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MicroRNA Circuits in Transforming Growth Factor- β actions and Diabetic Nephropathy

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Abstract

Diabetes is associated with significantly increased rates of kidney disease or diabetic nephropathy (DN), a severe microvascular complication that can lead to End-stage renal disease (ESRD). ESRD needs to be treated by dialysis or kidney transplantation and is also associated with cardiovascular disease and macrovascular complications. Therefore, effective renal protection is critical to reduce the rates of mortality associated with diabetes. Although key signal transduction and gene regulation mechanisms have been identified and several drugs are currently in clinical use, the rates of DN are still escalating, suggesting the imperative need to identify new biomarkers and drug targets. The recent discovery of microRNAs (miRNAs) and their cellular functions provide an opportunity to fill these critical gaps. Since miRNAs can modulate the actions of key factors involved in DN such as transforming growth factor-beta (TGF- β), they could be novel targets for the treatment of DN. This review covers the recent studies on the roles of miRNAs and miRNA circuits in TGF- β actions and in DN.

Keywords

microRNAs; TGF- β ; Diabetic Nephropathy; Fibrosis; Hypertrophy

Introduction

Diabetes is a major health care problem that is associated with several debilitating microvascular complications such as diabetic nephropathy (DN). Moreover, patients with DN are also highly susceptible to macrovascular diseases such as atherosclerosis, hypertension and stroke and there is a close correlation between chronic kidney disease (CKD) and cardiovascular disease (CVD) ^{1–2}. Epidemiologic studies show that 40~50% of patients who have end stage renal disease and need dialysis are diabetic. However, the underlying molecular mechanisms remain unclear. Therefore, in this review, we have focused on the emerging role of new mechanistic players in DN called microRNAs.

Accumulation of extracellular matrix (ECM) proteins such as collagen (fibrosis) and mesangial expansion (hypertrophy) in the kidney mesangium and tubular compartments,

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along with podocyte dysfunction, are major hallmarks of DN, and contributes to renal failure in diabetes mellitus^{3–8}. Transforming growth factor-beta1 (TGF- β) levels and signaling are enhanced in renal cells during the progression of DN. TGF- β plays a key role in mesangial fibrosis and hypertrophy under diabetic conditions by inducing the expression of ECM proteins such as collagen and fibronectin^{3, 5, 7, 9–11}. An antibody against TGF- β was effective to some extent in animal models of DN¹² and some trials targeting TGF- β itself and with antifibrotic drugs are ongoing¹³. However, due to the multifunctional nature of TGF- β , it is worthwhile to further investigate key molecular events downstream of TGF- β signaling in renal cells to uncover new targets for more effective prevention or treatment of DN.

microRNAs (miRNAs) are short (~21 nucleotides) non-coding RNAs that can regulate gene expression at the post-transcriptional level by blocking translation or promoting cleavage of their target mRNAs (Figure 1). As several reviews are available on miRNAs^{8, 14–16}, here we focus more on their role in DN. miRNAs play important roles in transcription, signal transduction and pathogenesis of human diseases, including cancer, diabetes and diabetic complications and interestingly, more than 60% of protein-coding genes are affected by miRNAs. Of particular interest in the renal field are reports demonstrating that a cluster of miRNAs are highly expressed in the kidney, and that there are differences in the miRNA expression profile in renal cortex versus medulla^{17–18}. In past few years, investigations of the specific roles of key miRNAs in the kidney have revealed their functional roles in TGF- β actions and diabetic kidney disease^{7, 19}. Notably, reports showed that podocyte-specific deletion of Dicer, a key enzyme involved in miRNA biogenesis, led to progressive glomerular and tubular damage along with proteinuria and other podocyte defects in mice^{20–22}. Moreover, these small RNAs have now been identified in plasma and urine of humans and animal models^{23–27}. There is thus increasing interest in evaluating their molecular mechanisms of actions and biological roles in the diabetic kidney in order to determine their value as therapeutic targets for DN.

