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microRNA-181a Downregulates Deptor for TGF β -induced Glomerular Mesangial Cell Hypertrophy and Matrix Protein Expression

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Abstract

TGF β contributes to mesangial cell hypertrophy and matrix protein increase in various kidney diseases including diabetic nephropathy. Deptor is an mTOR-interacting protein and suppresses mTORC1 and mTORC2 activities. We have recently shown that TGF β -induced inhibition of deptor increases the mTOR activity. The mechanism by which TGF β regulates deptor expression is not known. Here we identify deptor as a target of the microRNA-181a. We show that in mesangial cells, TGF β increases the expression of miR-181a to downregulate deptor. Decrease in deptor augments mTORC2 activity, resulting in phosphorylation/activation of Akt kinase. Akt promotes inactivating phosphorylation of PRAS40 and tuberlin, leading to stimulation of mTORC1. miR-181a-mimic increased mTORC1 and C2 activities, while anti-miR-181a inhibited them. mTORC1 controls protein synthesis via phosphorylation of translation initiation and elongation suppressors 4EBP-1 and eEF2 kinase. TGF β -stimulated miR-181a increased the phosphorylation of 4EBP-1 and eEF2 kinase, resulting in their inactivation. miR-181a-dependent inactivation of eEF2 kinase caused dephosphorylation of eEF2. Consequently, miR-181a-mimic increased protein synthesis and hypertrophy of mesangial cells similar to TGF β . Anti-miR-181a blocked these events in a deptor-dependent manner. Finally, TGF β -miR-181a-driven deptor downregulation increased the expression of fibronectin. Our results identify a novel mechanism

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CONFLICTS OF INTEREST

None

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involving miR-181a-driven dectator downregulation, which contributes to mesangial cell pathologies in renal complications.

Keywords

Glomerulosclerosis; miR-181a; dectator; mTORC1

INTRODUCTION

Pathologic features of chronic kidney disease include infiltration of inflammatory cells, leading to capillary rarefaction, podocyte depletion, tubular atrophy and fibrosis [1]. Recruited inflammatory cells secrete pro-fibrogenic cytokines including transforming growth factor β (TGF β), which directly and indirectly contribute to the phenotypic alterations of the resident kidney cells including glomerular mesangial cells [2]. Mesangial cells constitute one third of the glomerular cell population and respond to TGF β by undergoing hypertrophy and increasing synthesis of matrix proteins, leading to mesangial expansion [3]. The homodimeric TGF β binds to the constitutively active TGF β receptor II on the mesangial cell membrane to recruit TGF β receptor I, which phosphorylates the receptor-specific Smad 2 and Smad 3 at their C-termini. Phosphorylated Smads then bind to the co-Smad, Smad 4 and translocate to the nucleus to regulate transcription of select genes [4]. TGF β also activates Smad-independent non-canonical signaling that includes, PI 3 kinase, Erk1/2, JNK, p38 MAPK and mTOR [5].

microRNAs are noncoding RNAs that act as physiological and pathological regulators for post-transcriptional gene expression [6,7]. They bind predominantly to the 3' untranslated region of mRNAs with imperfect complementarity to suppress their translation. In human cells 1,657 miRNAs have been validated for RNA interference [8]. miRNAs are synthesized in the nucleus as primary transcripts, which undergo cleavage by the ribonuclease III Droscha to form precursor miRNAs. Pre-miRs are transported by exportin-5 to the cytosol where they are further processed by dicer to form mature miRNAs. Mature miRNAs then interact with the 3'UTR of target mRNA in an RNA-induced silencing complex to repress mRNA translation [7]. Recognition of miRNAs in renal injury came from studies in which renal podocyte-specific deletion of dicer in mouse resulted in proteinuria [9].

