or sometimes in long noncoding RNA (lncRNA) that are several hundred nucleotides in length, cleaved by the RNase-III, Drosha, to form pre-miRs and exported as pre-miRs to the cytosol by a transporter known as exportin-5 (Fig. 1A). Pre-miRs form a complex hairpin structure with areas of complementarity interspersed by areas of non-complementarity. These pre-miRs are ~70 nucleotides in length. They are subsequently loaded into a protein chaperone complex by the enzyme Dicer1, where they are cleaved into the mature 21- to 22-nt mature miR (Fig. 1A) (49, 57, 72, 107). The mature miR then complexes with protein chaperones, forming a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC), which includes the essential endonuclease activity of the enzyme argonaute-2 (AGO2) (23, 66) (Fig. 1A). This protein complex directly interacts with mRNA transcripts and binds by sequence complementarity to the mRNA transcripts. This interaction triggers two separate processes. One is the blockade of ribosomal function and therefore prevention of translation. Separately, the argonaute complex triggers degradation of the mRNA (23, 88). By both of these mechanisms, miRs in the argonaute-containing RISC complex silence gene expression (Fig. 1A). Computational analysis of complementary sequences to specific miRs observed that most complementary sequences are in the 3′-untranslated region (UTR) of...
The field of RNA interference heralded these rapid advances in our understanding of miR biology. Several years before the identification of miRs, the process of RNA interference was reported by Fire and coworkers (30, 82) to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms, demonstrating that this process was mediated through RNA degradation rather than mRNA synthesis. Shortly thereafter, scientists identified that small numbers of synthetic dsRNA molecules ~21 nt in length could effect RNA interference and prevent mRNA translation (74, 102). Therefore, the endogenous mechanism of miR action was discovered and manipulated several years before the identification of the endogenous effector dsRNA molecules.

Since the original descriptions of 15–50 distinct miRs in worms, the presence of >1,000 different miRs in mammalian cells is now widely accepted. Many of these miRs have poorly resolved functions, but an increasing number have been shown to profoundly affect cellular fate and function and prove to be every bit as powerful as transcription factors in controlling cellular processes.

Critical roles for miRs in organ patterning during embryogenesis as well as cell fate during cancer and even homeostatic functions were rapidly determined. Initial studies in worms and flies showed that a single miR such as let-7 or miR-14 was capable of silencing critical genes that regulate organogenesis or lipid metabolism (13, 80, 98). A major insight into miR function in mammals was made possible by deletion of the enzyme Dicer, which cleaves pre-miRs to mature and therefore bioactive miRs. Dicer mutation results in severe vascular patterning defects in early development and lethality by embryonic day 12.5 in mice and severe defects in organogenesis in fish (27, 34, 43, 101). Conditional deletion of Dicer in mice has yielded considerable additional insight into miR function in different organs and has implicated miRs as major regulators of cellular function, not only in development but also in a number of diseases (27, 40, 60). In addition to development, miRs were first shown in 2002 to be dysregulated as a result of proximate mutations in many human cancers. In particular, lymphomas were shown to have mutations close to miR-155 and miR-15 that contributed to their survival, either by deletion of the miR (miR-15, -16) or by overexpression due to proviral insertions into the genomic DNA (miR-155) (8, 12, 28, 48, 55, 68). In 2005, miR-21 was reported to be an important survival factor in glioblastomas (16, 38) and subsequently in hepatocellular carcinoma, renal cell cancers (RCC), and certain lymphomas (15, 17, 24, 35, 66, 67, 104), where hypomethylation of the miR-21 chromatin locus has been repeatedly noted to explain why it is overexpressed.

Although miRs are RNA molecules, their secondary structure renders them very stable and highly resistant to enzymatic degradation. Hence, although ssRNA is rapidly degraded extracellularly, miRs are readily detected in exosomes as well as unprotected in body fluids, where they have been shown to play active roles in cell-to-cell transmission, cell-to-cell signaling, as well as a secondary role as biomarkers (5, 16, 100, 103). miR expression is also regulated by a number of factors, including activity of the activating enzyme Dicer, epigenetic regulation of the pre-miR gene locus by factors such as histone modifications, and promoter methylation (7).

**A Role for miRs in Kidney Development and Cancer**

In the kidney, miRs have been shown to play critical roles in nephrogenesis and also shown to be dysregulated in renal cell carcinomas. Conditional mutation of the pre-miR processing enzyme Dicer1 only in the kidney epithelium or kidney stroma results in profound defects in nephrogenesis. When Dicer was mutated in the developing epithelium, premature termination of nephrogenesis due to loss of epithelial progenitors was noted, as well as disruption of branching morphogenesis, along with dysregulation of the epithelial cell cycle, associated with enhanced apoptotic cell death. Cyst formation was a frequent feature in the developing kidney, particularly when Dicer1 was mutated in the ureteric bud epithelium (69, 72). These defects were associated with loss of miR-200, -30, and let-7a from the kidney epithelium. Recent reports of Dicer1 mutation in the kidney stroma show profound patterning and differentiation defects of not only the stroma and its derivatives but also in the nephron and microvasculature (71). Such broad effects of a stromal-restricted mutation on nephrogenesis emphasize critical roles for the stroma in epithelial patterning, as well as microvascular patterning (which receives significantly less attention) in nephrogenesis. The defects in epithelial development included impaired polarized cell division and therefore nephron lengthening and impaired segmental differentiation. The defects in the endothelium included delayed vasculogen-
esis, abnormal branching, and abnormal dilatation of capillaries. The kidney defects were sufficient to bring about perinatal mortality at least in part due to failure of the kidney to function. Therefore, miRs critically coordinate many aspects of organ patterning in the kidney. Strikingly, the stromal cells that lack Dicer1 are hypovascularized rather than overactivated, and many signaling pathways are suppressed rather than derepressed (73). This may suggest Dicer1 has broader roles in cellular function than only activation of miRs, but it also points to miRs having major roles in suppressing endogenous inhibitors of cell function. Analysis of miRs enriched in stroma alongside those suppressed by Dicer1 mutation in stroma identified a limited number of miRs that appear to regulate stromal function. These include miR-199, -214, -32, -127, -136, -143, -451, -466, and -467. By contrast, other miRs active in development but enriched in the epithelium or endothelium include miR-451, -144, -192, -200, -223, and -126 (73).

