microRNA-29b prevents renal fibrosis by attenuating renal tubular epithelial cell–mesenchymal transition through targeting the PI3K/AKT pathway

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Received: 26 May 2020 / Accepted: 6 March 2021 / Published online: 19 March 2021
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Abstract
Purpose This study aimed to investigate the effects of miR-29b on renal interstitial fibrosis in the obstructed kidney of mouse with unilateral ureteral obstruction (UUO) via inhibiting phosphatidylinositol 3-kinase/protein kinaseB (PI3K/AKT) signaling pathway.

Methods Adult male CD-1 mice were intraperitoneally injected with vehicle or PI3K inhibitor LY294002 (3 mg/kg, 30 mg/kg) daily for 1 or 2 weeks after performing UUO or sham operation. The mice were sacrificed on days 7 and 14 after surgery. The rat proximal tubular epithelial cell (TEC) line NRK-52E was cultured in DMEM and treated with various concentrations angiotensin II (AngII). Obstructed and sham mouse kidneys were analyzed via HE, Masson and immunohistochemistry to assess the degree of renal fibrosis. Real-time quantitative polymerase chain reaction assays (RT-PCR) were performed to investigate changes in the levels of expression of miR-29b and Western blot was used to analyze the activation of PI3K/AKT signaling and expression of E-cadherin, α-smooth muscle actin (α-SMA).

Results Histologic analyses of obstructed kidney revealed that LY294002 attenuated the degree of renal fibrosis. In this study, loss of miR-29b accompanied with increased epithelial–mesenchymal transition (EMT) was observed in renal tubules of mice after UUO and cultured NRK-52E cells exposed to AngII. LY294002 also prominently decreased phosphorylation of AKT in vivo and vitro. By RT-PCR and Western blot analysis, LY294002 blocked the PI3K/AKT-induced loss of E-cadherin expression and de novo increase of the expression of α-SMA in a time- and dose-dependent manner. The overexpression of miR-29b markedly reversed the phenotype induced by AngII in NRK-52E cells and the downregulation miR-29b expression with an miR-29b inhibitor resulted in enhanced EMT. In addition, the PI3K/AKT signaling pathway was found to be suppressed in the presence of overexpression of miR-29b by direct hybridization with 3’-untranslated region (3’-UTR) of PIK3R2.

Conclusion Our findings suggested that miR-29b significantly prevented tubulointerstitial injury in mouse model of UUO by attenuating renal tubular epithelial cell–mesenchymal transition via repressing PI3K/AKT signaling pathway.

Keywords Unilateral ureteral obstruction · Angiotensin-II · NRK-52E cells · MicroRNA-29b · Phosphatidylinositol 3-kinase/protein kinase B · Epithelial–mesenchymal transition

Introduction
Renal tubulointerstitial fibrosis is a morphological hallmark of progressive chronic kidney disease (CKD), and is closely related to the loss of renal function [1, 2]. The major effector cells in the disease process are activated mesangial cells and fibroblasts, as well as myofibroblasts [3–5]. It has been confirmed that a considerable proportion of interstitial myofibroblasts are actually derived from tubular epithelial cells of the affected kidneys via epithelial–mesenchymal transition (EMT) [6–8]. Simply put, the EMT process constitues epithelial cells losing cell–cell contacts, apical–basal
polarity and epithelial-specific junctional proteins such as E-cadherin and finally transdifferentiating into a mesenchymal cell phenotype involving vimentin, α-SMA, and type I collagen expression [9].

A number of studies have shown that miRNAs play important roles in a variety of biological processes including EMT [10]. miRNAs are short, approximately 22 nucleotide endogenous small noncoding RNAs that silence gene expression through hybridization to the 3′-UTRs of mRNAs to posttranscriptionally regulate gene expression [11, 12]. The miR-29 family consists of three members, which are encoded by two different genomic loci in both human and rodent genomes. They all bind to the same set of target genes, because the members have the same binding seed sequence [13]. Smad3 knockout mice, in which renal fibrosis was reduced, had significantly increased expression of miR-29 [14]. However, the biological roles of miR-29b in renal fibrosis are poorly understood.

