Published in final edited form as:

Cell Signal. 2015 July ; 27(7): 1276–1285. doi:10.1016/j.cellsig.2015.03.007.

High glucose enhances microRNA-26a to activate mTORC1 for mesangial cell hypertrophy and matrix protein expression

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Abstract

High glucose milieu inhibits PTEN expression to activate Akt kinase and induces glomerular mesangial cell hypertrophy and matrix protein expression in diabetic nephropathy. Specific mechanism by which high glucose inhibits PTEN expression is not clear. We found that high glucose increased the expression of the microRNA-26a (miR-26a) in mesangial cells. Using a sensor plasmid with 3'UTR-driven luciferase, we showed PTEN as a target of miR-26a in response to high glucose. Overexpression of miR-26a reduced the PTEN protein levels resulting in increased Akt kinase activity similar to high glucose treatment. In contrast, anti-miR-26a reversed high glucose-induced suppression of PTEN with concomitant inhibition of Akt kinase activity. Akt-mediated phosphorylation of tuberin and PRAS40 regulates mTORC1, which is necessary for mesangial cell hypertrophy and matrix protein expression. Inhibition of high glucose-induced miR-26a blocked phosphorylation of tuberin and PRAS40, which lead to suppression of phosphorylation of S6 kinase and 4EBP-1, two substrates of mTORC1. Furthermore, we show that expression of miR-26a induced mesangial cell hypertrophy and increased fibronectin and collagen I ($\alpha 2$) expression similar to that observed with the cells incubated with high glucose. Anti-miR-26a inhibited these phenomena in response to high glucose. Together our results provide the first evidence for the involvement of miR-26a in high glucose-induced mesangial cell hypertrophy and matrix protein expression. These data indicate the potential therapeutic utility of anti-miR-26a for the complications of diabetic kidney disease.

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Keywords

MicroRNA; Diabetic nephropathy; mTOR; Mesangial cell pathology

1. Introduction

A major clinical manifestation of diabetic nephropathy is microalbuminuria, which is followed by macroalbuminuria, resulting in loss of renal function. The pathological changes of diabetic nephropathy comprise renal hypertrophy including glomerular hypertrophy with altered hemodynamics and increased matrix protein expression [1]. In addition, diabetic kidney disease is associated with progressive mesangial dysfunction and glomerulosclerosis [1, 2]. In fact accumulation of mesangial matrix correlates with the progression of diabetic kidney injury [3]. Furthermore, mouse that fails to undergo mesangial hypertrophy are resistant to glomerular injury in diabetes [4]. These results indicate a pivotal role of mesangial cells in the progression of diabetic nephropathy.

High glucose-stimulated Akt signal transduction pathway regulates mesangial cell hypertrophy and matrix expansion [5, 6]. Akt kinase is activated by the lipid phosphatidylinositol 3,4,5-trisphosphate (PIP₃), a product of PI 3 kinase. PTEN (phosphatase and tensin homolog deleted on chromosome 10) dephosphorylates PIP₃ to inactivate Akt kinase [7]. We have shown that PTEN is downregulated in high glucose-treated mesangial cells and in diabetic rodent glomeruli [6]. Furthermore, our data demonstrated that PTEN deficiency contributes to the mesangial cell hypertrophy and matrix protein expression by stimulation of Akt kinase [6].

The short 22-25 nucleotides long microRNAs (miRNAs) act as rheostat of gene expression. They predominantly bind to the specific recognition elements present in the 3' untranslated region of mRNAs with imperfect complementarity to suppress their translation [8]. miRNAs are synthesized in the nucleus as long transcripts, which are then processed by Drosha, an RNase III like enzyme, to pre-miRNAs with a stem-loop structure. Pre-miRNAs are then transported to the cytoplasm for further cleavage by the enzyme dicer to produce mature miRNA, which associates with Argonaute 2 in RISC (RNA-induced silencing complex) and binds to the 3'UTR of mRNAs to suppress their translation.