Increasing recognition for important roles for miRs in RCC has been reported. These cancers are widely believed to be of epithelial origin. An important role for miR-21 in regulating glycolysis has been implicated in RCC, although not mechanistically tested (17, 66). Glycolytic tumors have a marked survival advantage, and the miR-21 gene locus was identified as a hypomethylated site (activated) in those carcinomas with poor outcome, suggesting miR-21 is functioning as an RCC oncomir. In addition to miR-21, a characteristic pattern of dysregulated oncogenic miRs has been reported in RCC, which includes miR-21, -126, -451, -146, and -200 (4, 17, 81, 82, 88).

**Chronic Kidney Diseases Characterized by Injury and Fibrosis**

Chronic kidney disease (CKD) comprises a heterogeneous group of diseases characterized by progressive loss of kidney function. Although many differing triggers initiate the disease, including diabetes, hypertension, ischemic injury, xenobiotics, immune complex deposition, infections, as well as inherited/ genetic causes, there are common histological and transcriptional features shared by these disparate diseases once they enter the chronic phase (13, 17, 74). It is increasingly believed that common processes drive progression, regardless of the initiating insults. The chronically diseased kidney is characterized by parenchymal injury, inflammation, fibrosis, and capillary loss and may be considered a chronic wound-healing response that has not resolved (17, 27). Flattening and thinning of epithelial cells, thickening of basement membranes, and associated loss of function characterize the parenchymal injury. Glomerular injury is characterized by endothelial swelling and subsequent loss of capillaries, which are replaced by a form of fibrosis known as sclerosis. Similarly, capillary injury adjacent to tubules is characterized by progressive loss of capillaries. Current thinking is that injury to capillaries or the epithelium directly drives the fibrogenic process, and recruited leukocytes may serve to perpetuate the inflammatory process and maintain fibroblasts in a pathological state (17, 27, 60). In certain diseases, such as autoimmune diseases, leukocytes may initiate injury, particularly to the glomerular and peritubular capillary endothelium when immune complexes form (8, 17, 37, 55).

**Dysregulated miRs in Human CKD**

Because pathways regulated in development and in cancer are frequently activated in disease states, we reasoned that miRs may play a role in kidney disease, where reactivation of developmental pathways is widely reported. To identify abnormally expressed miRs and study the possible role of miRs in kidney disease, we collected kidney biopsies from patients with kidney transplants who had developed a fibrotic CKD of their transplant and collected biopsies from normal healthy kidneys that were being donated for transplantation. Isolated total RNA was subjected to hybridization to Agilent miR arrays, and relative miR levels were determined. By performing a simple analysis comparing fibrotic kidney miR levels with normal kidney miR levels, we identified 21 different miRs that were upregulated with a high degree of significance and 3 miRs that were downregulated (Fig. 2A). Although no kidney-specific miRs were identified, the signature of upregulated miRs included several miRs that had been associated with survival in cancers including miR-15, -21, -200, and -451 (1, 38, 48, 77). In a comparison of results from human CKD with kidney biopsies from short-term animal models of kidney injury with fibrosis triggered by obstruction (UUO) or ischemic injury (unilateral ischemia-reperfusion injury), 24 regulated miRs were identified that were shared by both fibrosing models (Fig. 2B). These miRs were all upregulated and included the following miRs that were also upregulated in human biopsies (Fig. 2A): let-7i, miR-15b, -21, -25, -132, -199, and -214. The finding that animal models shared many of the same miR changes with human biopsies characterized by fibrosis (Fig. 2A) suggested there may be a fibrotic miR signature in the kidney, that these dysregulated miRs may be playing roles in regulating the disease process either positively or negatively, and that the animal models could be used to interrogate their function.

**miR-21—A Central Regulator of Metabolic Activity in the Kidney**

miR-21 was identified as an upregulated miR in several distinct animal models of kidney disease and in both human acute kidney injury (AKI) and CKD tissue samples (17, 35, 37, 50, 104) (Fig. 2). In addition, it was also shown to be one of the more highly expressed miRs in the healthy kidney (17, 37) (Fig. 2). In hypertrophic cardiac tissue, miR-21 was reported to contribute to disease pathogenesis by stimulating MAPK signaling, whereas in the setting of malignancy miR-21 had been reported to enhance survival and prevent apoptotic cell death when overexpressed in malignant cells, suggesting it could serve as an oncomir (16, 21, 36, 100). The miR-21 gene locus is embedded in the VMP1 gene coding for a vesicle-associated protein, which is upregulated in settings of cell stress and has recently been described to regulate the formation of autophagosomes during cell stress (Fig. 3) (22, 69). miR-21 expression is induced by factors including hypoxia and transforming growth factor (TGF)-β stimulation of cells in vitro, in keeping with its role as a stress response miR (17, 37). In addition to upregulation in epithelial cells in the kidney and liver (hepatocytes), however, it is expressed at moderately high levels in quiescent conditions or in healthy tissue but appears to be inactive (4, 21). One study separated different cytosolic compartments from hepatocytes and found that miR-21 was within a vesicular
fraction in quiescent conditions but translocated to a more soluble fraction in response to cell stress (4, 36). The implication from these observations is that miR-21 can respond very rapidly to cell stress because it is already present within epithelial cells. Although miR-21 has all the hallmarks of playing important roles in disease, global deletion of the miR-21 gene has no effect on development or health of adult mice housed in sterile facilities over the course of more than a year, suggesting miR-21 may be functionally important only in stress settings.

