The TGF-β Route to Renal Fibrosis Is Not Linear: The miR-21 Viaduct

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The chronic kidney disease (CKD) riddle appeared solved 20 years ago when TGF beta (TGF-β) was identified as a potent inducer of renal fibrosis.[1] It not only stimulated extracellular matrix production but also efficiently transformed the phenotype of critical cellular mediators into profibrotic activists and induced the expression of additional fibrogenic molecules (plasminogen activator inhibitor-1, connective tissue growth factor, for example) to amplify the response. A multitude of studies on fibrogenic mechanisms all appeared to converge on TGF-β, its receptors, and its dose—both upstream and downstream.[2]

The impetus to translate these landmark scientific discoveries into badly needed human therapeutics encountered a significant obstacle when it became evident that TGF-β is a multifunctional growth factor that includes a critical role in immune surveillance. Indeed, TGF-β null mice die in infancy due to an aggressive multisystem autoimmune inflammatory disorder.[3] Hope for translational application resumed when a multitude of studies determined that several intracellular signaling pathways are potentially activated when TGF-β engages the type I plus type II receptor complex,[4] and that one pathway in particular was a major effector of its fibrogenic actions—phosphorylation and nuclear import of the transcription factors mothers against decapentaplegic homologues (Smad 2/3). Smad7, another member of this family, attenuates fibrotic responses. These discoveries raised the possibility that cell-membrane-permeable therapeutic agents might selectively block the profibrotic effects of TGF-β. As a proof of the concept of this study, renal fibrosis is less severe in Smad3 null mice.[5]

In this issue of *JASN*, Zhong and colleagues[6] identify microRNA-21 (miR-21) as another downstream target of Smad3 that executes TGF-β-induced renal fibrosis. It was identified as a potential candidate on the basis of upregulated expression in tubular epithelia exposed to TGF-β and its fibrosis-promoting effects in other solid organs. Using specific knockout mice, they show that Smad3, but not Smad 2, is necessary for miR-21 expression. They provide evidence that Smad3 functions as a miR-21 transcription factor by interacting with Smad binding sites 1 and 2 within the promoter region of the miR-21 gene in the presence of TGF-β.

Although miRNAs were first discovered in *Caenorhabditis elegans* in 1993, their role in fibrotic events has only recently become clear.[7,8] Now approximately 1000 miRNAs have been identified in humans, yet, together, they may influence the post-transcriptional control of the majority of the protein-encoding genes. miRNAs—small, genetically encoded but protein noncoding RNAs (18 to 24 nucleotides)—act in the cytoplasm to disrupt the translation of their target RNAs. They do so within the RNA-induced silencing complex by binding to a short region (7 nucleotides) in the 3′ untranslated region (UTR), which blocks translation or destabilizes the RNA, leading to its degradation. It is thought that each miRNA has as many as 10 to 100 target genes. Enhanced miR-21 expression was first reported in 2005 in human glioblastomas.[9] Antagomir-based inhibition studies now establish its role in cardiac and pulmonary fibrosis. In the Zhong study, ultrasound-microbubble-mediated gene transfer of a miR-21 knockdown plasmid attenuates fibrosis in mice with chronic kidney disease (CKD) induced by unilateral ureteral obstruction (UUO). Increased miR-21 is also reported in hypertensive nephropathy, anti-Thy1.1 glomerulonephritis, and diabetic nephropathy.[10,11]

What is remarkable is the fact that TGF-β regulates the expression of numerous miRNAs—inducing some and suppressing others—such that TGF-β is one of the most extensively investigated pathways in miRNA biology. TGF-β signaling may initiate the transcription of genes that encode the precursor (primary) nuclear miRNA (pri-miRNA). In the study by Zhong *et al.*, the finding of increased levels of kidney tissue pri-miR-21 is consistent with its transcriptional upregulation. However, TGF-β may also enhance the rate of processing of certain premature miRNAs, including miR-21, through a unique mechanism that employs Smad proteins. Two essential pri-miRNA processing steps involve cleavage by RNAase III enzymes. The enzyme, Drosha, mediates the initial nuclear step, and the cytoplasmic enzyme, Dicer, performs the second. Acting on the first step, TGF-β promotes the formation of an active microprocessor complex containing SMAD proteins, pri-miRNAs, and Drosha to generate premiRNA, the product that is exported into the cytoplasm by exportin-5 nuclear channels.[12]

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Initially selected on the basis of differential expression, miR-21 is now one of the most extensively investigated miRNAs thus far. It appears to be a ubiquitous oncogene, given its upregulated expression in the majority of cancers profiled, to date, and its biologic actions of enhanced proliferation and reduced apoptosis.[13] An emerging theme is that miR-21 has cell type-specific and unique disease-state effects. These variable responses may explain why the outcome of miRNA inhibition may not be the reverse of the effects of overexpression, and why differences in interrogation strategies (varied chemical methods to engineer miRNA inhibitory oligonucleotide antagomirs or use of genetically engineered mice) may yield conflicting results. For example, in a model of cardiac-stress-induced fibrosis, miR-21 is upregulated in fibroblasts, where it enhances fibrosis by stimulating MAP kinase signaling due to *Sprouty1* inhibition.[14] miR-21 inhibition significantly reduces cardiac fibrosis, yet when the same model is induced in miR-21 null mice, no differences are observed.[15] In a kidney model of ischemia-reperfusion injury, miR-21 was identified as one of nine differentially expressed miRNAs, upregulated primarily within proliferating tubular cells.[16] Although miR-21 improves cell survival *in vitro*, overexpression *in vivo* fails to prevent tubular cell death.