Mechanistic target of rapamycin (mTOR) is coded by a single gene and forms two distinct kinase complexes, mTORC1 and mTORC2, which contain common subunit mLST8 that interacts with the kinase domain [10]. Presence of PRAS40 and raptor in mTORC1 distinguishes it from mTORC2, while the latter uniquely contains rictor and mSin1 [10]. Recently, dectator has been identified as a component of both these kinase complexes [11]. Dectator acts as a negative regulator of mTORC1 and mTORC2. We have recently shown that TGF β downregulates dectator to increase mTOR activity [12]. However, the mechanism by which dectator is downregulated by TGF β is not known. Here we show that miR-181a induced by TGF β targets dectator mRNA 3'UTR to suppress its expression. We demonstrate that miR-181a regulates the TGF β -stimulated mTORC1 and mTORC2 activities involved in glomerular mesangial cell hypertrophy and fibronectin expression.

Materials and Methods

Reagents

Tissue culture materials and TRI reagent for preparing RNA were purchased from Thermo Fisher. Protease inhibitor cocktail and Na_3VO_4 were purchased from Sigma. Recombinant TGF β 1 was obtained from R & D Systems. Antibodies for phospho-Akt (Ser-473), phospho-Akt (Thr-308), phospho-GSK3 β (Ser-9), phospho-PRAS40 (Thr-246), phospho-tuberin (Thr-1462), phospho-S6 kinase (Thr-389), phospho-ribosomal protein s6 (rps6) (Ser-240/244), phospho-4EBP-1 (Thr-37/46), phospho-eEF2 kinase (Ser-366) and phospho-eEF2 (Thr-56), Akt, GSK3 β , PRAS40, S6 kinase, rps6, 4EBP-1, eEF2 kinase and eEF2 were purchased from Cell Signaling. Antibodies against tuberin and dectop were purchased from Santa Cruz. FLAG and actin antibodies were from Sigma. pMirTarget Dectop 3'-UTR luciferase (Dectop 3'-UTR-Luc) reporter plasmid was purchased from OriGene Technologies. OPTIMEM transfection medium, siRNA against dectop and the primers for detecting mature miR-181a, U6 RNA, miR-181a mimic and anti-miR-181a were obtained from Thermo Fisher. FuGENE HD transfection reagent was purchased from Promega.

Cell Culture

Rat glomerular mesangial cells prepared from rat were grown in DMEM with low glucose in the presence of 17% serum as described previously [13–15]. The cells were grown to confluency before incubation in the serum free DMEM for 18–24 hours. The cells were incubated with 2 ng/ml TGF β for 24 hours or as indicated.

RNA isolation and real time PCR

Total RNAs were prepared using the TRI reagent according to the vendor's protocol. cDNAs were prepared and used to detect miR-181a using the real time PCR machine (7500, Applied Biosystems). U6 primers were used to normalize the data as described previously [16,17].

Cell lysis and immunoblotting

After the incubation period, RIPA buffer was added to the cells for 30 minutes at 4°C. Lysed cells were scrapped off the plates and centrifuged at $14,000 \times g$ for 30 minutes at 4°C. The supernatant was collected and protein concentration was determined. Equal amounts of proteins were separated by SDS polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF membrane. The membrane containing the proteins was immunoblotted with indicated antibodies. HRP-conjugated secondary antibodies were used and the protein bands were visualized using chemiluminescence on X-ray film [13,16].

Protein synthesis and hypertrophy

Protein synthesis was determined using ^{35}S -Methionine incorporation as described previously [18]. Hypertrophy of mesangial cells were determined as a ratio of total protein content to the cell number as described previously [19,20].

Transfection

The mesangial cells were plated in complete medium. Next day the medium was changed to OPTIMEM and the cells were transfected with the plasmid vector, miR-181a mimic or anti-miR-181a using FuGENE HD according to the protocol provided by the vendor as previously described [16,19]. 4–6 hours post-transfection, complete medium was added. The transfected cells were serum starved and treated with TGF β as described above.

Luciferase activity

The mesangial cells were transfected with Deptor 3'-UTR-Luc reporter plasmid alone or along with miR-181a mimic. Transfected cells were incubated with TGF β as indicated. The luciferase activity was determined in the cell lysates using a luciferase assay kit as described previously [13,19].

Statistics

GraphPad Prism 6 Pro was used. Analysis of variance followed by Tukey's multiple comparison test was used to analyze the data. A p value of less than 0.05 vs was considered significant [15].