Aberrant expression of miRNAs has been reported in various diseases including diabetic kidney disease [9, 10]. Deletion of dicer, which produces the mature miRNAs, in the kidney glomerular epithelial cells, produces proteinuria and renal fibrosis, suggesting a critical role of miRNAs in development of renal disease [11]. For example, expression of miR-93 is inhibited while miR-192, miR-216a and miR-217 are increased in diabetic glomeruli and in cultured mesangial cells incubated with high glucose [12-14]. Similarly, many other miRNAs have been shown to be upregulated and downregulated in diabetic kidneys [10, 14]. In the present report, we show significant increase in miR-26a in mesangial cells in the presence of high glucose. We demonstrate that miR-26a targets PTEN to increase Akt signal transduction, which activates mTOR necessary for mesangial cell hypertrophy and matrix protein expression.

2. Materials and methods

2.1. Reagents

Tissue culture materials and first strand cDNA synthesis kit were obtained from InVitrogen. TRI Reagent, Na₃VO₄, Nonidet-P40, S6 kinase inhibitor PF-4708671 (SKI), anti-actin and – fibronectin antibodies were purchased from Sigma. Antibodies for phospho-Akt (Ser-473), phospho-Akt (Thr-308), Akt, phospho-GSK3 β (Ser-9), GSK3 β , phospho-S6 kinase (Thr-389), S6 kinase, phospho-s6 (Ser-240/244), s6, phospho-4EBP-1 (Thr-37/46), 4EBP-1, phospho-PRAS40 (Thr-246), PRAS40, phospho-tuberin (Thr-1462) and tuberin were obtained from Cell Signaling. PTEN and collagen I (α 2) antibodies were purchased from Santa Cruz. Detailed information about the antibodies is presented in Supplementary Table S1. The primers for detection of mature miR-26a and U6 (for normalization), the miRVana quantitative RT-PCR miRNA detection kit and anti-miR26a were purchased from Ambion. Eugene HD transfection reagent and luciferase assay kit were obtained from Promega. PTEN 3'UTR-Luc reporter plasmid was obtained from Genecopea. GFP-tagged miR-26a overexpression vector was a kind gift from Dr. Eric C. Holland (Memorial Sloan-Kettering Cancer Center, NY).

2.2. Cell culture

Rat glomerular mesangial cells, podocytes and mouse proximal tubular epithelial cells were grown as described previously [15, 16]. Confluent monolayer was serum starved for 24 hours to make them quiescent. These cells were incubated with 25 mM glucose for the indicated times. For osmotic control, 5 mM glucose plus 20 mM mannitol was used.

2.3. Cell lysis

After treatment, PBS-washed cell monolayer was lysed in RIPA buffer and centrifuged to collect the supernatant as described [17, 18]. Equal amounts of protein were separated by SDS polyacrylamide gel and immunoblotted with indicated antibodies [17, 18].

2.4. RNA isolation and real time PCR

Total RNAs were prepared using TRI Reagent according to vendor's protocol. cDNA was synthesized. The cDNAs were amplified using pre-miR-26a primers (Rat Forward: 5'-AAGGCCGTGGCCTTGTTCAA-3'; Rat Reverse: 5'-CAGGCCCGCGTCCCCGTGCA-3') (Mouse Forward: 5'-AAGGCCGTGGCCTCGTTCAA-3'; Mouse Reverse: 5'-CAGGCCCGCGTCCCCGTGCA-3'). For detection of mature miR-26a, qRT-PCR primers (Ambion) were used according to the manufacturer's protocol. U6 primers were used to normalize the data as described previously [15, 19]. Expression of green fluorescence protein (GFP) mRNA was detected as a surrogate for expression of miR-26a from the vector. The primers for detecting GFP mRNA are: Forward, 5'-ACGGCAAGCTGACCCTGAAG-3'; Reverse, 5'-GGGTGCTCAGGTAGTGGTTG-3'.

2.5. Protein synthesis and hypertrophy

Protein synthesis was measured by ³⁵S-methionine incorporation as described [18, 20]. Hypertrophy of mesangial cells was determined by the ratio of total protein to cell number

as described previously [5, 18, 20]. Also hypertrophy of the mesangial cells was determined by increased cell size using a flow cytometer. The cells were trypsinized, washed with PBS and resuspended in PBS. 1 μ g/ml propidium iodide was added prior to flow cytometry. Cytometry was carried out using a LSR II four laser system (BD Biosciences). The mesangial cell size was determined with FlowJo v7.6 software as described previously [21].

2.6. Transfection

Rat mesangial cells were transfected with miR-26a vector plasmid using Eugene HD as described previously [17, 18]. Anti-miR-26a and scramble RNA were transfected using the same protocol. Transfected cells were starved for serum and incubated with 25 mM glucose as described above.