In this review, we will mainly discuss recent advances in the roles of miRNAs in TGF- β actions and DN and the potential application of miRNAs as biomarkers and drug targets for DN.

miRNAs circuits in diabetic renal fibrosis

It was recently observed that TGF- β can induce the expression of key miRNAs in mouse renal mesangial cells (MMC) and in glomeruli of diabetic mice. Furthermore, these miRNAs could mediate the expression of fibrotic genes related to the pathogenesis of DN. In an initial screening, using mouse kidney glomerular mesangial cells (MMC), TGF- β could induce the expression of the Collagen type I alpha2 (*Col1a2*) gene by inhibiting the expression of the E-box repressors, Zeb1 and Zeb2⁷. Under basal conditions, these repressors negatively regulate *Col1a2* expression by binding to E-box elements in the far upstream region of the *Col1a2* promoter⁷. Zeb1 and Zeb2 are now widely recognized as general E-box repressors that bind to promoter E-box elements and repress genes such as E-Cadherin and collagens^{28–32}. In the same study, it was noted that miR-192, which is highly expressed in kidney, is upregulated in MMC treated with TGF- β and renal glomeruli from mouse models of diabetes [type 1 (streptozotocin (STZ)-induced) and type 2 (db/db)] relative to the corresponding controls⁷. Interestingly, miR-192 targets Zeb1 and Zeb2 and increases *Col1a2* gene expression in MMC, demonstrating that increased miR-192 in diabetic conditions induces fibrosis by inhibiting E-box repressors (Figure 1).

In addition, the expression of other miRNAs, miR-216a, miR-217 and key miR-200 family members were also increased in MMC treated with TGF- β and in kidney glomeruli from

mouse models of diabetes^{6, 33–34}. miR-216a and miR-217 are located in the second intron of the non-coding RNA RP23-298H6.1-001(RP23) and their expression depends on the host non-coding RNA⁶. The expression of RP23 is regulated by the E-box cluster in its promoter region and by miR-192 through Zeb1/2, namely mechanisms similar to that involved in Col1a2 regulation, suggesting signal amplification via miRNAs (Figure 1,2)⁶. Genomic structures of miR-216a, miR-217 and host non-coding RNAs are conserved from human to mouse. Furthermore, miR-216a can also increase collagen expression by another parallel mechanism involving the inhibition of Ybx1, an RNA binding protein and a component of Processing bodies³⁴. This led to subsequent enhancement of Tsc-22 translation and collagen transcription via interaction with Tfe3, an E-box activator³⁴.

The miR-200 family members are separated into two clusters (cluster1 and cluster 2, Figure 2) based on genomic structures. They are located in the introns of their host RNAs and also regulated by Zeb1/2, targets of miR-192, through E-boxes in the promoter of the host gene^{33, 35–36}. The genome structures of these miRNAs and host genes are conserved from human to mouse. miR-200 family also targets Zeb1/2 at their upstream E-boxes, and this process can lead to their auto-upregulation and further acceleration of the signaling pathways leading to collagen expression and renal fibrosis (Figure 2, 3).

Other miRNAs have also been implicated in renal fibrosis associated with DN. In one study, miR-377 was found to induce fibronectin (ECM protein) expression in MCs *via* downregulation of manganese superoxide dismutase and p21-activated kinase and furthermore, high glucose treatment of the MCs increased the expression of miR-192 (Figure 2)¹⁹. It was reported that miR-93 levels were lower in glomeruli from diabetic db/db mice compared to control mice, and also in high glucose treated podocytes and kidney microvascular endothelial cells. There was a parallel increase in the expression of Vascular Endothelial Growth Factor-A, a target of miR-93³⁷. Another report showed that, along with miR-192 and miR-200b, miR-29c is also upregulated in glomeruli from db/db mice, as well as in endothelial cells and podocytes treated with high glucose and activates Rho kinase by targeting Spry1 and leads to DN (enhanced ECM accumulation and podocyte apoptosis)³⁸. miR-192 also regulates E-cadherin gene expression in tubular epithelial cells through Zeb1/2³⁹. In addition, it was reported that miR-200a targets TGF- β 2 in cultured proximal-tubular epithelial cells, creating another circuit in the TGF- β response since TGF- β increased TGF- β 2 expression via decreases in miR-200a in these cells⁴⁰. It is possible that this decrease in miR-200a might also be due to miRNA decay as recently suggested⁴¹.

miRNA circuits in diabetic kidney glomerular hypertrophy

Akt kinase is activated by TGF- β in diabetic kidneys and plays important roles in fibrosis and hypertrophy in glomerular MC^{7, 9–10, 42–49}. Akt activation is related to cancer development and diabetes by regulating many downstream cellular targets such as mTOR, GSK β and FoxO proteins⁵⁰. Since these downstream targets are major regulators of protein synthesis, apoptosis and cell survival, Akt activation is a major mediator of cellular growth and hypertrophy under disease conditions. One report suggested that TGF- β activates phosphatidylinositol-3-kinase (PI3K)/Akt pathway through direct interaction of TGF- β receptor and PI3K. However, the mechanisms (especially long-term chronic activation) of Akt activation by TGF- β were not fully understood. As described above, two miRNAs (miR-216a and miR-217) were increased in TGF- β -treated MMC and in kidney glomeruli from diabetic mice. Notably, it was observed that both these miRNAs target phosphatase and tensin homolog (PTEN), a known upstream inhibitor of the PI3K/Akt pathway, demonstrating a mechanism by which these two miRNAs upregulated by TGF- β can activate Akt and downstream signaling through inhibition of PTEN (Figure 2)⁶. Thus, Akt activation by the miRNA circuit induced by TGF- β could play a significant role in