RESULTS

Identification of miR-181a to regulate deptor level

We have shown previously that TGF β decreases deptor [12] and confirmed it in the present study in rat glomerular mesangial cells (Supplementary Fig. S1). To determine the mechanism of deptor downregulation, we considered the involvement of miRNAs. TargetScan and miRanda analyses of the 3'UTR of deptor mRNA revealed the presence of the recognition element for miR-181a in the human and mouse deptor (Fig. 1A). We also identified a conserved miR-181a binding site in the rat deptor mRNA 3'UTR (Fig. 1A). The predicted minimum free energy (ΔG) for binding of miR-181a seed sequence and deptor 3'-UTR for human, mouse and rat is less than -6 kcal/mol (Supplementary Fig. S2). These data support the critical energy requirement for optimal repression of target protein expression [21]. TGF β significantly increased the expression of miR-181a in a time-dependent manner (Fig. 1B). To evaluate if miR-181a targets the deptor 3'-UTR, we transfected a reporter plasmid which contains the deptor 3'-UTR fused to the luciferase cDNA (Deptor 3'-UTR-Luc) into mesangial cells. Incubation of these cells with TGF β significantly inhibited the luciferase activity, similar to its effect on deptor expression (Figs. 1C and 1D). Furthermore, cotransfection of miR-181a mimic with the Deptor 3'-UTR-Luc markedly inhibited the luciferase activity, suggesting the responsiveness of the deptor 3'UTR to this miRNA (Fig. 1E). Indeed, expression of miR-181a mimic significantly inhibited the levels of deptor similar to TGF β (Fig. 1F). These results indicate that miR-181a inhibits deptor expression.

miR-181a controls TGF β -stimulated mTORC2 activity

Deptor was identified as an inhibitory component of mTOR complexes [11]. To determine whether deptor controls mTORC2 activity, we determined the phosphorylation of its substrate Akt at Ser-473 [22]. We used a plasmid vector expressing FLAG-tagged deptor.

Expression of dector inhibited the TGF β -induced phosphorylation of Akt at Ser-473 (Fig. 2A). Similarly, phosphorylation of Akt at Thr-308 was also inhibited by dector (Fig. 2A). Phosphorylation of Akt at Ser-473 and Thr-308 induces its full kinase activity [23]. This was confirmed by finding that TGF β increased the phosphorylation of an Akt substrate GSK3 β and expression of dector inhibited it (Fig. 2B). Similarly, dector suppressed the phosphorylation of two other Akt substrates, PRAS40 and tuberlin, which regulate the activation of mTORC1 (Fig. 2C and 2D). We have shown above that TGF β increases miR-181a to inhibit dector expression in mesangial cells. Therefore, we examined the effect of miR-181a mimic on mTORC2 activity. Similar to TGF β , expression of miR-181a mimic increased the phosphorylation of Akt, resulting in enhanced phosphorylation of GSK3 β , PRAS40 and tuberlin (Fig. 2E – 2H). Furthermore, miR-181a-induced phosphorylation of Akt and its substrates was significantly inhibited by the expression of dector (Figs. 2I – 2L), suggesting that miR-181a-targeted dector may regulate the mTORC2 to increase Akt activity. To confirm this observation, we used anti-miR-181a, which increased the expression of dector in the presence or absence of TGF β (Supplementary Fig S3). Transfection of anti-miR-181a significantly inhibited the TGF β -induced phosphorylation of Akt at Ser-473 and Thr-308, which resulted in attenuation of phosphorylation of GSK3 β , PRAS40 and tuberlin (Figs. 3A – 3D). Importantly, the inhibition of these TGF β -induced phosphorylations by anti-miR-181a was prevented by the expression of siRNA targeting dector (Figs. 3E – 3H). These results conclusively demonstrate that TGF β -induced miR-181a reduces dector and enables stimulation of mTORC2 and hence Akt kinase activity.

miR-181a regulates TGF β -stimulated mTORC1 activity for phosphorylation of mRNA translation initiation factors