In the study by Zhong *et al.*, miR-21 knockdown significantly reduces fibrosis in association with fewer αSMA^+ interstitial myofibroblasts, interstitial collagen I, and fibronectin, and lowers mRNA levels encoding TGF-β1. A key question that deserves further consideration is the nature of the cell-specific effects of miR-21 during renal fibrosis. While the TGF-β/Smad/miR-21 axis is clearly associated with enhanced matrix production by tubular cells, it also served a beneficial function—enhancing tubular cell survival. This is important because tubular cell death, leading to nephron atrophy, is the root cause of declining renal function in fibrosis-mediated CKD.[1] Although not specific to miR-21, mice with a renal proximal tubular cell-specific genetic deficiency of the pri-miRNA processing enzyme, Dicer, develop less tubular damage after ischemia-reperfusion, suggesting the global effect of miRNA expression in this model is detrimental.[17] Future studies will need to differentiate tubular cell-specific effects of miR-21 from effects on interstitial fibroblasts, macrophages (miR-21 is increased during monocyte differentiation),[18] and capillaries (mir-21 induces endothelial cell inflammatory adhesion molecules and inhibits angiogenesis)[19,20], and include experiments in animal model systems that make it possible to measure effects of miR-21 inhibition on tubular cell density and renal function. Since miRNAs do not encode proteins, their localization in tissue requires *in situ* hybridization techniques. Studies in the UUO model performed by Zhong *et al.* indicate both tubular and interstitial cells express miR-21. Although their work emphasizes effects on the former, modulating interstitial myofibroblast activities would be consistent with the important role shown for fibroblasts in models of miR-21-induced cardiac[14] and pulmonary fibrosis,[21] and the fact that interstitial myofibroblasts may be a primary source of interstitial scar-forming extracellular matrix proteins in CKD.

A key step to elucidating the molecular basis of cell-specific and context-dependent effects of miR-21 and other miRNAs will be the identification of their target genes. This is not a trivial undertaking, as their bp binding only requires partial complementarity—between its *seed nucleotides* (2–8) and the binding site within the 3′UTR of the target gene. This minimal requirement likely explains why a single miRNA can interfere with the translation of a multitude of genes. Many of their potential targets are hypothetical candidates, selected by computational analysis of the 3′UTR region of potential target genes. Relatively few studies provide evidence of direct interactions. Presently, more targets are known for miR-21 than most miRNAs; many involve the TGF-β family, extracellular matrix-receptor interactions, mitochondrial apoptosis, and the p53 tumor suppressor protein.

While a growing body of evidence suggests that miRNAs participate in fibrotic processes, the current miR-21 story is almost certainly the first chapter of a large novel that has yet to be written. Although the highlighted paper focuses on Smad3, other TGF-β signaling pathways are likely involved, and miR-21 may be induced by TGF-β-independent mechanisms (for example, a STAT3 mechanism has been reported).[22] A more global version of the miRNA CKD molecular signature will eventually emerge. This will be a complicated story; miR-192, for example, is both downregulated[23] or upregulated[24,25] by TGF-β, and, in both directions, has been implicated in the pathogenesis of CKD. Still other miRNAs, such as miR-29a and miR-200a, appear to be constitutively expressed in the kidney and downregulation by TGF-β, with detrimental effects, presumably due to the derepression of harmful genes.[26,27] To complicate matters further, the repressive effects of miRNAs are generally modest ($< 50\%$ reduction in protein expression), which can make it challenging to detect clear biologic effects in complex *in vivo* model systems. Furthermore, it has been suggested that some miRNAs may even enhance the post-transcription expression of target genes. Much of the effect of these changes may depend on context and timing.

Will miR-21 emerge as a serum or urine biomarker for chronic kidney disease? miRNAs are known to circulate in a stable soluble form in serum, and profiling studies have recently been reported for patients with acute kidney injury.[28] Genetically determined polymorphisms in the 3′ UTR of genes will become of greater interest if they modify miRNA binding, as recently reported for the +1166A/C variant in the human angiotensin II type 1 receptor gene.[29] For now, miR-21 is high on the list of noncoding, small, regulatory RNAs that promote kidney fibrosis, but many questions await answers.

DISCLOSURES

None.

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See related article, "Smad3-Mediated Upregulation of miR-21 Promotes Renal Fibrosis," on pages 1668–1681.

Making a Tubule the Noncanonical Way

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Tubulogenesis is an essential feature during formation of multiple organ systems. The genitourinary, cardiovascular,

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