By contrast, in the setting of kidney injury lasting 1–2 wk, the kidneys from miR21−/− mice showed marked protection against the development of fibrosis (2, 17). This was associated with marked preservation of epithelial integrity and protection from epithelial cell death, suggesting miR-21 was contributing to both epithelial disease in response to injury, as well as promoting fibrosis (Fig. 3).

miR-21 has several hundred predicted gene targets based on the identification of complementary sequences in the UTR of miRNAs (see Fig. 1). When healthy kidneys were analyzed for the engagement and degradation of those potential target mRNAs by comparing miR21−/− kidney mRNA levels with those from miR21+/+ kidneys, investigators found very few of the potential target mRNAs were being silenced. However, when the same study was performed in kidneys that had become diseased, >100 predicted target genes were now shown to be actively silenced in the kidney (17, 92) (Table 1). The degree of silencing of a single type of mRNA by miR-21 was in the range of two- to fourfold only. Strikingly, however, the genes targeted for silencing by miR-21 were not involved in inflammation and fibrosis; rather they were predominantly involved in metabolic and mitochondrial functions of cells (Fig. 4). Many of the gene targets were functionally interrelated. One striking pathway regulated by miR-21 is oxidoreduction activity, which occurs principally in mitochondria, whereas another involved the fatty acid oxidation (FAO) pathway in peroxisomes and mitochondria (17, 110) (Fig. 4). FAO is highly regulated by the transcription factor peroxisome proliferator-activated receptor (PPAR)α and its coactivated factor PPARγ coactivator-1α (PGC1α). PPARα was one of the matched gene targets for miR-21 in kidney disease as well as multiple factors and enzymes that are also regulated by PPARα activity in the nucleus (Fig. 3). Therefore, miR-21 plays a major regulatory role in FAO by regulating multiple FAO effector genes, including, as well as in concert with, PPARα. In addition to inhibiting FAO, the target genes for miR-21 indicated that miR-21 silences a range of mitochondrial functions and favors cells toward glycolytic metabolism (17, 87). The functional consequence of miR-21 activity is to decrease the capacity of...
mitochondria to generate ATP and increase their capacity to produce high levels of mitochondrial reactive oxygen species (ROS) (Fig. 3). Although there are a number of other well-recognized potential target genes, including TIMP3, SMAD7, SPROUTY1, and PTEN, there was no convincing evidence that, in these kidney disease models, these genes were suppressed by miR-21 activity (6, 17, 37). Nevertheless, miR-21 was identified because of its ability to promote injury and fibrosis in the kidney. Therefore, the studies suggest that by suppressing FAO, suppressing mitochondrial function, and enhancing mitochondrial ROS generation (Fig. 3) miR-21 drives fibrosis indirectly, through augmenting deleterious epithelial responses to stress or injury. Although the kidney epithelium, which is rich in mitochondrial content, is the obvious cell target for miR-21 activity, studies indicate miR-21 is widely expressed in disease settings and that miR-21 controls FAO and mitochondrial function not only in epithelial cells but also in fibroblasts and podocytes (37, 64) (Fig. 3). There is evolving literature pointing to a metabolic switch to glycolysis as a critical step in activation of fibroblasts in a number of settings (65, 77). Therefore, it is likely that miR-21 regulates a metabolic switch that is also directly important in fibrogenesis.

It should be noted that although many studies show a pathological role for miR-21 in kidney disease progression, there are two examples where miR-21 has been reported to be protective (54, 99). In one of these, miR-21 was reported to play a role in ischemic preconditioning, where miR-21 expression protects against subsequent injury. It makes sense that activated miR-21 plays a physiological role in protecting cells from acute injury by shutting down metabolic functions, which could therefore contribute to ischemic preconditioning. In chronic disease settings, however, persistent activation of miR-21 is detrimental, due to chronic loss of cell function and persistent activation of fibrogenesis.

Anti-miR Oligonucleotides—A Novel Class of Therapeutics with High Penetration of the Kidney

In parallel with the discovery of gene silencing by RNA has been the development of anti-RNA oligonucleotides that are able to enter cells in whole animal and suppress translation of mRNA (Fig. 1B). Several advances been made in RNA stability in vivo by molecular modification of the RNA backbone to