Our data above show that miR-181a facilitates mTORC2-dependent phosphorylation of two negative regulators of mTORC1, PRAS40 and tuberlin in a dector-dependent manner. Phosphorylation of these proteins results in their inactivation leading to activation of mTORC1. First, we determined the role of dector in mTORC1 activity. To measure the mTORC1 activity, we used phosphorylation of its two endogenous substrates, S6 kinase and 4EBP-1, which positively and negatively regulate the initiation phase of mRNA translation, respectively [10]. TGF β increased the phosphorylation of both these proteins that was inhibited by the expression of dector (Figs. 4A and 4B). Since phosphorylation of S6 kinase by mTORC1 increases its activity towards the small ribosomal protein s6, we determined its phosphorylation. As shown in Fig. 4C, expression of dector blocked the TGF β -stimulated phosphorylation of rps6. Next, we examined the role of miR-181a in mTORC1 activation. Expression of miR-181a mimic increased the phosphorylation of S6 kinase, rps6 and 4EBP-1 similar to those found with TGF β (Figs. 4D, 4E and 4F). Furthermore, dector significantly inhibited the miR-181a mimic-induced phosphorylation of these proteins (4G – 4I), suggesting that this microRNA may regulate mTORC1 activity in response to TGF β via dector. To confirm this observation, we transfected anti-miR-181a, which led to inhibition of TGF β -stimulated mTORC1 activity as judged by the phosphorylation of S6 kinase, rps6 and 4EBP-1 (Figs. 5A – 5C). siRNA against dector reversed this inhibition by anti-miR-181a (Figs. 5D – 5F).

miR-181a regulates phosphorylation of mRNA translation elongation factors

In addition to the initiation phase, mTORC1 is known to control the elongation phase of mRNA translation via S6 kinase by phosphorylation of eEF2 kinase, which phosphorylates its substrate eEF2 [24]. mTORC1-induced eEF2 kinase phosphorylation inhibits its activity leading to reduced phosphorylation of eEF2, which facilitates peptide elongation [25]. In mesangial cells, TGF β increased the phosphorylation of eEF2 kinase resulting in dephosphorylation of eEF2. Rapamycin inhibited these reactions (Supplementary Figs. S4A and S4B). We examined the role of miR-181a-targeted dector in this process. Expression of dector inhibited the phosphorylation and dephosphorylation of eEF2 kinase and eEF2, respectively (Figs. 6A and 6B). In contrast, miR-181a mimic, respectively, increased and decreased the phosphorylation of eEF2 kinase and eEF2 similar to the TGF β treatment (Figs. 6C and 6D). However, dector significantly blocked the miR-181a mimic-induced phosphorylation and dephosphorylation of eEF2 kinase and eEF2, respectively (Figs. 6E and 6F). In conjunction with these results, we found that anti-miR-181a significantly inhibited the TGF β -stimulated eEF2 kinase phosphorylation, which resulted in the inhibition of the dephosphorylation of eEF2 (Figs. 6G and 6H). Furthermore, these effects of anti-miR-181a were prevented by the expression of siRNA against Dector (Figs. 6I and 6J). These results conclusively demonstrate that miR-181a-targeted dector regulates the elongation phase of the mRNA translation.

miR-181a regulates mesangial cell hypertrophy

We reported previously that TGF β -induced mesangial cell protein synthesis and hypertrophy are mediated by dector-dependent mTORC1 regulation [12,26]. We have shown above that miR-181a-targeted dector controls key events in the initiation and elongation phases of translation via dector. Therefore, we determined the role of this microRNA in protein synthesis and hypertrophy. We found that expression of dector significantly inhibited TGF β -induced protein synthesis and hypertrophy of mesangial cells (Figs. 7A and 7B). In contrast, expression of miR-181a mimic induced protein synthesis and hypertrophy similar to that with TGF β (Fig. 7C and 7D). Also, expression of dector inhibited the miR-181a mimic-induced protein synthesis and hypertrophy (Figs. 7E and 7F). To confirm the role of miR-181a, anti-miR-181a significantly blocked TGF β -induced protein synthesis and hypertrophy (Figs. 7G and 7H). Furthermore, expression of siRNA against dector reversed the anti-miR-181a-induced inhibition (Figs. 7I and 7J). These results demonstrate that miR-181a-targeted dector reduction regulates TGF β -induced mesangial cell hypertrophy.