2.7. Luciferase activity

Mesangial cells were transfected with the PTEN 3'UTRLuc reporter plasmid along with the miR-26a expression vector or anti-miR-26a. The luciferase activity was determined in the cell lysates using a luciferase assay kit as described previously [15, 22]. The data are presented as the mean luciferase activity/microgram protein as arbitrary unit $\pm$ SE.

2.8. Statistics

Analysis of variance with Student-Newman-Keuhls analysis was used to determine the significance of the data. The mean $\pm$ SE of indicated measurements is shown. A p value of less than 0.05 was considered as significant.

3. Results

3.1. High glucose increases miR-26a to target PTEN in glomerular mesangial cells

We have recently shown that PTEN is downregulated in diabetic glomeruli. Also, high glucose decreases the levels of PTEN in mesangial cells [6]. To investigate the mechanism of high glucose-induced PTEN downregulation, we considered the involvement of miRNAs. PTEN is targeted by multiple miRNAs [23]. Bioinformatic analysis of the 3'UTR of PTEN revealed the presence of recognition element for miR-26a, which is conserved in human, mouse and rat (Fig. 1A). The predicted minimum free energies (ΔG) for binding of miR-26a to the 3'UTR of human, mouse and rat PTEN mRNA are comparable (-24.4 , -22.5 and -22.8 kcal/mol, respectively) (Supplementary Fig. S1A). Moreover, the free energy of binding of the seed sequence of miR-26a to the corresponding recognition element present in the PTEN 3'UTR is significantly lower than -6 kcal/mol, which supports the critical free energy requirement for optimal repression of target protein expression (Supplementary Fig. S1B) [24]. In support of the notion that PTEN is a direct target of miR-26a, we examined the expression of this miRNA in glomerular mesangial cells. High glucose significantly increased the expression of pre-miR-26a and mature miR-26a (Figs. 1B and 1C). Similarly, high glucose enhanced the levels of both these species of miR-26a in proximal tubular epithelial cells (Fig. 1D and 1E) and podocytes (Fig. 1F and 1G). To determine if miR-26a targets the PTEN 3'UTR, we transfected a reporter plasmid which contains the PTEN 3'UTR fused to the luciferase cDNA (PTEN 3'UTR-Luc) into mesangial cells. Incubation of these cells with 25 mM glucose significantly inhibited the luciferase activity (Fig. 1H).

Cotransfection of these cells with a plasmid vector expressing GFP-tagged miR-26a also markedly inhibited the reporter activity similar to high glucose treatment (Fig. 1H). High glucose treatment did not have any further inhibitory effect. To examine the specificity of miR-26a action, we cotransfected anti-miR-26a with the PTEN 3'UTR-Luc into mesangial cells prior to incubation with high glucose. As expected, high glucose suppressed the luciferase activity. However, anti-miR-26a, which inhibited high glucose-stimulated miR-26a levels, significantly reversed the high glucose-induced suppression of luciferase activity to its basal level (Fig. 1I and Supplementary Fig. S2). These data suggest that high glucose-induced increase in miR-26a may regulate PTEN expression.

To investigate the role of miR-26a in PTEN expression, we expressed the GFP-tagged miR-26a in mesangial cells. High glucose reduced the expression of PTEN. Expression of miR-26a inhibited PTEN levels in cells grown in 5 mM glucose, similar to that obtained with high glucose alone (Fig. 2A and Supplementary Fig. S3A). PTEN inhibits Akt phosphorylation [7]. Thus the reduction in PTEN levels by miR-26a was associated with increased Akt phosphorylation at both Thr-308 and Ser-473 similar to high glucose treatment (Fig. 2B and Supplementary Fig. S3B). miR-26a in the presence of high glucose did not show further increase in Akt phosphorylation (Fig. 2B). Phosphorylation of Akt at these sites increases its kinase activity. GSK3 β is a substrate of Akt. Therefore, we tested its phosphorylation. Expression miR-26a increased the phosphorylation of GSK3 β similar to that found with high glucose and high glucose with miR-26a (Fig. 2C and Supplementary Fig. 3C).