<table>
<thead>
<tr>
<th>Metabolism/Mitochondrial Biogenesis</th>
<th>Acat1</th>
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<tr>
<td>Acetyl-CoA C-acetyltransferase</td>
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<tr>
<td>Mpv17 like</td>
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<tr>
<td>Pyruvate glycerol kinase 5</td>
<td></td>
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<tr>
<td>Acyl-CoA synthetase medium chain</td>
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<td>PPARα</td>
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<td>Sperm mitochondrial protein</td>
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<td>Flavin containing mono-oxygenase 2</td>
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<td>Choline phosphotransferase</td>
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<td>Coenzyme A synthase</td>
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<td>Peroxisome biogenesis gene</td>
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<tr>
<td>Alkylglycerone phosphate</td>
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<td>Aldehyde dehydrogenase</td>
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<tr>
<td>Glucosamine 6 phosphate dehydrogenase</td>
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<tr>
<td>Mitochondrial acyl transferase</td>
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<td>Fat storage-inducing membrane 2</td>
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<td>Acyl cobinding domain protein</td>
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<tr>
<td>Phosphoglucomutase 3</td>
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<td>Cytochrome B5</td>
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<td>Fatty acid desaturase</td>
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<td>Acyl-glycerol phosphate acyltransferase 3</td>
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<td>Pyruvate dehydrogenase 1</td>
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<td>Acyl-glycerol phosphate acyltransferase 5</td>
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<td>Shuttling protein</td>
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<td>Alcohol dehydrogenase</td>
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Table 1. List of 24 seed-matched target genes that are highly overrepresented in the diseased mouse kidney when miR-21 is absent

miR, microRNA; PPAR, peroxisome proliferator-activated receptor.
MicroRNAs IN KIDNEY DISEASE

Significant up-regulated GO biological processes in miR-21 KO UUO

- Log (P value)

-10 -20 -30 -40 -50

0 5 10 15 20 25

1. Cellular amino acid metabolic process
2. Cellular amine metabolic process
3. Carboxylic acid metabolic process
4. Organic acid metabolic process
5. Cofactor metabolic process
6. Acid and derivative metabolic process
7. Coenzyme metabolic process
8. Lipid metabolic process
9. Cellular lipid metabolic process
10. Sulfur metabolic process
11. Fatty acid metabolic process
12. Oxidation reduction
13. Cellular Ketone metabolic process
14. Organic acid metabolic process
15. Carboxylic acid metabolic process
16. Oxoeic acid metabolic process

Fig. 4. Graph showing the most significantly enriched gene ontology (GO) pathways in the diseased mouse kidney when miR-21 is absent. KO, knockout.

convert highly unstable RNA molecules into highly stable RNA molecules that are resistant to the activity of RNase H (Fig. 1C). These modifications include the use of 2′-O-methyl RNA molecules, the replacement of the phosphodiester backbone with a phosphorothioate backbone, and the use of locked bicyclic nucleic acids in certain places in the oligonucleotide (1, 6) (Fig. 1C). Additionally, the 2′-O-methoxyethyl (MOE) and 2-oxy-methyl (OMe) modifications to the backbone enhance affinity for complementary nucleotides, thereby enhancing drug-like properties (50). Attaching these modified RNA molecules to lipids or other moieties has helped direct them to a variety of different tissues or specific cell types (37). Moreover, modification of the native RNA backbone has markedly reduced the type I interferon response that cells have in response to extracellular RNA or DNA. The oligonucleotides are designed to bind by Watson-Crick sequence complementarity to the active site of the specific RNA that is being targeted (Fig. 1B), usually in the 3′-UTR.

In tracking studies of radiolabeled or fluorescently tagged oligonucleotides, the RNA oligonucleotides have a predilection to distribute widely throughout the body to intracellular compartments. However, the greatest concentration of anti-miR oligonucleotides is found in the kidney and liver following systemic delivery (Fig. 5A). One possible reason for this is that these organs are both critical in removal of waste products from the body. In the kidney, the proximal tubule concentrates the oligonucleotides most strongly, but endothelial cells, macrophages, podocytes, and fibroblasts all concentrate readily detectable levels of the oligonucleotide drug (Fig. 5A). One interesting aspect of the behavior of the drug is that the healthy glomerulus appears devoid of intracellular uptake, but the diseased glomerulus readily concentrates the drug in all cell types, including the podocyte, indicating that anti-oligonucleotide therapy may benefit glomerular as well as tubulointerstitial diseases of the kidney. The latest generation of anti-miR oligonucleotides can be delivered by weekly subcutaneous injection without loss of activity. Recent studies using anti-miR-21 oligonucleotides have demonstrated their utility and specificity in animal models of AKI as well as a chronic disease known as Alport syndrome (17, 37) (Fig. 5). Alport syndrome is an inherited single-gene disorder of the collagen-IV gene, affecting the kidney and ear, leading to deafness and kidney failure. The disease shows strong similarity to other chronic progressive diseases, including focal segmental glomerulosclerosis, hypertensive nephropathy, and IgA nephropathy. Although Alport nephropathy is caused by mutations in the capillary basement membrane, the abnormal basement membrane is thought to trigger cell stress in the endothelium, podocytes, and tubular cells, which secondarily triggers fibrosis and recruitment of leukocytes. Mice with an engineered mutation in one of the collagen-IV genes develop a progressive kidney disease, which is highly similar to the human disease, and succumb to kidney failure by 11 wk of age. As in other kidney diseases in mice, miR-21 was elevated in the kidneys of these mutant mice, but the elevated levels preceded any histological abnormalities in the kidneys, consistent with miR-21 being regulated by cell stress. Weekly delivery of anti-miR21 oligonucleotides to diseased mice after disease onset, profoundly retarded the progression of disease, normalized tubular functions, and led to an increase in life expectancy of nearly 50% (37) (Fig. 5, B–D). The transcriptional changes in kidneys as a result of anti-miR-21 were significant protection of mitochondrial function and marked activation of PPARα and PGC1α signaling pathways, which include FAO. In primary cell cultures from Alport kidneys, the anti-miR-21 oligos directly protected cells from mitochondrial dysfunction in the setting of induced cell stress, directly inhibited fibroblasts from activation under typical activating stimuli (Fig. 3), and showed highly similar effects on the kidney as genetic deletion of miR-21 (16, 36).