The PI3K/AKT pathway of mammals plays diverse roles in cell growth, proliferation, survival, and metabolism [15]. Activated PI3K generates several phosphoinositides, leading to AKT activation through phosphorylation at Thr308 and Ser473 by phosphoinositide-dependent kinase-1 [16]. In the present study, we investigated the role of PI3K/AKT signaling activation and inhibition in EMT in renal fibrosis progression.

The purpose of this study was to investigate the ability of miR-29b to prevent the pathogenesis of tubulointerstitial fibrosis in the obstructed kidneys of mice with UUO by repressing PI3K/AKT signaling pathway.

Materials and methods

Animals

Adult male CD-1 mice (30–35 g) were acquired from Charles River Laboratories, Vitalriver, Beijing, China. After 1 week of acclimation, mice were anesthetized with chloral hydrate (400 mg/kg body weight, intraperitoneally). The left ureter was then obstructed via double-ligation with 4–0 silk as described previously [17]. Sham-operated mice underwent identical surgical procedures, except for ureteral ligation, and were used as controls. In both experimental sets, the mice were randomly divided into the same five subgroups: (1) Sham-vehicle; (2) UUO-vehicle; (3) Sham 30 mg/kg/day LY294002; (4) UUO 3 mg/kg/day LY294002; and (5) UUO 30 mg/kg/day LY294002 (N = 6, each subgroup). All mice were sacrificed by cervical dislocation after anesthetized with 400 mg/kg of chloral hydras on day 7 or day 14. We checked the mice, the stopped heartbeat and dilated pupil showing that the mice were died and then, the kidneys were harvested.

Cell culture, treatment and transfection

NRK-52E cells, a rat renal tubular epithelial cell line, were obtained from American Type Culture Collection (CRL-1571; Manassas, VA, USA). Routine culture of NRK-52E cells was performed in Dulbecco’s modified Eagle’s medium (DMEM) (low glucose) supplemented with 5% fetal bovine serum (FBS) (both from Gibco-BRL, Gaithersburg, MD, USA) and grown at 37 °C with a 95% air, 5% CO2 atmosphere in a CO2 incubator. They were then treated with Ang II (100 nmol/l) for 0, 12, 24 or 48 h. Another batch of cells was stimulated with Ang II at different concentrations (0, 10, 100 and 1,000 nmol/l) for 24 h. The LY294002 group was treated with LY294002 at the concentrations of 100, 500 and 1,000 nmol/l for 60 min. Control cells were incubated for equivalent periods under the same conditions. Cells were transfected with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s instructions. The cells were divided into five groups: the blank control group (no transfection), the upregulation group (transfection with miR-29 mimics), the mimic control group (transfection with miRNA synthesized randomly), the down-regulation group (transfection with miR-29b inhibitor), and the inhibitor control group (transfection with miRNA synthesized randomly). After transfection, all groups were incubated with 100 nmol/l Ang II for 24 h and then harvested for use in relevant experiments.

Morphometric and immunohistochemical analyses

Samples were sectioned transversely with a thickness of 4 µm, deparaffinized in xylene, and rehydrated through step-wise decreasing ethanol gradients. Samples were stained with hematoxylin–eosin (HE) and Masson’s trichrome. The severity of the renal fibrosis was determined by the counting of Masson’s trichrome-positive areas. Blue granular and linear deposits were considered positive for collagen staining.

For immunohistochemistry of paraffin-embedded tissue, deparaffinized and rehydrated sections were subjected to microwave-based antigen retrieval, followed by quenching in 1% hydrogen peroxide solution for 15 min. After blocking with 10% goat serum blocking buffer for 1 h and separately staining overnight with antibodies against E-cadherin (1/200; Cell Signaling) and α-SMA (1/100; Maixin Bio, Fujian, China), the sections were further incubated with peroxidase-conjugated secondary antibodies and DAB, and then counterstained with hematoxylin. Images were acquired by light microscopy (400×).
RT-qPCR analysis of miR-29b, α-SMA and E-cadherin expression