miR-181a controls fibronectin expression

We showed previously that TGF β increases the expression of the matrix protein fibronectin in mesangial cells [18,27]. To determine the mechanism, we examined the role of dector. Expression of dector significantly inhibited the expression of TGF β -stimulated fibronectin expression in mesangial cells (Fig. 8A). On the other hand, miR-181a mimic markedly increased the expression of fibronectin similar to TGF β while dector inhibited miR-181a mimic-induced fibronectin expression (Figs. 8B and 8C). Furthermore, anti-miR-181a markedly abrogated the TGF β -stimulated fibronectin (Fig. 8D). Importantly, this inhibitory effect of anti-miR-181a was prevented by the expression of siRNAs targeting dector (Fig.

8E). Together our results demonstrate that TGF β -induced miR-181a regulates the mesangial cell fibronectin expression by modulating the expression of deptor.

DISCUSSION

In this report, we show that miR-181a is a mediator of TGF β in initiating the noncanonical mTOR signaling. We present the first clue that TGF β -stimulated miR-181a targets deptor to suppress its expression for increasing the mTORC1 and mTORC2 activity. We show that miR-181a-targeted deptor regulates the initiation and elongation factors of mRNA translation that contribute to mesangial cell hypertrophy induced by TGF β . Finally, we demonstrate the involvement of miR-181a and deptor in TGF β -induced fibronectin expression.

A role of TGF β in renal fibrosis including hypertrophy and matrix protein expression is established [28]. Along with canonical Smad signaling, TGF β utilizes the non-canonical PI 3 kinase/Akt/mTOR pathway for these phenomena [26,29]. In fact, in fibrotic disorder such as diabetic nephropathy, which is promoted by the action of TGF β , we and others have shown that rapamycin prevented both mTORC1 and mTORC2 in mice that resulted in reduction in renal hypertrophy and matrix protein expansion [30–33]. When calcineurin inhibitors were substituted by rapamycin in patients with chronic allograft nephropathy, a significant number of patients developed proteinuria [34]. Also, in renal transplant patients who were treated with rapamycin showed increased proteinuria due to renal damage [35]. In fact, long term treatment of mice with rapamycin showed renal atrophy [31,36]. Similarly, rapamycin produced loss of renal function in puromycin aminonucleoside-induced nephropathy [37]. Also, loss of mTORC1 as well as mTORC2 in the glomerular cells leads to significant renal injury with albuminuria [36,38]. Thus complete inhibition of mTOR activity by rapamycin may not be an ideal therapeutic option for TGF β -mediated renal fibrosis.

Deptor was identified as a partner of both mTORC1 and mTORC2 [11]. In mice deptor gene in chromosome 15 has been linked to obesity [39]. In a murine polygenic model, high expression of deptor in the white adipose tissue correlated with obesity [40]. Furthermore, deptor overexpression in mice promoted white adipose tissue expansion, suggesting its role in fat cell growth. In obese human, level of deptor correlated with the degree of obesity [40]. In certain cancers, deptor acts as an oncogene [41]. However, in many cancers, deptor is downregulated. Transcriptional inhibition and proteasomal degradation of deptor have been reported as mechanisms of downregulation in cancer cells. In conjunction with this notion, we confirmed our previous observation that TGF β in mesangial cells reduced the expression of deptor [12]. However, as a mechanism, we identified the involvement of TGF β -induced increase in miR-181a, which targeted the 3' UTR of deptor.