In other studies, delivery of anti-miR-21 oligonucleotides to mice with a model of diabetic kidney disease also had beneficial effects on glomerular function and retarded the progression of diabetic kidney disease (109). Moreover, these compounds have been given to small and large animals for many weeks without obvious deleterious effects. Therefore, anti-miR-21 oligonucleotides represent a novel class of therapeutics to treat fibrosing kidney diseases, now entering phase II clinical trials. Although the safety of delivering a modified oligonucleotide to the body has been raised, phase III clinical trials are underway using antisense oligonucleotides to treat a number of diseases, and have so far proven safe. These oligonucleotides have a similar backbone structure to anti-miR oligonucleotides. In addition, anti-miR-21 oligonucleotides have proven to be safe in long-term studies in primates and more recently in humans.

Other Dysregulated miRs That Play Pathological Roles in the Kidney

An increasing number of publications have identified pathological or beneficial roles for miRs in regulating kidney diseases (Table 2), and many of these overlap with the miRs identified in fibrogenesis. In the following, we will highlight those miRs that have been shown to play additional roles in fibrogenic disease.

miR-214 has also been shown to be expressed at high levels in human kidney disease and animal models of kidney disease (21, 36) (Fig. 2). miR-214 is cotranscribed with the miR-199a family on a single IncRNA strand which is on the complementary strand of an intron of the dynamin-3 gene. Dynamin-3 plays roles in cell trafficking of vesicles along microtubules
and in cell motility. Genetic mutation of the coregulated 199a/214 pre-miR strand (known as Dnm3os) indicated that the RNA is expressed predominantly in mesenchymal, skeletal, and vascular smooth muscle cells, and osteoblasts during embryogenesis rather than in the epithelium. Although Dnm3os mutant mice were born in Mendelian ratios, they were small, gained weight poorly, and had a median survival of 2–3 wk with substantial musculoskeletal defects, including impaired ossification, muscle weakness, and reduced adipogenesis. Defects in other organs were not reported. On the other hand, selective global deletion of the miR-214 gene only, or miR-199a only in mice, leads to a Mendelian ratio at birth and normal fertility without overt organ disease or musculoskeletal disease (22, 37). Nevertheless, when kidney disease was induced in the form of obstruction of the ureter, miR-214-deficient mice were highly protected from the development of fibrosis (21). miR-214 and miR-199a genes appear to be regulated by activation of the mesenchymal transcription factor TWIST and also hypoxia through the actions of hypoxia-inducible factor-1α (36). The effect of miR-214 on cell function in the kidney has been less well studied, but in other tissues has been reported to be expressed widely in disease settings including in injured or activated epithelial cells, stromal cells, and the endothelium (2, 87). Inhibition of miR-214 function in primary human kidney stromal cell cultures resulted in cells that were hypoactivated, hypomigratory, and hypoproliferative in response to activating stimuli (73). Similarly, blockade of miR-214 in cancer cells has rendered them hypomigratory and more resistant to cytotoxic stress (92), potentially by enhancing BCL family proteins or the AP2 family of transcription factors (110), and inhibition of miR-214 stimulates beneficial angiogenesis (87) potential by enhancing expression of Quaking (QKI), an RNA binding protein that enhances RNA translation. Recent studies in biomechanical stress-induced cardiac fibrosis showed that miR-214 along with miR-199a potently regulated pathological responses to disease and that antagonomiRs against miR-214, and to a lesser extent miR-199a3p, blocked cardiac fibrosis (6, 64). Studies of human hepatic stellate cells indicate that miR-214 directly stimulates TGF-β-mediated pathological responses and inhibits matrix gene expression (65). Although the mechanism of action of miR-214 in fibrosis is less well elucidated than that for miR-21, several recent studies have identified critical target genes in injury and fibrosis. In cardiac disease, PPARβ has been recognized as an important cellular target (6). This transcriptional regulator, like PPARα, plays important roles in regulating FAO and mitochondrial function, and, like PPARα, has been reported to play a protective role in the

Fig. 5. Anti-miRNA modified oligonucleotides block specific miRNA function and have excellent drug like properties with high penetrance to kidney cells in vivo. (A) Fluorescence images (X400) of the kidney showing the distribution of a single injection of anti-miR21-Cy3 (red) subcutaneously (25 mg/kg in 50 μl, 48 h previously). Note that the compound is concentrated in tubule epithelium but is also detected in glomerular cells, macrophages (F4/80), endothelium (CD31) and fibroblasts (PDGFRβ). Arrowheads show areas of co-localization. Effect of Anti-miR21 on (B) Fibrosis, (C) Blood urea nitrogen concentration (D) and survival (I) in Col4a3−/− mice with Alport nephropathy. (Adapted from Ref. 37). ****P < 0.0001.
kidney following acute injury (37). Moreover, polymorphisms at the PPARD locus have been associated with CKD in several independent studies (42). Thus one of the ways in which miR-214 may act in kidney fibrosis is by regulating metabolic responses, similarly to miR-21. The phosphatase and AKT signaling pathway inhibitor, known as PTEN, is also a recognized target for miR-214 in the setting of cancer cell function. The AKT signaling pathway has been shown to be an important mechanism in chronic disease of the kidney. Further studies are required to understand the mechanism of action of miR-214.

An important but unresolved question is the pathological importance of miR-199a. This gene forms a pre-miR, which after RNA processing gives rise to two distinct miRs, miR-199a5p and miR-199a3p. miR-199b5p and miR-199b3p are identical but transcribed from a distinct chromosome. Several studies in non-renal tissue have pointed to discrete roles for these mature miRs in migration and matrix deposition, but they share significant overlapping gene targets with miR-214, and their importance has not been validated well in kidney. One study on human kidney fibroblast cells indicated that both miR-199a3p and miR-199a5p enhanced proliferation and cell activation but that, miR-199a-3p was more potent than miR-199a-5p in overall activity. (73). Other studies have indicated miR-199a5p plays a role in fibrogenesis in the lung, particularly by inhibiting expression of caveolin-1 (61). The significance of the miR-199a genes in kidney disease merits further investigation.

