miR-21 promotes renal fibrosis in diabetic nephropathy by targeting PTEN and SMAD7

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Abstract
The cytokine transforming growth factor (TGF)-β1 plays a central role in diabetic nephropathy (DN) with data implicating the miRNA (miR) miR-21 as a key modulator of its prosclerotic actions. In the present study, we demonstrate data indicating that miR-21 up-regulation positively correlates with the severity of fibrosis and rate of decline in renal function in human DN. Furthermore, concomitant analyses of various models of fibrotic renal disease and experimental DN, confirm tubular miR-21 up-regulation. The fibrotic changes associated with increased miR-21 levels are proposed to include the regulation of TGF-β1-mediated mothers against decapentaplegic homolog 3 (SMAD3) and phosphoinositide 3-kinase (PI3K)-dependent signalling pathways via co-ordinated repression of mothers against decapentaplegic homolog 7 (SMAD7) and phosphatase and tensin homolog (PTEN) respectively. This represents a previously uncharacterized interaction axis between miR-21 and PTEN–SMAD7. Targeting of these proteins by miR-21 resulted in de-repression of the respective pathways as reflected by increases in SMAD3 and V-Akt murine thymoma viral oncogene homolog 1 (AKT) phosphorylation. Many of the changes typically induced by TGF-β1, including phosphorylation of signalling mediators, were further enhanced by miR-21. Collectively, these data present a unified model for a key role for miR-21 in the regulation of renal tubular extracellular matrix (ECM) synthesis and accumulation and provide important insights into the molecular pathways implicated in the progression of DN.

Key words: biopsy, diabetic nephropathy, fibrosis, micro ribonucleic acid (RNA), signalling, transforming growth factor (TGF)-β1.

INTRODUCTION
Progression of diabetic nephropathy (DN) is characterized by the gradual scarring of the renal glomerulus and fibrosis of the tubulointerstitial region. The degree of fibrosis of the diabetic kidney strongly associates with progression to end-stage renal disease (ESRD). Although mesangial expansion and podocyte effacement and loss are the primary drivers of glomerulosclerosis, tubulointerstitial fibrosis (TIF) is primarily mediated by renal fibroblasts and tubular epithelial cells [1,2]. Transforming growth factor (TGF)-β1 plays a central role in the onset and progression of the structural changes seen in the fibrotic diabetic kidney [3]. The fibrotic effects of TGF-β1 are primarily mediated by the well characterized mothers against decapentaplegic homolog 3 (SMAD3)-dependent pathway with other pathways such as those mediated by V-Akt murine thymoma viral oncogene homolog 1 (AKT) also participating [4]. Intracellularly, these pathways are negatively regulated by mothers against decapentaplegic homolog 7 (SMAD7) and phosphatase and tensin homologue (PTEN) respectively [5,6]. TGF-β1 also mediates its effects via small non-coding RNAs, aptly named miRNA (miR), which have rapidly emerged as important regulatory molecules [7–10]. The role of a number of miRs is well established in DN, including the miR-29 family,
the miR-200 family and miR-192 [8]. These miRs are known to interact with and modulate various components of the TGF-β1-induced fibrotic programme.

Among these, miR-21 has been extensively studied because many of its targets are relevant to DN and especially related to the modulation of TGF-β1 and AKT activation [11,12]. miR-21 has been associated with fibrosis in experimental models of lung, heart and kidney sclerosis [9–14]. Expression of miR-21 was recently studied in kidney biopsies of American–Indian patients with early stage of DN [glomerular filtration rate (GFR) above 90 cc/min] where it was up-regulated only in the glomerular fraction and correlated with the level of proteinuria [15].

In the present study, we aimed to study miR-21 expression in kidney biopsies from diabetic patients with advanced fibrotic stages of DN and to explore the potential pathogenic role of differentially expressed miR-21 in human kidney. We hypothesized that miR-21 is associated with the development of DN and that co-ordinated targeting of SMAD7–PTEN is essential to the role of miR-21 in TGFβ-mediated fibrotic signalling. The interactions of these particular signalling cascades with miR-21 or the extent to which these interactions individually contribute to the fibrotic milieu have not been previously delineated [13,14,16,17].

RESEARCH DESIGN AND METHODS

Kidney biopsy and histological assessment

Formalin-fixed paraffin-embedded (FFPE) kidney specimens (n=43) including diagnostic kidney biopsies of patients with DN (n=35) and normal living kidney donors (n=8) were obtained from the pathological archives of the Department of Pathology at Rabin Medical Centre. The study was approved by the hospital Institutional Ethics Committee [18].