mesangial hypertrophy in diabetes. The miR-200 family has also been reported to control PI3K signaling by targeting *Fog2*, an inhibitor of PI3K⁵¹. Since PI3K/Akt activation in diabetic kidney cells induces hypertrophy and fibrosis^{6, 34, 42–43}, upregulation of miR-200 family members may also activate Akt through inhibition of *Fog2* to promote mesangial hypertrophy. Together, this cascade of miRNAs initiated by miR-192 may therefore further augment Akt activation, hypertrophy and fibrosis in MC (Figure 2). In addition, a recent report showed that miR-21 can also target PTEN in MC to activate Akt kinase and contribute to MC hypertrophy and renal pathology⁵² (Figure 2).

Interestingly, evidence shows that p53 and miR-192 can regulate each other to form another signal loop (Figure 2,3)^{53–54}. Since p53 regulates several genes related to cell cycle regulation, such as p21 (cyclin-dependent kinase inhibitor)⁵⁵, it is possible that the inhibition of cell cycle progression by miRNA actions may promote renal glomerular hypertrophy. Furthermore, as p53 also upregulates Eukaryotic translation Elongation factor 1-alpha 1 (EF1α)⁵⁶, increased levels of EF1α might enhance protein synthesis and induce hypertrophy in the diabetic glomeruli. Recent reports showed that miR-200 family members are also regulated by p53^{57–58}.

In MMC, diabetic conditions (high glucose) induce *TGF-β1* through binding of E-box activators such as Upstream Stimulatory Factors (USFs) to an E-box element in the *TGF-β1* promoter^{59–61}. Interestingly, *TGF-β1* gene itself is upregulated by TGF-β1 in MMC through the same E-box element in the promoter³³. Recent data demonstrate that this autoregulation is also mediated by the E-box repressors Zeb1/2 targeted by the miRNA circuit (miR-192 to miR-200 family) (Figure 2, 3).

Therefore, these miRNA-mediated amplifying circuits encompassing miR-192, miR-200 family, miR-216a, miR-217 as well as other miRNAs can serve as signal modulators to enhance signaling to accelerate fibrosis and hypertrophy in TGF-β-mediated chronic diseases like DN (Figure 2, 3).

Regulation of miRNAs by TGF-β

There is much interest in examining the molecular mechanisms of miRNA expression under disease states. Two major mechanisms may be involved in TGF-β-induced miR-192. One is the classical regulation by Smad3^{62–63} and another by p53^{53–54, 64}, since the miR-192 promoter has Smad and p53 binding sequences and is upregulated by Smad3 or p53 (Figure 1). Enhancement of miR-192 maturation (Drosha processing) by p53 and Smad has also been suggested (Figure 1)^{65–66}. Subsequently, miR-200b/c can be induced by miR-192 through E-boxes in their promoters^{35–36}.

However, some reports have also shown that the expression of miR-192 and miR-200 family members are decreased in cancer or renal epithelial cell lines treated with TGF-β1 for long time periods along with decreased expression of the epithelial marker E-cadherin, in the process of epithelial-to-mesenchymal transition (EMT)^{36, 67–71}. In the NRK52E epithelial cell line, one report showed that miR-192 levels were increased by 2ng/ml TGF-β1 at 1–24 hr⁶³, while another showed that miR-192 levels were decreased by 10ng/ml TGF-β1 at 3 days³⁹. Therefore, cell specific as well as temporal regulation mechanisms for miR-192 are likely. Because type I collagen induces EMT^{72–73}, the initial induction of collagen may subsequently induce EMT at later time points (possibly by downregulation of these miRNAs). A recent report showed that TGF-β increases miR-451–5p targeting Par-3 (cell adhesion molecule not related to E-Cadherin) and results in tubular epithelial cell dysfunction, suggesting other non-E-Cadherin- related mechanisms in tubular injury⁷⁴. As EMT is not relevant in mesangial cells, it is also likely that the response of miRNAs to TGF-β may be cell-type specific and context dependent.