To confirm the involvement of miR-26a in regulation of PTEN expression, we transfected mesangial cells with anti-miR-26a. As expected high glucose decreased the levels of PTEN (Fig. 2D). However, the expression of anti-miR-26a prevented the reduction in expression of PTEN induced by high glucose (Fig. 2D, compare lane 4 with lane 2; Supplementary Fig. S3D and S3E). Consequently, anti-miR-21 inhibited high glucose-induced phosphorylation of Akt at both activating sites (Fig. 2E and Supplementary Figs. S3F and S3G). Accordingly, anti-miR-26a inhibited phosphorylation of the Akt substrate GSK3 β similar to that observed with Akt inhibitor MK2206 (Figs. 2F and 2G; Supplementary Figs. S3H - S3J). These results suggest that high glucose-induced expression of miR-26a regulates PTEN-mediated Akt activation in mesangial cells.

3.2. miR-26a regulates mTORC1 activity in response to high glucose

We have shown recently that Akt kinase regulates high glucose-induced mTORC1 activity in mesangial cells via phosphorylation and inactivation of the tumor suppressor protein tuberlin, which acts as an inhibitor of the kinase activity [19, 25]. Similarly, PRAS40, a component of the mTORC1, acts as the negative regulator of mTORC1 activity [26]. Akt kinase-mediated phosphorylation of PRAS40 results in its inactivation [5]. We tested the role of miR-26a in phosphorylation of tuberlin and PRAS40. High glucose increased the phosphorylation of both tuberlin and PRAS40 (Figs. 3A, 3B and Supplementary Figs. S4A and S4B). Similarly, expression of miR-26a alone enhanced phosphorylation of both these proteins (Figs. 3A and 3B). miR-26a in the presence of high glucose did not have any additive effect. To confirm these observations, we used anti-miR-26a. Inhibition of miR-26a

blocked the high glucose-induced phosphorylation of tuberin and PRAS40 (Figs. 3C, 3D and Supplementary Figs. S4C - S4F). Since phosphorylation of PRAS40 induces its inactivation, which results in mTORC1 activation, we examined the effect of miR-26a on mTORC1 activation. S6 kinase and 4EBP-1 are two direct substrates of mTORC1 [27]. Therefore, we used their phosphorylation as an indicator of mTORC1 activation in mesangial cells. Expression of miR-26a increased the phosphorylation of S6 kinase and 4EBP-1 similar to that obtained with high glucose alone (Figs. 4A, 4B and Supplementary Figs. S5A and S5B). In contrast, anti-miR-26a inhibited high glucose-induced activation of mTORC1 activity (Figs. 4C, 4D and Supplementary Figs. S5C – S5F). Since phosphorylation of S6 kinase by mTORC1 increases its activity, we tested the phosphorylation of its substrate s6, a subunit of the small ribosome. High glucose increased the phosphorylation of s6 (Figs. 4E and 4F). Expression of anti-miR-26a significantly inhibited the high glucose-induced phosphorylation similar to treatment with the S6 kinase inhibitor (SKI) (Figs. 4E, 4F and Supplementary Fig. S5G – S5I). These results indicate that miR-26a induced by high glucose contributes to activation of mTORC1.

3.3. miR-26a induces mesangial cell hypertrophy and increases matrix protein expression

We have shown previously that high glucose induces mesangial cell hypertrophy via activation of mTORC1 [5, 18]. We showed above that miR-26a regulates high glucose-stimulated mTORC1 activity (Fig. 4). Therefore, we tested the effect of miR-26a on mesangial protein synthesis induced by high glucose treatment. Expression of miR-26a significantly increased protein synthesis similar to that induced by high glucose (Fig. 5A). miR-26a in the presence of high glucose did not have any additive effect. In contrast, inhibition of miR-26a by anti-miR transfection significantly blocked high glucose-induced protein synthesis (Fig. 5B and Supplementary Fig. S6A). We also determined the mesangial cell hypertrophy by the ratio of total protein content to the cell number [6, 18, 20]. Expression of miR-26a alone significantly induced mesangial cell hypertrophy analogous to high glucose treatment (Fig. 5C). miR-26a in the presence of high glucose did not have any additive effect on hypertrophy (Fig. 5C). On the other hand, anti-miR-26a significantly inhibited mesangial cell hypertrophy induced by high glucose (Fig. 5D and Supplementary Fig. S6B). To confirm this observation, we determined the hypertrophy of mesangial cells by determining the increase in cell size by flow cytometry, using forward scatter as the parameter of cell size. High glucose increased the mesangial cell size (Figs. 5E and 5F). Expression of miR-26a increased the cell size similar to that observed with high glucose treatment (Fig. 5E and Supplementary Fig. S6C). In contrast, expression of anti-miR-26a blocked the high glucose-induced increase in cell size (Fig. 5F and Supplementary Fig. S6D).