Renal biopsy was performed according to clinical indications and in order to exclude the co-existence of other types of kidney disease, the presence of atypical features including short duration between the diagnosis of diabetes and the onset of nephropathy or the absence of concomitant diabetic retinopathy. Cases were defined by the presence of histological changes consistent with DN and the absence of other potential causes of renal disease [18,19].

In situ hybridization

In situ hybridization (ISH) of miR-21 was performed on eight kidney biopsies with mild fibrosis, eight with severe fibrosis and eight normal kidneys. A miR-21 probe was used for ISH as per the manufacturer’s recommendations (Exiqon). A scrambled probe was used for negative control and U6 was used as a positive control probe (Exiqon) [20].

Laser capture microdissection

Laser capture microdissection (LCM) was performed using the PALM MicroBeam instrument (PALM). FFPE blocks used in immunohistochemistry (IHC)/ISH and for miR analysis were cut into sections (5-μm thick) on to PALM membrane slides (PALM), baked and deparaffinized with xylene, lightly stained with haematoxylin and eosin and air-dried. All the glomeruli or the tissue surrounding the captured glomeruli were microdissected and captured on PALM adhesive caps tubes followed by total RNA extraction using the RNeasy FFPE Kit (Qiagen).

Experimental kidney disease models

miR-21 expression was assessed in renal cortices from three rodent models of renal fibrosis as previously described [20] (Supplementary Methods). These comprised two models of DN, the streptozotocin (STZ)-induced diabetic ApoE−/− mouse and the STZ-induced diabetic uni-nephrectomized (UNx) Sprague–Dawley rat. A third model of non-diabetic renal disease was utilized, specifically adenine-induced renal fibrosis.

Detection of tubulointerstitial fibrosis and immunohistochemistry

Immunohistochemistry was performed on serial, 4-μm sections of FFPE kidneys or renal biopsies, as previously described [21]. Sections were processed in parallel with the appropriate control tissues. IHC staining intensity was ascertained via standard procedures [22]. TIF was detected by Masson’s Trichrome staining [23].

Cell culture, treatments and transfections

Rat proximal tubule epithelial cells (PTC; A.T.C.C.) were maintained and passaged in DMEM (Dulbecco’s modified Eagle’s medium; Life Technologies) supplemented to 25 mM glucose with 10% FBS. For treatments, media contained only 2% FBS and treatments were for 72 h. TGF-β1 (R&D systems) was used at 10 ng/ml. The phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (LY, Cell Signalling Technology) was used at 50 nM. TGF-β1 and LY were added simultaneously where indicated. For transfection, PTCs were seeded in 12-well plates and were transfected with 100 nM of precursor-miR (pre-miR; Life Technologies) or 50 nM locked nucleic acids (LNA, Exiqon) in Opti-MEM (Life Technologies) using Oligofectamine (Life Technologies). Negative control pre-miR and LNA were used at 50 nM. Cells were transfected with 25 nM anti-SMAD3 siRNA (si-SMAD3, Sigma–Aldrich) or scrambled control siRNA (si-Ctrl, Sigma–Aldrich) for 48 h. Following transfection, media were changed to 2% FBS DMEM without antibiotics after 5 h at which time appropriate treatments were added. Cells were harvested for RNA or protein 3 days later.

RNA extraction and qRT-PCR

RNA was extracted from PTCs and renal cortical tissue samples using TRIzol (Ambion). RNA from human renal biopsies was extracted using RNeasy mini columns (Qiagen). DNase treatment and cDNA synthesis were performed as previously described [24]. Gene expression was determined utilizing TaqMan reagents (Life Technologies) with fluorescence signals being normalized to 18s rRNA utilizing the ddCT method [25].

miR assay

miR expression was measured using TaqMan miRNA assays (Life Technologies). Fluorescence was normalized to U6 small-RNA for PTC. Pre-amplification was performed for human tissue samples using TaqMan PreAmp Master Mix with Megaplex PreAmp Primers, Human Pool A and B (Life Technologies).
Western blotting and densitometry
Denatured cell lysates (40 μg) were run on 4%–12% pre-cast gels and transferred to PVDF membranes. Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.05% Tween (BTBS-t) and primary antibodies applied overnight with 0.5% BTBS-t. After washing, horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated with membranes for 1 h with BTBS-t. Secondary antibodies were detected with chemiluminescent peroxidase substrate and imaged with the GelDoc XRS (BioRad).