In those cancers where increased expression of deptor induces tumorigenesis, it activates the Akt kinase [42]. Similarly, deptor-mediated Akt activation is necessary for its role in adipogenesis in the obese mouse model and in skeletal muscle during insulin resistance where a metabolic shift from oxidative to glycolysis is observed [43]. In contrast to these results, our data show that overexpression of deptor in mesangial cells significantly blocked

the TGF β -stimulated Akt phosphorylation at the mTORC2 targeted hydrophobic motif site Ser-473. These results are consistent with its role in inhibiting the mTORC2 activity [11,22]. Guertin et al showed that the Thr-308 phosphorylation by PDK-1 was not affected by the ablation of mTORC2 [23,44]. Interestingly, deutor-mediated inhibition of mTORC2 in mesangial cells also inhibited the PDK-1-dependent Akt Thr-308 phosphorylation. It was reported that the hydrophobic motif site of Akt serves as the docking site for PDK-1, which phosphorylates Akt at Thr-308 [45]. Thus, our results demonstrating lack of Akt Thr-308 phosphorylation by deutor overexpression indicate that deficiency of Akt Ser-473 phosphorylation may block PDK-1 binding and hence inhibits phosphorylation at Thr-308.

Negative regulatory role of various microRNAs in renal hypertrophy and fibrosis due to upregulation of TGF β has been established. Downregulation of miR-29 is associated with renal fibrosis in the TGF β -dependent mouse models of fibrosis [46]. In fact, overexpression of miR-29 prevented the renal fibrosis in the models of unilateral ureteral obstruction and diabetic nephropathy [47]. This action of miR-29 was due to its ability to target different TGF β -responsive extracellular matrix-related genes [48]. Similarly, the expression of miR-200a is suppressed in these models of renal fibrosis [49]. On the other hand, multiple microRNAs have been shown to play positive role in kidney hypertrophy and fibrosis [7]. Thus Natarajan and coworkers noted increased expression of miR-192, which resulted in enhanced expression of miR-216 and 217 in the mesangial cells and in the diabetic kidneys [50,51]. The expression of miR-216 and miR-217, which directly target the tumor suppressor protein PTEN, leads to activation of Akt [51]. We showed previously that TGF β inhibited the expression of PTEN to induce renal cell hypertrophy and fibronectin expression in mesangial cells [18]. In fact, more recently, we have identified two different microRNAs, miR-21 and miR-214 which are upregulated by TGF β in diabetic renal fibrosis and they in turn downregulate the expression of PTEN to activate Akt/mTORC1 signaling that contributes to the hypertrophy and matrix protein expression in mesangial cells [15,27]. In the present study, we show that another microRNA, miR-181a is increased in response to TGF β in mesangial cells.

miR-181 is a family of four microRNAs (a, b, c and d), which are coded from three different chromosomes with identical seed sequence in the mature microRNAs [52]. miR-181 family is evolutionally conserved among the vertebrates, indicating their important role in physiology. miR-181 was initially found to be highly expressed in the T and B cell lineages although its expression is abundant in lung, brain and bone marrow [53]. miR-181 acts as a tumor suppressor in acute myeloid leukemia [54]. Also, miR-181a regulates antigen sensitivity of T cells during development. [55]. Recent work has revealed that miR-181a controls the lymphatic endothelial cell fate [56]. Furthermore, in vascular smooth muscle cells, angiotensin II downregulates miR-181a, which controls their adhesion to collagen by regulating the expression of osteopontin [57]. Thus miR-181a contributes to the pathophysiology of atherosclerosis. Similarly, in cardiac fibroblasts, angiotensin II decreased the expression of miR-181 family suggesting its role in fibrosis [52,58]. In contrast to these observations, we found that the profibrotic TGF β increased the expression of miR-181a in glomerular mesangial cells, which contributes to glomerular hypertrophy and glomerulosclerosis. In fact, we for the first time demonstrate a significant role of miR-181a in regulating the TGF β -induced mesangial cell hypertrophy and fibronectin expression.