An important pathologic feature of diabetic kidney disease is glomerular sclerosis, which results from increased matrix protein expression by the mesangial cells [1, 3]. We determined the effect of miR-26a on the expression of two matrix proteins fibronectin and collagen I ($\alpha 2$). Incubation of mesangial cells with high glucose increased the expression of both fibronectin and collagen I ($\alpha 2$) (Fig. 6). Exogenous expression of miR-26a elevated the abundance of both these matrix proteins similar to that obtained with high glucose (Figs. 6A, 6B and Supplementary Figs. S7A and S7B). In contrast, anti-miR-26a inhibited the high

glucose-induced expression of fibronectin and collagen I ($\alpha 2$) (Figs. 6C, 6D and Supplementary Figs. S7C – S7F). These results suggest that miR-26a contributes to two major pathologic effects of diabetes on the glomerulus, i.e., mesangial cell hypertrophy and matrix protein expression.

4. Discussion

In human and mouse, miR-26 family of miRNA comprises three members, miR-26a1, miR-26a2 and miR-26b. Mature miR-26a1 and miR-26a2 possess identical sequence while miR-26b contains two different nucleotides. In the rat, one miR-26a locus is present in the intron 4 of Ctdspl gene (carboxyterminal domain RNA polymerase II polypeptide A small phosphatase) (Supplementary Fig. S8) [28]. In the present study, we identified miR-26a be upregulated in response to high glucose in rat glomerular mesangial cells. We present evidence that miR-26a suppresses the expression of the tumor suppressor protein PTEN to increase the Akt signal transduction. We show that miR-26a inactivates PRAS40 and tuberlin, the negative regulators for mTORC1. Finally our results demonstrate that miR-26a regulates high glucose-induced mesangial cell hypertrophy and matrix protein expression in mesangial cells (Fig. 7).

Role of PTEN in glomerular hypertrophy and matrix expansion is established [6, 13]. Although the expression of PTEN is regulated at the level of transcription, miRNA-mediated posttranscriptional downregulation of PTEN has been reported [29, 30]. For example, in many cancers, different miRNAs including miR-18a, miR-32, miR-21, miR-214, miR-205 and several others have been shown to directly target PTEN to promote oncogenesis [22, 31-34]. Overexpression of miR-17-miR-92 cluster containing the PTEN-targeting miR-17-5p and miR-19 in the mouse produced a lymphoproliferative disorder along with glomerular hypertrophy and mesangial expansion; the latter two changes also represent two pathologic features of diabetic nephropathy [35]. miR-216, miR-217 and miR-21, which target PTEN, are also increased in the glomeruli of diabetic mice and in cultured mesangial cells exposed to high glucose [13, 15]. Consistent with these results, here we show increased expression of an intronic miRNA, miR-26a, in high glucose-treated glomerular mesangial cells (Figs. 1C, 1E and 1G). In fact we demonstrate that miR-26a targets PTEN 3'UTR to regulate PTEN protein levels in mesangial cells (Figs. 1H, 1I and Fig. 2A).

Both up- and down-regulation of miR-26a has been reported in various tumors. Thus, for many tumors such as bladder, breast, thyroid, hepatocellular, pancreatic carcinomas and rhabdomyosarcoma and Burkitt lymphoma, miR-26a acts as a tumor suppressor and its expression is significantly reduced as compared to the normal tissues [36, 37]. On the other hand miR-26a acts as an oncomiR and its expression is increased in high grade glioma [36]. Similar to this observation, during normal myogenesis, miR-26a was found to increase gradually [36]. In mice with autoimmune glomerulonephritis, and in patients with lupus nephritis and IgA nephropathy, expression of miR-26a is significantly decreased. However, the urinary excretion of miR-26a was significantly increased in autoimmune glomerulonephritic patients and in mice [38]. In mice, in response to myocardial infarction and in human with acute coronary syndrome, the expression of miR-26a is elevated [39]. In fact, overexpression of miR-26a inhibited endothelial cell proliferation, migration and