Statistical analysis
All statistical analyses were performed utilizing GraphPad Prism software. Correlative analyses were evaluated using Pearson’s correlation test. Comparisons of both mRNA and protein expression datasets were evaluated using Tukey one-way ANOVA. Experiments with only one treatment were assessed by Student’s t-test. A P-value < 0.05 was considered statistically significant. Significance between groups is indicated for each figure. Data are presented as mean ± S.E.M.

RESULTS
miR-21 levels correlate with tubulointerstitial fibrosis and estimated glomerular filtration rate (eGFR) in human DN biopsies
Renal biopsies were collected from a total of 35 patients with varying degrees of type 2 diabetes mellitus (T2DM) associated renal damage and eight control subjects (Supplementary Table S1; Supplementary Figure S1a). Renal biopsies revealed significantly greater miR-21 expression in patients displaying fast progression (median time to dialysis 18 months) in the rate of renal function decline compared with control or with those diabetic subjects with slow progression (time to dialysis 60 months) of declining renal function (Figure 1A). Patients were further divided into normal, mildly fibrotic and moderate to severely fibrotic groups based on histological analysis of the renal architecture and pathology assessment of the degree of tubulointerstitial sclerosis (Figures 1B, 2A and 2B). Again, miR-21 levels were increased with increasing renal fibrosis. Patients with moderate to severe fibrosis displayed the highest levels of miR-21 (Figure 1B). Approximately 80% of T2DM subjects in each group were being administered angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB) at the time of study. Although miR-21 levels were reduced in these patients, miR-21 expression was still significantly higher than that seen in control subjects (Figure 1C).

eGFR was found to be negatively correlated with increasing miR-21 levels (Figure 1D). miR-21 levels were also positively correlated with proteinuria in DN subjects only, whereas glomerulosclerotic injury was positively correlated with miR-21 expression in all subjects (Figures 1E and 1F).

Cellular localization of miR-21 in human DN biopsies
Localization of miR-21 expression in biopsy sections was determined via ISH and compared with the degree of fibrosis as determined by Masson’s Trichrome staining, α-smooth muscle actin (αSMA) and SMAD7 mRNA (Figures 2A–2C). Increased miR-21 probe binding (Figure 2A) in biopsies from both mild and moderate to severe fibrosis was observed predominantly in tubulointerstitial regions of the biopsies and correlated with degree of TIF and glomerular sclerosis index (GSI; Supplementary Table S1). Providing further support for this observation, miR-21 levels were studied in 12 selected biopsies with a high degree of fibrosis by LCM. miR-21 was significantly up-regulated in the tubulointerstitial fraction in DN patients compared with controls (Figure 2D). miR-21 levels were significantly lower in the glomerular fraction and did not change between biopsies from control and DN groups.

miR-21 is consistently up-regulated in experimental models of renal fibrosis
Renal cortical tissue from a number of experimental models of kidney fibrosis revealed elevated miR-21 expression compared with control. In the STZ-induced diabetic ApoE−/− mice, a model representing early DN, miR-21 was increased ∼2-fold in the renal cortex when compared with control mice (Figure 3A). In a model of more advanced DN, UNx STZ-induced diabetic Sprague–Dawley rats, a ∼3.5-fold increase in renal miR-21 levels was observed compared with non-diabetic UNx controls (Figure 3B). In both models, increased miR-21 was associated with increased expression of collagen and FN1 (Fibronectin 1) (Figures 3C and 3D).

GSI and TIF were also increased in diabetic UNx rats when compared with control UNx rats (Figure 3E). Finally, in a model representing severe, non-diabetic fibrotic renal disease, C57Bl/6 mice administered adenine by gavage (20,26), miR-21 expression was increased ∼3-fold in whole cortex from these animals when compared with controls and was associated with increased collagen deposition as indicated by Masson’s Trichrome staining (Figures 3F and 3G). These data, in addition to those from human renal biopsies, support a role for miR-21 in fibrotic renal disease of both a diabetic and a non-diabetic nature, thus highlighting a potential role for miR-21 in the pathogenesis of fibrosis in DN.