### Table 2. Summary of publications highlighting miRs that play a role in kidney disease

<table>
<thead>
<tr>
<th>miRs</th>
<th>Level During Disease</th>
<th>Target</th>
<th>Outcome/Function</th>
<th>Models</th>
<th>Reference(s)</th>
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</thead>
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<tr>
<td>Let-7b</td>
<td>Down</td>
<td>TGFBR1</td>
<td>SMAD3, ECM decrease</td>
<td>Diabetic mice (STZ), NRK52E cells</td>
<td>89</td>
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<tr>
<td>miR-21</td>
<td>Up</td>
<td>PPARGa, MPV17L, and FAO</td>
<td>Increased fibrosis, p-Akt, mTORC1, hypertrophy, COL1α2, FN increase</td>
<td>Alport mouse, human biopsy, HMCs</td>
<td>37, 25</td>
</tr>
<tr>
<td>miR-25</td>
<td>Down/Up</td>
<td>TIMP1, MMP9</td>
<td>Microalumunuria, TGF-β, NF-κB increase</td>
<td>db/db mice</td>
<td>111</td>
</tr>
<tr>
<td>miR-29a/b/c</td>
<td>Down</td>
<td>COL1 and COL4</td>
<td>COL1, COL4, decrease</td>
<td>HK-2 cells, Podocytes</td>
<td>26, 59</td>
</tr>
<tr>
<td>miR-29a</td>
<td>Down</td>
<td>COL1a1/2</td>
<td>COL1, COL4, decrease</td>
<td>HK-2 cells</td>
<td>26</td>
</tr>
<tr>
<td>miR-29b</td>
<td>Down</td>
<td>TGFB</td>
<td>Podocyte dysfunction</td>
<td>db/db mice</td>
<td>19</td>
</tr>
<tr>
<td>miR-29c</td>
<td>Up</td>
<td>SFRI1/IFH1a</td>
<td>Albuminuria, ECM increase</td>
<td>db/db mice, db/db mice</td>
<td>63</td>
</tr>
<tr>
<td>miR-93</td>
<td>Down</td>
<td>VEGFA</td>
<td>COL4α3, VEGF, and FN decrease</td>
<td>db/db mice, podocytes, and endothelial cells</td>
<td>62</td>
</tr>
<tr>
<td>miR-124</td>
<td>Up</td>
<td>Integrin-α3β1</td>
<td>Urinary podocyte nephrin, podocin, albumin increase</td>
<td>Diabetic rats (STZ)</td>
<td>58, 87</td>
</tr>
<tr>
<td>miR-126</td>
<td>Up</td>
<td>Endothelium</td>
<td>Vascular integrity, Microalumunuria and renal fibrosis increase</td>
<td>Renal cell carcinoma, db/db mouse</td>
<td>79, 88, 41</td>
</tr>
<tr>
<td>miR-135a</td>
<td>Up</td>
<td>TRPC1</td>
<td>Microalumunuria and renal fibrosis increase</td>
<td>Dicer KO</td>
<td>73</td>
</tr>
<tr>
<td>miR-136</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Stromal function</td>
<td>Dicer KO</td>
<td>73</td>
</tr>
<tr>
<td>miR-144</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Stromal function</td>
<td>Dicer KO</td>
<td>73</td>
</tr>
<tr>
<td>miR-146</td>
<td>Up</td>
<td>AD-2</td>
<td>Inflammation</td>
<td>Renal cell carcinoma</td>
<td>83, 33</td>
</tr>
<tr>
<td>miR-155</td>
<td>Up</td>
<td>B cells</td>
<td>Increased inflammation</td>
<td>Lupus</td>
<td>85, 96</td>
</tr>
<tr>
<td>miR-192</td>
<td>Up</td>
<td>SIP1</td>
<td>COL1α1 and COL1α2 increase</td>
<td>STZ mice, db/db mice</td>
<td>47, 78</td>
</tr>
<tr>
<td>miR-195</td>
<td>Up</td>
<td>BCL2</td>
<td>Caspase-3, caspase-8 increase</td>
<td>Diabetic mice (STZ), podocytes, MMCs</td>
<td>20, 31</td>
</tr>
<tr>
<td>miR-199</td>
<td>Up</td>
<td>TGFB</td>
<td>Increased fibrosis</td>
<td>Fibroblast activation and human biopsy</td>
<td>18, 61</td>
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<tr>
<td>miR-200a</td>
<td>Down</td>
<td>TGF-β2</td>
<td>COL1, COL4, FN decrease</td>
<td>NRK52E cells, STZ mice, db/db mice</td>
<td>38, 90, 44</td>
</tr>
<tr>
<td>miR-200b/c</td>
<td>Up</td>
<td>ZEB1</td>
<td>TGFβ, COL1a2, and COL4α1 increase</td>
<td>MMC</td>
<td>73, 75</td>
</tr>
<tr>
<td>miR-214</td>
<td>Up</td>
<td>FOG</td>
<td>Increased fibrosis</td>
<td>Human biopsy</td>
<td>21, 110</td>
</tr>
<tr>
<td>miR-215</td>
<td>Up</td>
<td>CTNNB1P</td>
<td>β-Catenin, FN, α-SMA increase</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>miR-216a</td>
<td>Up</td>
<td>PTEN, YB1</td>
<td>COL1α2 increase, MMC survival and hypertrophy</td>
<td>MMC</td>
<td>46</td>
</tr>
<tr>
<td>miR-217</td>
<td>Up</td>
<td>PTEN</td>
<td>MMC survival and hypertrophy</td>
<td>MCC</td>
<td>45</td>
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<tr>
<td>miR-221</td>
<td>Up</td>
<td>Unknown</td>
<td>Inflammomas</td>
<td>RCC</td>
<td>88</td>
</tr>
<tr>
<td>miR-223</td>
<td>Up</td>
<td>NLRP3/IL1b</td>
<td>Inflammomas</td>
<td>Diabetic mice (STZ), MMCs, HMCs</td>
<td>94</td>
</tr>
<tr>
<td>miR-377</td>
<td>Up</td>
<td>PAK1, SOD</td>
<td>FN increase</td>
<td>MMCs and RCC</td>
<td>82, 108</td>
</tr>
<tr>
<td>miR-451</td>
<td>Down</td>
<td>YWHAZ</td>
<td>p38MAPK, ECM decrease</td>
<td>Dicer KO</td>
<td>73</td>
</tr>
<tr>
<td>miR-466</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Stromal function</td>
<td>Dicer KO</td>
<td>73</td>
</tr>
<tr>
<td>miR-467</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Stromal function</td>
<td>Dicer KO</td>
<td>73</td>
</tr>
<tr>
<td>miR-541</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Stromal function</td>
<td>Dicer KO</td>
<td>73</td>
</tr>
<tr>
<td>miR-1207-5p</td>
<td>Up</td>
<td>TGFB</td>
<td>TGF-β1, PAI-1, FN increase</td>
<td>HK-2 cells, podocytes, and mesangial cells</td>
<td>3</td>
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</table>