angiogenesis [39]. On the other hand, expression of miR-26a is reduced in myocardial hypertrophy and in humans with atrial fibrillation [40, 41]. Thus, *in vivo* expression of miR-26a decreased vulnerability to atrial fibrillation in a mouse model [41]. In cultured cardiomyocytes, angiotensin II decreased miR-26a levels [40]. Conversely, overexpression of miR-26a inhibited cardiomyocyte hypertrophy. In contrast to these results, expression of miR-26a is significantly increased in mesangial cells, concomitant with hypertrophy in response to high glucose (Figs. 1 and 5A, 5C and 5E). Expression of anti-miR-26a inhibited high glucose-induced mesangial cell hypertrophy (Figs. 5B, 5D and 5F). These results conclusively indicate a pathological role of increased miR-26a in mesangial cell hypertrophy.

GSK3 β has been identified as a direct target of miR-26a in cardiomyocyte [40]. Also a role of miR-26a-regulated GSK3 β has been postulated in controlling cardiomyocyte hypertrophy [40]. Suppression of GSK3 β by miR-26a expression inhibited myocardial hypertrophy [40]. GSK3 β is an endogenous substrate of Akt kinase, which inactivates its kinase activity. In the present study, we could not detect any downregulation of GSK3 β protein levels in mesangial cells treated with high glucose or in the presence of overexpression of miR-26a (Fig. 2C). These results are not unusual as miRNAs are known to act in a cell-specific manner. In fact, expression of miR-26a increased the phosphorylation and hence inactivation of GSK3 β , which may contribute to renal cell hypertrophy (Figs. 5A and 5C) similar to that observed with suppression of this kinase in cardiomyocyte by overexpression of miR-26a [40, 42].

The pathologic hypertrophic response of kidney is mediated by the pivotal kinase mTORC1 [25, 43-46]. The mTORC1 is maintained in its basal state by the negative regulator of its kinase activity, tumor suppressor tuberous sclerosis complex (TSC), which comprises TSC1, TSC2 and TBC1D7 [25, 47]. Phosphorylation of TSC2 (tuberin) in this complex inactivates its tumor suppressor function, resulting in activation of mTORC1 [25]. Recently it has been shown that phosphorylation of tuberin is significantly increased in kidneys of patients with diabetic nephropathy [48]. Similar to tuberin, PRAS40, a component of mTORC1, also inhibits its kinase activity [26]. PRAS40 when phosphorylated by Akt kinase undergoes inactivation, leading to activation of mTORC1 [27]. Recently, we have shown that phosphorylation of both tuberin and PRAS40 in mesangial cells increases the mTORC1 activity [5, 19]. Our results presented in Fig. 3 show that miR-26a regulates high glucose-induced phosphorylation of tuberin and PRAS40.

Recently, we have demonstrated the presence of elevated mTORC1 activity in the kidneys of mice with type 1 and type 2 diabetes [46, 49]. Moreover, we showed that inhibition of mTOR activity ameliorated renal hypertrophy and matrix protein expression [46, 49]. Similar to these *in vivo* studies, we also demonstrated a critical role of mTORC1 in high glucose-induced mesangial cell hypertrophy [5]. Now for the first time we show that expression of miR-26a increased mTORC1 activity similar to that found in the presence of high glucose (Figs. 4A and 4B). Alternatively, inhibition of miR-26a suppressed mTORC1 activity in response to high glucose (Fig. 4C and 4D). Furthermore, our data demonstrate that anti-miR-26a inhibits the S6 kinase activity similar to that observed with a S6 kinase inhibitor (Figs. 4E and 4F). These results conclusively demonstrate that miR-26a regulates high glucose-stimulated activation of mTORC1. Consistent with the requirement of this

kinase for mesangial cell hypertrophy our results demonstrate that miR-26a contributes to the hypertrophy of mesangial cells (Fig. 5).

Glomerular hypertrophy represents a major feature of diabetic kidney disease. In response to hyperglycemia, mesangial cells undergo hypertrophy, which is regulated by mTORC1 [5, 18]. Many miRNAs are found to be upregulated in the glomeruli of mouse models of diabetes [14]. Among these upregulated miRNAs, miR-216a and miR-217 increase Akt kinase activity by directly targeting PTEN [13]. Interestingly, miR-200 family has been shown to target Fog2, an inhibitor of PI 3 kinase [50]. Consistent with our previous report showing that increased PI 3 kinase-mediated activation of Akt kinase contributes to mesangial cell hypertrophy, elevated miR-200 in mesangial cells promoted mesangial cell hypertrophy [5, 51]. In line with these indirect modes of Akt activation, in the present report we show activation of Akt by increased miR-26a, which targets PTEN and promotes the downstream signal transduction to activate mTORC1 necessary for mesangial cell hypertrophy (Figs. 2-5).