miR-21 is induced by TGF-β1 and alters TGF-β1 induced Col1 (Collagen 1), CollIV and FN1 expression in PTCs
PTCs maintained in high glucose (25 mM) were subjected to 72 h treatment with TGF-β1, resulting in up-regulation of ColI, CollIV, FN1 and miR-21 (Figures 4A and 4B). These data, together with the human renal biopsy data, confirm that miR-21 is up-regulated in tubular epithelial cells (Figure 2C). To delineate the role of miR-21 in PTCs, cells were transfected with miR-21 precursor mimics (pre-miR) or miR LNA inhibitors under high glucose conditions. Ectopic expression of miR-21 increased expression of CollIV and TGF-β1 (Figures 4C and 4D). Consistent with a role in TGF-β1 signalling, miR-21 enhanced the effect of TGF-β1 on a number of extracellular matrix (ECM) genes and pro-fibrotic growth factors (Figures 4C and 4D).

As observed with miR-21 up-regulation, LNA repression of miR-21 levels did not affect basal gene expression (Figures 4E and 4F).
miR-21 is positively associated with the rate of progression of DN and degree of tubulointerstitial pathology

(A) miR-21 expression in normal and in T2DM subjects with slow or fast decline in eGFR and those with late presenting CKD (chronic kidney disease). **P < 0.01, slow compared with fast; ††P < 0.01, normal compared with late; ‡‡P < 0.005, normal compared with fast.

(B) miR-21 expression in normal and DN with mild and moderate to severe TIF. *P < 0.05, moderate to severe compared with mild; †P < 0.05, normal compared with mild; **P < 0.005, normal compared with moderate to severe.

(C) miR-21 expression in control and DN subjects receiving and not receiving ACE/ARB treatment. ***P < 0.001, normal compared with ACE/ARB, normal compared with no ACE/ARB; †††P < 0.005, ACE/ARB compared with no ACE/ARB.

(D) Pearson correlation ($R^2 = 0.29, P = 0.0008$) between miR-21 expression and eGFR.

(E) Pearson's correlation ($R^2 = 0.18, P = 0.02$) between miR-21 and proteinuria for subjects with DN (mg/day).

(F) Pearson correlation ($R^2 = 0.32, P = 0.001$) between miR-21 and percentage global glomerulosclerosis.
miR-21 is associated with increased renal pathology and up-regulated in microdissected tubules

**Figure 2**  
**A** HC, IHC and in situ analysis of human renal biopsies from control and DN subjects with mild or moderate to severe TIF. Scramble is a non-specific RNA probe and U6 is a positive control in situ probe.  
**B** SMAD7 mRNA expression in normal and in DN subjects.  
**C** Quantification of α-SMA IHC in control and DN subjects with mild or moderate to severe fibrosis. *P < 0.05, normal compared with mild; **P < 0.005, normal compared with moderate to severe; †P < 0.05, mild compared with moderate to severe.  
**D** miR-21 expression in laser-capture micro-dissected tubulointerstitial fraction and glomeruli from control and DN subjects. ***P < 0.001, control tubuli compared with DN tubuli.

and 4F). However, the TGF-β1 mediated up-regulation of the genes was attenuated with miR-21 inhibition (Figures 4E and 4F). These data suggest that miR-21 mediates the effects of TGF-β1 in PTCs in hyperglycaemic conditions in a manner that could be conducive to the development and progression of renal fibrosis.

**miR-21 targets known fibrotic signalling proteins**

SMAD7 and PTEN, the repressors of SMAD3-dependent and PI3K-dependent TGF-β1-signalling respectively, are known targets of miR-21 [12,14,27–29]. Ectopic expression of miR-21 in PTCs reduced SMAD7 protein under basal conditions but also prevented the TGF-β1-induced increase in SMAD7 protein (Figure 5A). Consistent with these observations, SMAD3 phosphorylation was increased by miR-21 and TGF-β1-induced SMAD3 phosphorylation was further enhanced (Figure 5B).

PTEN protein was decreased by miR-21 (Figure 5C). Additionally, this miR further enhanced the TGF-β1-induced down-
Figure 3  miR-21 expression is positively associated with tissue pathology in experimental renal disease
regulation of PTEN (Figure 5C). AKT phosphorylation was increased by exogenous miR-21 (Figure 5D) and elicited greater TGF-β1-induced phospho-AKT levels than TGF-β1 alone. These data, combined with those assessing SMAD7 regulation (Figure 5A), demonstrate that miR-21 plays an important role in the regulation of these distinct regulatory proteins and therefore TGF-β1-mediated fibrotic signalling pathways in PTCs [2].