See the text for additional abbreviations.
miR-155 has been reported to be elevated in a number of human chronic kidney diseases, including diabetic nephropathy, lupus nephritis, anti-neutrophil cytoplasmic antibody-associated vasculitis, and hypertensive kidney disease (85). It is expressed in the endothelium, stromal cells, as well as tubular epithelium. Although its role in kidney disease remains to be fully elucidated, several studies have shown miR-155 contributes to B cell activation and antibody production, in part by inhibiting the phosphatase SHIP. SHIP plays important roles in myeloid cell activation in addition to B cells, and therefore miR-155 is likely to play a role in monocyte and neutrophil activation in the kidney. In fact, miR-155 amplifies LPS responses in macrophages as well as activation by oxidized lipids, suggesting broader roles in regulating inflammatory signaling. Given the broad activating effects of miR-155 in models of lupus nephritis and the efficacy of anti-miRs in these models, miR-155 may evolve as a potential target for autointerimmunity or immune complex-activated kidney diseases.

miR-223 is upregulated in a range of human kidney diseases, as well as animal models (see above and Fig. 2). The function of miR-223 is less well understood than other miRNAs. It has been shown to directly target the inflammasome effector molecule NLRP3 and suppress expression of NLRP3 in myeloid cells (9, 39) in settings of infection or pathogen pattern recognition receptor activation. In preliminary studies from our laboratory, anti-miRs against miR-223 had no overt effect on the fibrogenic process during the ureteral obstruction model of kidney disease. However, this particular model of fibrogenic disease progresses without a substantial role in innate immune signaling whereas the role of innate immunity in kidney diseases, particularly ischemic kidney disease, is well accepted (14). Therefore miR-223 may serve in beneficial ways in kidney disease to limit activation of the inflammasome.

The miR-200 family includes miR-200a, -200b, -200c, -141 and -429, all of which have the same seed sequence; that is, the critical sequences that recognizes the 3'-UTR sequence is identical. In short-term models of kidney injury and fibrosis, the family is broadly downregulated (97). In kidney cancers, miR-200 family members are also downregulated, but our studies of CKD biopsies indicate miR-200c is downregulated whereas miR-200a is upregulated. Therefore, the significance of this target in chronic fibrogressive human disease requires further studies. The miR-200 family has been implicated in maintaining epithelial health by inhibiting the process of the epithelial-to-mesenchymal transition (EMT) and may play inhibitory roles in metastasis. They have been shown to silence the ZEB family of transcription factors that regulate EMT and epithelial migration. Furthermore, in vitro studies using kidney epithelial cell lines indicate RNA mimics of these miRs delivered intracellularly inhibit both the EMT process and ZEB1/2 transcription factor activity (97). The absolute importance of the EMT process in human epithelial disease has recently been questioned, and therefore further studies into the importance of the miR-200 family in human injury repair remain to be determined.

The miR-29 genes include miR-29a, -29b, and -29c, which are coded by distinct genomic loci but have identical seed sequences. They have been reported to be downregulated or abnormally regulated in many diseases of the kidney (29). In fibrotic kidney transplant biopsies, miR-29a was modestly upregulated, however (Fig. 2), and in other series miR-29 was not reported to be downregulated. miR-29 is one of the signature miRs of fibrotic diseases. In the setting of skin fibrosis, myocardial disease, and lung disease, miR-29 has been shown to be downregulated. Downregulation of the miR-29 loci is under the regulation of c-MYC, GLI, NF-kB, TCF/LEF, and SMAD3 (70). Analyses of target genes for miR-29 reveal that many target genes are matrix genes, including collagens, laminins, fibrillin, elastin, and integrin-β1 (51). In fact, miR-29 has the potential to reduce expression of as many as 20 collagen genes. Therefore, a miR-29 function in healthy tissues is to provide tonic suppression of production of matrix and matrix turnover. However, miR-29 also slows cell growth and may trigger apoptosis in certain cancer cells, such as acute myeloid leukemia, potentially by suppressing genes that normally silence the transcription factor P53 (76, 86). This proapoptotic effect of miR-29 may be triggered by DNA damage. Therefore, miR-29 has the potential to both suppress matrix deposition and drive cell death. Potentially, this could be beneficial if the cells that undergo apoptosis are fibrogenic cells, but a potential downside is deletion of cells that are critical to organ function. Although miR-29 has not been studied in great detail in the kidney, it is likely that miR-29 will be an important target to tackle fibrogenesis. Studies that separate antifibrotic effects from proapoptotic effects in will help elucidate whether miR-29 is a useful target in kidney diseases.