In an effort to determine the mechanism of dector expression, we identified the miR-181a-mediated direct downregulation of dector, which contributes to the activation of both mTORC2 and mTORC1 in mesangial cells. We and others have reported a role of mTORC1 in the pathologic hypertrophy of the kidney to which TGF β contributes significantly [18,27,31,32,59]. Under basal state, mTORC1 activity is negatively regulated by two proteins tuberin and PRAS40. The tumor suppressor protein tuberin in complex with hamartin and TBC1D7 inhibits the Rheb-GTP formation, thereby block the mTORC1 activity [10]. Phosphorylation of tuberin inactivates its GAP function towards Rheb-GTP, resulting in activation of mTORC1 [60]. Importantly, phosphorylation of tuberin is significantly increased in the kidneys of patients with fibrotic disorder such as diabetic nephropathy [61]. Similarly, the unphosphorylated mTORC1 component PRAS40 blocks its kinase activity. When phosphorylated by Akt, it dissociates from the mTORC1 complex, leading to its activation [10,62]. A role of PRAS40 in cardiac hypertrophy has been reported where expression of this protein in cardiomyocyte blocked cardiac hypertrophy [63]. Furthermore, cardiomyocyte-specific overexpression of PRAS40 inhibited mTORC1 activity resulting in decrease in pathological hypertrophy including diabetic cardiomyopathy [63,64]. We have recently shown that phosphorylation of PRAS40 contributes to the renal cell hypertrophy driven by TGF β in diabetic nephropathy and *in vitro* in mesangial cells [20,27]. Phosphorylation-deficient mutant of PRAS40 inhibited the mTORC1 activity, resulting in inhibition of pathological mesangial cell hypertrophy [20]. Our results here show that miR-181a increases the phosphorylation of tuberin and PRAS40 similar to TGF β . In fact, miR-181a directly contributes to the phosphorylation of these two mTORC1 inhibitors by targeting dector.

We and others have established a role of mTORC1 in TGF β -driven renal hypertrophy and fibrosis [18,30–32,65]. Inhibition of mTORC1 by rapamycin blocked renal hypertrophy and matrix protein expression [30,32]. We also showed a role for the mTORC1 substrate S6 kinase in the renal cell hypertrophy and matrix protein expression [66]. More recently, using rapamycin in a mice model of increased mTORC1 activity in the kidney, ribosomal protein s6 phosphorylation by S6 kinase was shown to contribute to hypertrophy [67]. However, in this model, rapamycin impaired kidney function leading to premature death of the animals, suggesting a deleterious effect of this macrolide in kidney. In the present study, we demonstrate a significant role of miR-181a in activating the mTORC1 and S6 kinase, where the latter phosphorylates the rps6 similar to TGF β . Also, our data show direct requirement of miR-181a to suppress dector expression in TGF β -stimulated mTORC1 activity. Furthermore, we present the first evidence that inhibition of miR-181a blocked TGF β -induced mesangial cell hypertrophy and fibronectin expression. Thus our results demonstrate a mechanistic basis for dector suppression that includes upregulation of miR-181a by TGF β . Therefore, targeting miR-181a may provide a therapeutic option for renal diseases where TGF β -driven mesangial cell hypertrophy and matrix protein expression contribute to pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

TGFβ increases miR-181a to target deceptor. (A) Sequences of miR-181a and imperfect complementarity of miR-181a with the 3'UTR of human (hsa), mouse (mmu) and rat (rno) deceptor mRNA 3'UTR. (B) Expression of miR-181a in mesangial cells. Mesangial cells were incubated with TGFβ (2 ng/ml) for indicated periods. Total RNAs were used to detect miR-181a as described in the Materials and Methods. Mean ± SE of quadruplicates is shown. *p < 0.01 vs control. (C) Mesangial cells were transfected with Deceptor 3'UTR-Luc reporter plasmid. The transfected cells were incubated with 2 ng/ml TGFβ. The luciferase activity was determined as described in the Materials and Methods. Mean ± SE of quadruplicates is shown. *p < 0.01 vs control. (D) Lysates of mesangial cells incubated with TGFβ (2 ng/ml) were immunoblotted with deceptor and actin antibodies. (E) Deceptor 3'UTR-Luc reporter plasmid was co-transfected with miR-181a mimic or scramble RNA. The cells lysates were used to determine the luciferase activity. Mean ± SE of quadruplicates is shown. *p < 0.001 vs scramble. (F) Mesangial cells were transfected with scramble or miR-181a mimic. The cells were incubated with TGFβ (2 ng/ml). The cell lysates were immunoblotted with deceptor and actin antibodies.