Apart from mesangial cell hypertrophy, expression of matrix proteins is a pathologic feature of diabetic kidney disease. Many miRNAs regulate expression of fibronectin and collagens in renal cells [14]. For example, miR-377 increases fibronectin expression via targeting SOD2 in mesangial cells [52]. Similarly, increased miR-21 and miR-29c by high glucose enhanced the expression of fibronectin [15, 53]. Furthermore, collagen IV ($\alpha 1$) has been shown to be a direct target of miR-29a. High glucose inhibits the expression of miR29a, thus increases the levels of collagen IV ($\alpha 1$) [54]. On the other hand, miR-192 by directly targeting Zeb1/2 transcriptional repressors increases the expression of collagen I ($\alpha 2$) in mesangial cells [55]. Using an alternate mechanism by inhibiting Ybx1, miR-192 also increases collagen I ($\alpha 2$) expression in these cells [56]. Here we show an additional miRNA, miR-26a, regulates the expression of both fibronectin and collagen I ($\alpha 2$) in mesangial cells in response to high glucose (Fig. 6).

Our results demonstrate that increased levels of miR-26a by high glucose act as a potent mediator of the signaling circuit involving PTEN-Akt-mTORC1 to induce mesangial cell hypertrophy and matrix protein expression, two main characteristics of diabetic nephropathy (Fig. 7). Targeting miR-26a may provide an effective therapy to prevent diabetic complications of kidney.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by the NIH RO1 DK50190 and VA Research Service Merit Review 5I01BX000926 grants to GGC. GGC is a recipient of VA Senior Research Career Scientist Award. NGC AND BSK are supported by VA Merit Review grants 5I01BX000150 and 5I01BX001340, respectively.

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Highlights

- High glucose increases microRNA-26a in renal glomerular mesangial cells.
- miR-26a targets 3' UTR of PTEN mRNA to suppress its protein expression.
- High glucose-induced miR-26a enhances Akt kinase activity to increase mTORC1 activity.
- miR-26a increases mesangial cell hypertrophy and matrix protein expression in response to high glucose.
- Our study suggests miR-26a as a therapeutic target for kidney disease in diabetes.

Figure 1.

High glucose-stimulated miR-26a in glomerular mesangial cells target PTEN. (A) Incomplete sequence complementarity between miR-26a and its target sites in the 3' UTR of human (hsa), mouse (mmu) and rat (rno) PTEN mRNAs. Glomerular mesangial cells (B and C), proximal tubular epithelial cells (D and E) and podocytes (F and G) were incubated with 25 mM glucose (HG) for 24 hours. 5 mM glucose plus 20 mM mannitol was used as control (NG). Total RNAs from these cells were used in real time qRT-PCR to detect the pre-miR-26a (panels B, D and F) and mature miR-26a (panels C, E and G) as described in the

materials and methods. N = 6 for both panels. *p = 0.002 vs NG for panels B - G. (H and I) Mesangial cells were transfected with PTEN 3' UTR-Luc reporter plasmid along with GFP-tagged miR-26a expression vector (panel H) or anti-miR-26a (panel I). Transfected cells were incubated with 25 mM glucose (HG) or 5 mM glucose plus 20 mM mannitol (NG) for 24 hours. The cell lysates were used to measure luciferase activity as described in the materials and methods. N = 6 for both panels. *p < 0.001 vs NG; **p < 0.001 vs HG in panel I. The bottom panel in panel H shows the expression of GFP mRNA in parallel samples. For panel I, the inhibition of miR-26a by the anti-miR is shown in Supplementary Fig. S2.