Mutations in *p53* and *Smad* genes have been noted in cancer cell lines and immortalized cell lines^{75–77}. Evidence shows that mutant p53 can alter TGF- β 1 responses⁷⁸. Moreover, renal injury and fibrosis were attenuated in *p53* or *Smad3* knockout mice^{63, 79}. Since miR-192 is regulated by Smad3 and p53^{54, 63}, these data suggest that TGF- β 1 effects on key miRNAs such as miR-192 and miR-200 family may also depend on the status of the *p53* and *Smad* genes. Thus the effects in primary MMC may be different from those in cancer or immortalized kidney cell lines, and might account for some of the cell-type discrepancies.

Other kidney disease models

As mentioned above, increased levels of miR-192, miR-200 family members and other miRNAs have been observed in glomeruli from mouse models of type 1 (STZ-induced) and type 2 (db/db) diabetes. Targeted deletion of Dicer from proximal tubules could protect against renal ischemia-reperfusion injury⁸⁰ and miR-192 levels were decreased in these KO mice. Another report showed lower levels of miR-192 in mice that survived after ischemia-reperfusion, also suggesting that lowering miR-192 might be protective against renal ischemia-reperfusion injury⁸¹. Interestingly, increased levels of these miRNAs have also been reported in human kidneys from patients with hypertensive nephrosclerosis, IgA nephropathy and lupus nephritis^{82–84} and some animal models of diabetic or non-diabetic kidney injury^{38, 63, 80, 85–86}, further supporting the pro-fibrotic role of miRNA circuits initiated by miR-192. On the other hand, one report showed decreased miR-192 levels associated with severity of DN and fibrosis in diabetic patients, although normal levels of miR-192 in healthy kidneys were not provided⁸⁷. Further studies with in vivo models will help determine the specific roles of miR-192 and other renal miRNAs in various renal cells and in the pathology of DN and other kidney diseases. Recently, TGF- β 1/Smad3-mediated increase of miR-21 and decrease of miR-29 were reported in the unilateral ureteral obstruction (UUO) model of fibrosis^{88–89}.

Amplification and acceleration of signal transduction by miRNA circuits in the diabetic kidney

As discussed above, amplification and acceleration of signals by miRNA circuits can operate in diabetic mouse glomeruli and MMC treated with TGF- β . For example, cascades from miR-192 to miR-216a and miR-217, and from miR-192 to miR-200 family can amplify signals from a single miRNA to several downstream miRNAs (Figure 2). Moreover, miR-200 family members can further augment accelerate their own expression through inhibition of Zeb1/2 (Figure 2,3). Therefore, miR-192 can be an initiator and miR-200 can be a propagator of the signaling to accelerate downstream effects. Due to the downstream cumulative effects of these miRNA circuits and cascade loops, small fluctuations in the expression of a single miRNA may make a major impact to worsen or accelerate chronic kidney diseases like DN. Thus it is important to curb the actions of these “fine tuners” of disease progression.

miRNAs as biomarkers for disease diagnosis

Due to the rapid recent technological advances in miRNA detection and quantification, including microarrays, quantitative PCRs and next generation sequencing, there is tremendous interest in developing miRNAs as potential sensitive biomarkers for human diseases and tissue injury^{23–26, 90}. One of the key reasons is that miRNAs are stable and easily quantified in a non-invasive manner in plasma and urine. Thus, they could serve as important biomarkers for the early detection of diabetic renal injury which would be extremely helpful for the clinical nephrologist. Recent reports showed that circulating miRNAs in blood are sensitive biomarkers for cancer, tissue injury and heart

failure^{23–26,90}. Levels of microRNAs in the urinary sediment of patients with IgA nephropathy have also been studied²⁷. Circulating miRNA levels were recently evaluated in chronic kidney diseases^{91–92}. Thus there appears to be great promise in evaluating the profiles and levels of circulating miRNAs in biofluids as diagnostic biomarkers of various human diseases in general and renal disorders in particular (Figure 4).