miR-126 is believed to be restricted in expression to endothelial cells and found to be highly expressed in capillaries and large blood vessels. miR-126 functions by regulating factors that control angiogenesis (95). It promotes endothelial proliferation and endothelialization of large vessels. The miR-126 gene is regulated by and encoded by an intronic region of the epidermal growth factor like-7 (EGFL7) gene. EGFL7 is restricted to endothelial cells. One of the main targets of miR-126 is EGFL7 itself. EGFL7 is known to be involved in cell migration and blood vessel formation and believed to function by interacting with Notch receptors and the Notch receptor ligand Delta-like 4 (84). miR-126 is also reported to silence VEGFA, DLK1, transcription factors including IRS1, HOXA11, as well as the chemokine CXCL12. During tissue repair, endothelial cells release miR-126 within apoptotic bodies, inducing CXCL12-dependent vascular protection (106) by recruiting bone marrow progenitor cells to the site of tissue injury, where they promote vascular repair. Furthermore, miR-126 overexpression in bone marrow cells has recently been shown to promote vascular integrity following kidney injury by promoting bone marrow progenitor cell trafficking to the injured kidney, allowing for vascular mobility and supporting recovery of the kidney microvasculature (11).

miR Mimetics: Future Therapeutics with Far-Reaching Possibilities

The miR-29 and miR-200 families are broadly downregulated in human fibrotic disease of the kidney and other epithelial organs including the skin, lung, and liver. In addition, although miR-126 and miR-223 are upregulated in disease, their roles are mainly beneficial to the injured tissue. By contrast, miR-21, -214, -199, and -155 have all been demonstrated to drive pathological states, and organs have shown benefit from inhibiting their action by use of anti-miRs. The
technological advances in anti-miR technology have resulted in anti-miRs that can function as candidate therapeutics. Similar efforts have been made to generate miR mimics. These are dsRNA molecules which separate intracellularly to ssRNA, and one strand loads into the RISC and functions as a miR. Several modifications can be made to the RNA to favor the loading of the mature miR strand over the other, with the RISC. Several innovative methods have been used to improve delivery and stability, including the use of three strands of RNA in which two have the LNA technology to enhance stability. Although such mimics have been widely used in cultured cells, their development as drug candidates has been hampered by delivery, triggering of TLR responses in vivo, and efficacy at the target organ. Although mimic technology lags behind anti-miR approaches, there is an increasing desire to target certain dysregulated miRs by augmenting their endogenous functions.

Discussion

A growing body of evidence indicates that miRs play critical roles in the function of epithelial, stromal, endothelial cells, and leukocytes in diseases of the kidney. Many of the dysfunctional miRs identified in fibrosing diseases of the kidney have also been shown to play roles in nephrogenesis and in kidney cancers. In addition, several miRs appear to play similar roles in fibrosing diseases of the skin, liver, kidney, and lung. miR-21 has probably been the most studied of the pathogenic miRs in kidney disease and detrimentally impacts the kidney by disrupting mitochondrial functions. Because miRs regulate multiple functionally related gene targets simultaneously, modifying a single miR with a drug has the possibility of affecting numerous cellular processes rather than simply regulating a single signaling pathway. In addition, many miRs are stress activated and appear to be inactive in situations of cell health. The genes coding such miRs are frequently embedded in, or are on the opposite strand of, important regulatory or stress-induced protein-coding genes such as those involved in migration or trafficking. This lack of activity in healthy tissues provides an important advantage in the development of safe therapies which target miRs. Since many of the candidate therapeutic targets identified to date in kidney disease also play important roles in regeneration or homeostasis, design of successful therapies for CKD has been held back by safety problems that have been encountered. Anti-miRs therefore offer the promise of enhanced safety as well as efficacy.

miRs are central coordinators of gene expression and are readily targetable, particularly to the kidney and liver; therefore, there is great potential for rapid transition from identification of pathological miR targets to drugs in clinical trials. There is currently a large unmet need for AKI and CKD. The latter affects as many as 10% of the adult population, leads to kidney failure or death, and currently there are very few therapies to halt the progression of disease. Safe new treatments that target the mechanisms of disease progression are urgently required. AKI leading to rapid organ failure is one of the most rapidly growing diseases in the United States in the hospital setting. There is currently no approved treatment for AKI other than supportive care, and it frequently is a cause of substantial morbidity, and even mortality, or the development of CKD. Anti-miR delivery to the kidney in the acute setting is also a promising new therapeutic strategy.

Over the next few years it is likely that we will see evidence in humans that anti-miR oligonucleotides slow progression of kidney disease since anti-miR-21 therapy is entering clinical trials for the treatment of Alport syndrome. Additional miR targets will become established as drug targets for kidney disease, and miRs in the urine will likely prove to be robust biomarkers of kidney disease severity, progression, and potentially disease stratification.

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MicroRNAs IN KIDNEY DISEASE


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