**Fibrotic consequences of the SMAD7–miR-21 and PTEN–miR-21 axes**

In order to delineate the role of miR-21-mediated repression of SMAD7 and PTEN in TGF-β1-mediated signalling, the effect of miR-21 on SMAD7 and PTEN was abrogated by either inhibition of PI3K with LY294002 (LY) [5] or siRNA for SMAD3. The rationale for this approach is depicted in Figure 7(A).

LY treatment reduced Coll expression even in the presence of TGF-β1, a known inducer of Coll expression, indicating that the expression of Coll is mainly dependent on PI3K signalling (Figure 6A) rather than SMAD3 and therefore dependent on the effect of miR-21 on PTEN rather than SMAD7. In contrast, TGF-β1-induced PAI1 (plasminogen activator inhibitor 1) expression was further up-regulated by LY treatment (Figure 6B) and even further enhanced by miR-21 overexpression, indicating the importance of both SMAD7 and PTEN in the regulation of PAI1 expression and therefore ECM accumulation. Finally, PI3K inhibition did not attenuate FN1 induction by TGF-β1 or the combination treatment of TGF-b1 and miR-21 (Figures 6C and 6H). Interrogation and specific targeting of the PTEN–miR-21 axis reveals this interaction is required for miR-21 augmentation of TGF-β1-induced Coll expression and is also an important negative regulator of TGF-β1-induced PAI1 expression.

SMAD3 siRNA reduced Coll expression in control and TGF-β treated cells (Figure 6D). miR-21 significantly enhanced Coll expression in both the presence and the absence of TGF-β, even in the presence of SMAD3 siRNA. Taken together with the findings above, these data provide evidence that the PTEN–miR-21 axis is a significant contributor to TGF-β1-mediated Coll up-regulation.

SMAD3 siRNA also reduced the expression of PAI1 under control conditions and significantly attenuated TGF-β1-mediated induction of PAI1 (Figure 6E). This is in contrast with PI3K inhibition which greatly enhanced PAI1 expression (Figure 6B). These data indicate that despite the negative effect of PI3K on PAI1 expression, SMAD3 is still required for the expression of PAI1. Finally, SMAD3 inhibition had minimal impact on FN1 expression and only partially attenuated the effect of miR-21 on TGF-β1-mediated induction of FN1 (Figure 6F). Examination of the SMAD7–miR-21 axis demonstrates this interaction is required for both baseline expression and miR-21–TGF-β1-induced expression of Coll and PAI1.

**The PTEN–SMAD7–miR-21 axis is required for TGF-β1-induced fibrotic response**

To determine if the ability of miR-21 to enhance TGF-β1-mediated fibrotic response in PTCs is indeed mediated by repression of PTEN and SMAD7, both pathways were simultaneously inhibited (Figure 7A). Co-repression of these signalling intermediates resulted in reduced baseline expression of Coll indicating a dependence on the miR-21–TGFβ axis for increased Coll expression (Figure 6G).

Conversely, PAI1 expression was increased 3.5-fold above baseline when both pathways were inhibited in the presence of the combination of miR-21 and TGF-β1 (Figure 6H). This is significantly greater than the expression induced by miR-21 alone confirming the importance of the inhibitory role of PI3K signalling on PAI1 and thus PAI1-mediated ECM accumulation [30]. Additionally, simultaneous repression of both of these pathways resulted in restoration of FN1 expression to baseline levels (Figure 6I). Simultaneous repression of both miR-21–PTEN and miR-21–SMAD7 interactions reveals that these pathways are essential to miR-21-augmentation of TGF-β1-induced Coll and FN1 expression.

**DISCUSSION**

There is increasing interest in the role of miRs in various chronic diseases including diabetic complications. miRs mediate post transcriptional regulation of genes via repressing translation of target mRNA [31,32]. In the present study, we studied the expression of miR-21 in human DN kidney biopsies with advanced renal fibrosis and also the role of miR-21 in TGF-β1-mediated expression of fibrotic genes induced under hyperglycaemic conditions [33,34].