Therapeutic Applications with miRNA inhibitors

Since increasing evidence shows that miRNAs may play major modulatory roles in TGF- β 1 actions and DN, the next step is to evaluate potential clinical applications and therapeutic strategies based on miRNA inhibition. Because miR-192 appears to be a master miRNA which controls other renal miRNAs, this miRNA could be a valuable target for prevention or treatment of DN. Reports showed specific and efficient reduction of miR-192 in vivo in normal mice injected with locked nucleic acid (LNA)^{6,93–94}-modified antimiR-192 (LNA-antimiR-192). This miR-192 inhibitor also reduced downstream miRNAs (miR-216a, miR-217 and miR-200 family) and functional indices of renal fibrosis and hypertrophy, namely collagens and TGF- β 1 and Akt activation in these mice, similar to the effects in cultured MMC^{6,33}. Furthermore, injection of LNA-antimiR-192 into STZ-injected type 1 diabetic mice ameliorated renal fibrosis⁹⁵. Interestingly, low doses of the cancer drug, paclitaxel, ameliorated fibrosis in the remnant kidney model by down-regulating miR-192⁹⁶. Another report showed reduced rates of DN progression in db/db mice injected with 2'-O-methyl antisense oligonucleotides targeting miR-29c³⁸. Together, these observations suggest that synthetic inhibitors of specific miRNAs can be developed in the future as novel much needed therapies of DN. Since clinical trials with anti-miRNAs for diseases such as hepatitis C are already ongoing⁹⁷, it is possible that targeted anti-miRNA therapies could also be developed for the treatment of acute and chronic human renal disorders, and that specific circulating miRNAs can serve as biomarkers for the early detection of renal diseases (Figure 4).

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Figure 1. Biogenesis and Mechanism of Action of microRNAs and miR-192

MicroRNA transcripts initially originate as primary miRNAs (Pri-miRNAs) that are then processed into Pre-miRNAs by the Drosha enzyme, which are further cleaved to result in double-strand RNA duplexes. The miRNA duplexes are then unwound by the action of a second enzyme, Dicer, and the mature miR guide strand is loaded into the RNA-induced silencing complex (RISC). miRNAs in the RISC complex then guide the recognition of target RNAs to induce their downregulation depending on the type of complementarity. In the case of miR-192, transcription of pri-miRNAs is enhanced by Smad3 or p53 and processing into pre-miRNA (by Drosha) may also be enhanced by p53 and Smad3. Mature miR-192 interacts with 3'UTR of targets Zeb1/2 and leads to inhibition of translation or induces degradation of the target mRNAs in Processing bodies (P-body).

Figure 2. Mesangial fibrosis and hypertrophy caused by amplification of signal cascades triggered by key miRNAs and downstream effector miRNAs

Scheme shows amplification of signals by miRNA circuits can operate in diabetic mouse glomeruli and MMC treated with TGF- β . Cascades originating from miR-192 to miR-216a and miR-217, and from miR-192 to miR-200 family can amplify signals from a single miRNA to several downstream miRNAs. Amplified signals through miR-192 and miR-200 family increase the expression of ECM (collagens) genes via Zeb1/2 and activate Akt via Fog2/PI3K pathway. Another amplified signal through miR-192 and miR-216a/217 activates Akt via Pten and Activated Akt also enhances ECM gene expression. In addition, other key miRNAs such as miR-21 and miR-377 can also contribute to these events. Therefore, these miRNA-mediated amplified signals increase fibrosis and hypertrophy related to DN.

Figure 3. Acceleration of renal gene expression by signaling loops involving TGF- β and miRNAs Signaling loops initiated by TGF- β accelerate downstream action and effects via miRNAs.

A) TGF- β increases miR-192 expression via Smad3 or p53. TGF- β 1 gene expression is itself auto-upregulated by miR-192 through Zeb1/2. B) miR-192 enhances p53 levels via inhibition of Mdm2 (inhibitor of p53). p53 increases transcription of miR-192 through p53 responsive elements on the promoter. C) miR-200 family members can further augment their own expression through inhibition of Zeb1/2. Initial expression of miR-200 family can also be induced by miR-192 through Zeb1/2. D) The promoter of the non-coding RNA RP23 (the host gene of miR-216a and miR-217) also contains E-boxes⁶ and increased miR-216a also activates its own promoter through Tsc-22 and Tfe3. miR-216a/Ybx1 pathway can amplify the signaling initiated by TGF- β and miR-192.

Figure 4. Schematic model for the use of miRNAs biomarkers for human renal disease diagnosis or early detection through miRNA profiling in biofluids, and for therapeutic purposes with chemically modified oligonucleotides targeting specific miRNAs

Circulating miRNAs in biofluids are potential diagnostic biomarkers of various human diseases in general and renal disorders in particular. Moreover, synthetic chemical inhibitors of specific miRNAs are very effective to reduce the target miRNAs and downstream signaling in animal models of human diseases^{6, 33, 38, 93–95}. Therefore, key circulating miRNAs will be useful for early detection of renal diseases and furthermore, anti-miRNA therapies could also be developed for the treatment of acute and chronic renal disorders.