Figure 2.

miR-181a and deceptor regulate TGFβ-induced Akt activation. (A – D) Mesangial cells were transfected with FLAG-tagged deceptor expression plasmid. The cells were incubated with TGFβ (2 ng/ml). (E – H) The mesangial cells were transfected with miR-181a mimic. The cells were then incubated with TGFβ. (I – L) The mesangial cells were transfected with miR-181a mimic and FLAG-tagged deceptor as indicated. The cell lysates were immunoblotted with indicated antibodies.

Figure 3. miR-181a targets deptor to regulate TGFβ-stimulated Akt activity. (A – D) The mesangial cells were transfected with scramble or anti-miR-181a. (E – H) The mesangial cells were co-transfected with anti-miR-181a and siRNA against deptor. The transfected cells were incubated with TGFβ. The cell lysates were immunoblotted with indicated antibodies.

Figure 4.

miR-181a and deptor regulate TGFβ-induced mTORC1 activation. (A – C) Mesangial cells were transfected with FLAG-tagged deptor expression plasmid. The cells were incubated with TGFβ (2 ng/ml). (D – F) The mesangial cells were transfected with miR-181a mimic. The cells were then incubated with TGFβ. (G – I) The mesangial cells were co-transfected with miR-181a mimic and FLAG-tagged deptor as indicated. The cell lysates were immunoblotted with indicated antibodies.

Figure 5. miR-181a regulates TGFβ-stimulated mTORC1 by targeting deptor. (A – C) The mesangial cells were transfected with scramble or anti-miR-181a. (D – F) The mesangial cells were co-transfected with anti-miR-181a and siRNA against deptor. The transfected cells were incubated with TGFβ. The cell lysates were immunoblotted with indicated antibodies.

Figure 6. miR-181a regulates TGFβ-induced elongation factor phosphorylation. (A – D) Mesangial cells were transfected with FLAG-tagged deptor expression plasmid (panels A and B) or miR-181a mimic (panels C and D). The cells were incubated with TGFβ (2 ng/ml). (E and F) The mesangial cells were co-transfected with miR-181a mimic and FLAG-tagged deptor expression vector. (G and H) Mesangial cells were transfected with anti-miR-181a. (I and J) The mesangial cells were co-transfected with anti-miR-181a and siRNA against deptor. The cells were incubated with TGFβ (panels A – D and G – J). The cell lysates were immunoblotted with indicated antibodies.

Figure 7.

miR-181a regulates mesangial cell protein synthesis and hypertrophy via deceptor. (A – D) Mesangial cells were transfected with FLAG-tagged deceptor (panels A and B) and miR-181a mimic (panels C and D). The cells were incubated with TGFβ (2 ng/ml). (E and F) miR-181a mimic was co-transfected with FLAG-tagged deceptor into mesangial cells. (G – J) Mesangial cells were transfected with anti-miR-181a (panels G and H) and anti-miR-181a plus siRNAs against deceptor (panels I and J). The cells were incubated with TGFβ. Protein synthesis was determined as ³⁵S-Methionine incorporation (panels A, C, E, G and I). Hypertrophy was determined as the ratio of protein to cell number (B, D, F, H and J). Bottom parts in panels A, B, E and F show the expression of transfected FLAG-tagged deceptor and actin. In panels I and J expression of deceptor and actin is shown. Mean ± SE of triplicates is shown. *p < 0.001 vs control; **p < 0.001 vs TGFβ or miR-181 mimic (in panels A, B and E – J); @p < 0.001 vs TGFβ plus anti-miR-181a (in I and J).

Figure 8.

miR-181a-targeted deceptor regulates TGFβ-induced fibronectin expression. Mesangial cells were transfected with FLAG-tagged deceptor (panel A) or miR-181a mimic (panel B) or anti-miR-181a (panel D). The cells were incubated with TGFβ. (C) The mesangial cells were co-transfected with miR-181a mimic and FLAG-tagged deceptor expression vector. (E) Mesangial cells were co-transfected with anti-miR-181a and siRNAs against deceptor. The cells were incubated with TGFβ. The cell lysates were immunoblotted with indicated antibodies.