Increased miR-21 was detected in the tubular regions of human kidney biopsies. Furthermore, in laser-capture microdissected tubuli, increased miR-21 expression correlated with TIF, glomerulosclerosis and declining renal function. Consistent with the findings in human DN, increased miR-21 was also consistently observed in experimental models of diabetic and non-diabetic fibrotic renal disease. The data presented in the present study not only confirm a pro-fibrotic role in the kidney for miR-21, but importantly defined the mechanism of action of this miR which appears to involve targeting the negative regulators of TGF-β1-induced prosclerotic SMAD3- and AKT-mediated signalling pathways in tubular epithelial cells, namely SMAD7 and
Figure 4  

**miR-21 is up-regulated by and enhances TGF-β-mediated fibrotic gene expression**

(A) mRNA expression for the indicated genes from control and TGF-β treated NRK-52E proximal tubular cells. (B) miR-21 expression from control and TGF-β treated NRK-52E cells. RNA expression for ECM genes (C) and pro-fibrotic growth factors (D) from control, TGF-β treated, miR-21 mimic transfected and miR-21 transfected/TGF-β treated NRK-52E cells. *P < 0.05, control compared with all groups; †P < 0.05, TGF-β compared with miR-21 + TGF-β. mRNA expression for ECM genes (E) and pro-fibrotic growth factors (F) from control, TGF-β treated, miR-21 inhibitor transfected miR-21 inhibited/TGF-β treated NRK-52E cells. *P < 0.05, control compared with all groups; †P < 0.05, TGF-β compared with miR-21 inhibitor + TGF-β.
**Figure 5** **SMAD7 and PTEN protein translation is modulated by miR-21 levels**

Immunoblot detection of SMAD7 (A), pSMAD3 (B), PTEN (C) and pAKT (D) from whole cell lysates of NRK-52E treated as indicated. Densitometric analysis of SMAD7/PTEN levels relative to β-actin and pSMAD3/pAKT relative to total SMAD3–AKT respectively for control, TGF-β treated, miR-21 transfected and miR-21 transfected/TGF-β treated are shown below each blot. *P < 0.05, control compared with all; †P < 0.05, TGF-β compared with TGF-β–miR-21.
Figure 6  

miR-21 mediates its effects via SMAD3-dependent and PI3K-dependent signalling

mRNA expression of Coll (A), PAI1 (B) and FN1 (C) from control, TGF-β treated, LY294002 and LY294002/TGF-β treated NRK-52E cells. Cells were transfected with control or miR-21 mimic oligonucleotides as indicated. *P < 0.05, control compared with all; †P < 0.05, LY294002 compared with LY294002 + TGF-β; ‡P < 0.05, miR + TGF-β compared with miR + TGF-β + LY294002; §P < 0.05, miR-NC + TGF-β compared with miR-21 + TGF-β + LY294002 compared with miR-21 + TGF-β + LY294002. RNA expression of Coll (D), PAI1 (E) and FN1 (F) from control, TGF-β treated, SMAD3 siRNA transfected and SMAD3 transfected/TGF cells-treated NRK-52E. *P < 0.05, control compared with all; †P < 0.05, miR-NC + TGF-β compared with all + TGF-β, Tukey one-way ANOVA; ‡P < 0.05, miR + TGF-β compared with miR + siSMAD3 + TGF-β; §P < 0.05, miR-21 compared with miR-21 + siSMAD3; ¶P < 0.05, siSMAD3 + TGF-β compared with miR-21 + siSMAD3 + TGF-β. RNA expression of Coll (G), PAI1 (H) and FN1 (I) from control cells, TGF-β treated, miR-21 transfected, miR-21 transfected/TGF-β treated, miR-21 transfected/LY294002/TGF-β treated, miR-21–SMAD3 siRNA transfected/TGF-β treated and miR-21–SMAD3 siRNA transfected/LY294002/TGF-β treated. Data were analysed via Tukey one-way ANOVA. *P < 0.05, control compared with all; †P < 0.05, miR-NC + TGF-β compared with all + TGF-β; ‡P < 0.05, miR-21 + TGF-β compared with all + miR-21 + TGF-β; §P < 0.05, miR-21 + TGF-β compared with miR-21 + siSMAD3 + LY294002 + TGF-β; ¶P < 0.05, miR-21 + siSMAD3 + TGF-β compared with miR-21 + siSMAD3 + LY294002 + TGF-β.
PTEN. These targets contribute individually and cooperatively to induce many of the fibrogenic changes typical of DN and which are regulated by miR-21.