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Figure 2. miR-26a regulates PTEN-mediated Akt activation in response to high glucose. (A – C) Glomerular mesangial cells were transfected with GFP-tagged miR-26a expression vector. The cells were incubated with high glucose (HG) for 24 hours. The cell lysates were immunoblotted with PTEN (panel A), phospho-Akt (Thr-308), phospho-Akt (Ser-473) (panel B) and phospho-GSK3β (Ser-9) (panel C) and indicated antibodies. The bottom parts show expression of GFP mRNA in parallel samples. Quantification of the protein bands for these panels are shown in Supplementary Fig. S3A – S3C. (D – F) Glomerular mesangial cells were transfected with anti-miR-26a or scramble RNA. The cells were incubated with

high glucose (HG) as described above and the cell lysates were immunoblotted with indicated antibodies. For these panels, quantification of the protein bands and the inhibition of miR-26a expression by anti-miR transfection are shown in Supplementary Fig. S3D – S3I. (G) Mesangial cells were incubated with MK2206 for 1 hour prior to incubation with high glucose (HG). The cell lysates were immunoblotted with indicated antibodies. The quantification of the protein bands is shown in Supplementary Fig. S3J.

Figure 3. miR-26a regulates high glucose-stimulated phosphorylation of tuberin and PRAS40. Mesangial cells were transfected with miR-26a expression vector (panels A and B) or anti-miR-26a (panels C and D). The cells were incubated with high glucose (HG). The cell lysates were immunoblotted with indicated antibodies. Expression of GFP mRNA in the parallel samples of panels A and B is shown in the bottom parts of A and B. Quantification of panels A, B, C and D are shown in Supplementary Figs. S4A - S4C and S4E, respectively. Expression of miR-26a for the panels C and D is shown in Supplementary Fig. S4D and S4F.

Figure 4. miR-26a controls high glucose-induced mTORC1 activity. Rat mesangial cells were transfected with miR-26a expression vector (A and B) or anti-miR-26a (C – E) or treated with S6 kinase inhibitor PF4708671 (SKI) as indicated. Phosphorylation of S6 kinase (Thr-389), 4EBP-1 (Thr-37/46) [activation of mTORC1] and phosphorylation of s6 (Ser240/244) [activation of S6 kinase] were tested by immunoblotting with indicated antibodies. Bottom parts in panel A and B show expression of GFP mRNA as surrogate of miR-26a expression. Quantification of Figs. 4A, 4B, 4C, 4D, 4E and 4F are shown in Supplementary Figs. S5A, S5B, S5C, S5E, S5G and S5I respectively. miR-26a expression for panels C – F is shown in parallel samples in Supplementary Fig S5D, S5F, S5H and S5J, respectively.

Figure 5. miR-26a regulates high glucose-induced protein synthesis and hypertrophy of mesangial cells. The cells were transfected with miR-26a expression vector (A, C and E) or anti-miR-26a (B, D and F). The cells were incubated with high glucose. ³⁵S-methionine incorporation was measured as described in materials and methods (A and B). Mesangial cell hypertrophy was determined as ratio of protein content to cell number as described in the materials and methods (C and D). Mean $\pm$ SE of triplicate measurements is shown. * $p < 0.01$ and 0.05 vs control in panels A and C, respectively. In panels B and D, * $p < 0.001$ vs

control; $**p < 0.001$ vs HG in panel B; $**p < 0.01$ vs HG in panel D. In panels E and F, the mesangial cell size was determined by flow cytometry as described in the materials and methods. [21] Bottom panels in A and C show expression of GFP mRNA as surrogate of miR-26a expression. For panel E the GFP expression is shown in Supplementary Fig. S6C. For panels B, D and F, the miR-26a expression is shown in Supplementary Fig. S6A, S6B and S6D.

Figure 6. miR-26a regulates fibronectin and collagen I ($\alpha 2$) expression in response to high glucose. Mesangial cells were transfected with GFP-tagged miR-26a expression vector (panels A and B) or anti-miR-26a (panels C and D). The cells were incubated with high glucose for 24 hours. The cell lysates were immunoblotted with fibronectin, collagen I ($\alpha 2$) and actin antibodies. The bottom parts in panels A and B show expression of GFP mRNA in parallel samples. Quantification of the protein bands for these panels are shown in Supplementary Figs. S7A, S7B, S7C and S7E respectively. The expression of miR-26a for panel C and D is shown in parallel samples in Supplementary Fig. S7D and S7F.

Figure 7. Schematic showing the results described in this paper. High glucose increases the expression of miR-26a to inhibit PTEN levels, resulting in Akt kinase-dependent mTORC1 activation to induce mesangial cell hypertrophy and matrix protein expression.