The present work adds to a number of studies demonstrating involvement of miR-21 in human renal pathology [12,35,36] although the present study has particularly focused on DN. miR-21 is reported to be up-regulated in allograft nephropathy with serum levels being increased independently of disease progression and also more recently in Alport syndrome [37]. It is therefore likely that miR-21 dysregulation is a common feature of renal disease where there is concomitant fibrosis. This is supported by our observations of increased miR-21 levels not only in a number of experimental models of diabetic renal disease but also in a model of non-diabetic renal disease. Despite a number of recent publications demonstrating increased miR-21 in mesangial cells and podocytes [12,13,35,36,38], our human in vivo data have demonstrated elevation of miR-21 primarily in the tubular compartment in DN. Thus, the present study has focused on the role of miR-21 in proximal tubule cells in the context of elevated glucose levels.

In PTCs, TGF-β1 increases miR-21 expression in conjunction with the expression of pro-fibrotic and ECM genes. Exogenous miR-21 augments these changes and represses miR-21 dampens the effects of TGF-β1. These data indicate that miR-21 plays a pivotal role in the regulation of processes downstream of TGF-β1. The SMAD- and AKT-dependent signalling pathways are both well-characterized and directly mediate TGF-β1-induced signalling thus contributing to the pathogenesis of DN [2]. Importantly, the negative regulators of these pathways, SMAD7 and PTEN respectively, are targeted and repressed by miR-21. The co-ordination of flux through these pathways with respect to miR-21 has not been investigated previously. Interrogation of the miR-21–PTEN axis via SMAD3 knockdown and the miR-21–PTEN axis via the use of the PI3K inhibitor LY294002 revealed that both of these pathways contribute to the regulation of classic effects of TGF-β1 including modulation of ColI, PAI1 and FN1 expression in PTCs.

Indeed, when both SMAD3 and AKT pathways were inhibited, both ColI and FN1 expression were not elevated by either miR-21 or TGF-β1 alone or in combination. Conversely, PAI1 expression was still elevated by combination treatment with miR-21 and TGF-β1 when both SMAD3 and AKT pathways were inhibited. Given that PAI1 possesses multiple SMAD3 promoter elements and that SMAD3 translation may not be fully inhibited by siRNA [39], this outcome is not unreasonable especially considering the changes in PAI1 expression seen under PI3K inhibition. PI3K–PTEN signalling acts as a negative regulator of FN1 and this pathway is also absolutely required for ColI expression. In all cases, miR-21 is seen to amplify the effect of TGF-β1 and its role in signalling through either pathway. The observation that TGF-β1 has some pro-fibrotic effects independent of SMAD3 and AKT, may indicate that yet another miR-21 targeted pathway mediates some of the pro-fibrotic effects of TGF-β1.

From our experiments, it is clear that miR-21 is an important player in the TGF-β1 signalling environment. Indeed, miR-21 levels were elevated concordantly with increasing disease pathology in human renal biopsies and experimental models of early/late DN and non-DN. It was shown that miR-21 is consistently up-regulated in the tubular compartment in models of diabetic and non-diabetic renal fibrosis. The observation of increased miR-21 in association with renal fibrosis could reflect a functional interaction between miR-21 and regulators of important signalling pathways in tubular cells. These interactions form part of a previously uncharacterized signalling system involving miR-21, PTEN and SMAD7. This system acts to co-ordinately regulate the TGF-β1-mediated fibrotic response of PTCs by concomitant de-repression of both SMAD3- and AKT-dependent signalling. In summary, this comprehensive approach to the study of the function of miR-21 in renal fibrosis provides important insight into the onset and progression of DN and could assist in defining alternative renoprotective strategies in diabetes.

**CLINICAL PERSPECTIVE**

- The present study analysed expression and correlation of miR-21 to fibrosis and renal function in biopsy material from DN subjects. Delineation of the role of miR-21 targets in PTCs was also performed.
- The results have highlighted the complexity of miR-21 action in TGF-β1-mediated fibrotic signalling in DN and also added
a level of understanding to the interplay of miR-21 and TGF-β1 which had been previously unrecognized.

- These findings are important in developing targeted therapeutics against the development and progression of DN.

**AUTHOR CONTRIBUTION**

Michal Herman-Edelstein, Radko Komers, Jay Jha, Shinji Hagiwara and Phillip Kantharidis researched data. Catherine Winbanks, Paul Gregorevic, Phillip Kantharidis and Mark Cooper reviewed/edited manuscript. Aaron McClelland researched data and wrote manuscript.

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