Total RNA, including miRNA, was extracted from kidney tissues and cultured cells using TRIzol solution (Invitrogen) in accordance with the manufacturer’s protocol. Total RNA was reverse transcribed into cDNA with an M-MLV kit (Invitrogen, CA, USA). Primers for miR-29b, α-SMA and E-cadherin, which were designed using Primer Premier (v5.0) and were based on the relevant sequences deposited in GenBank (Table 1). RT-PCR reaction was performed using the ABI 7500 Fast RealTime PCR System (Applied Biosystems, Bedford, MA, USA) to determine the relative quantification of RNA according to the manufacturer’s protocol. The relative expression levels were calculated according to $2^{-\Delta \Delta C_t}$, with U6 as the internal control for miRNA and GAPDH as the endogenous control for mRNA [18].

Western blot analysis

The kidney tissues and cells in each group were lysed, and the total protein was extracted. Equal amounts of protein (40 μg) were electrophoresed on 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to PVDF, blocked and then incubated with the following primary antibodies against the following antigens at 4 °C overnight: PI3K, p-PI3K, AKT, p-AKT, E-cad, α-SMA (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (Bioworld Technology, Inc., Atlanta, GA, USA). The filter membrane was washed with PBS buffer three times and incubated with a horseradish peroxidase (HRP)–labeled secondary antibody for 1 h at room temperature. With GAPDH expression as a reference, the relative protein expression was described as the ratio of the staining intensity of the target band to the staining intensity of the reference band.

Dual-luciferase reporter activity assay

The targeting relationship between miR-29b and PI3K was predicted by miRanda (www.micronRNA.org) and validated by the dual luciferase reporter assay. The wild-type or mutant-type seed region of PIK3R2 (PI3KR2, PI3K regulatory subunit 2, also known as p85-β) was synthesized and cloned into the pMIR-REPORT luciferase vector (Ambion, Inc., Grand Island, NY, USA). NRK-52E cells at the logarithmic growth phase were inoculated into 96-well plates. When the cell density reached 70%, either the miR-29b mimic or control was cotransfected with the constructed wild-type or mutant luciferase reporter vector into NRK-52E cells using Lipofectamine 2000 (Invitrogen). After 6 h of culture, the cells were cultured in new medium containing 10% fetal bovine serum (FBS) for 48 h. Luciferase activity was detected by the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer’s protocol.

Statistical analysis

All experiments were performed in triplicate, and continuous variables are presented as the means ± SEM. The data were analyzed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA). All data were tested for significant differences by one-way analysis of variance followed by Fisher’s Least Significant Difference (LSD) test. Differences were considered statistically significant at a P value less than 0.05.

Results

LY294002 attenuated the histological changes in the obstructed kidney

The obstructed kidney was characterized by severe tubular dilatation, tubular atrophy, and widened interstitial space, with a large number of interstitial cells and infiltrating leukocytes (Fig. 1b, f). The use of LY294002 (30 mg/kg/d) significantly improved renal tubular interstitial injury after UUO (Fig. 1d, h). However, the effect of 3 mg/kg/day LY294002 was not prominent (Fig. 1c, g). In contrast, there were no changes in the sham-operated and contralateral kidney, regardless of the use of LY294002 (Fig. 1a, e).

The positive staining for α-SMA and E-cadherin appeared as fine brown granularity (Fig. 1i, m). The immunohistochemistry results of α-SMA, a marker of myofibroblasts, showed that the total integral optical density (IOD) expression of α-SMA in the kidney tissues from mice having increased after UUO (Fig. 1d, h). However, the effect of 3 mg/kg/day LY294002 was not prominent (Fig. 1c, g). In contrast, there were no changes in the sham-operated and contralateral kidney, regardless of the use of LY294002 (Fig. 1a, e).

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of the epithelial marker E-cadherin showed that the total E-cadherin IOD in the kidneys of animals decreased after UUO (Fig. 2b). The E-cadherin IOD in the obstructed kidney of the UUO-30 mg/kg group was higher than that of the UUO control group on the seventh day (Fig. 2b) ($p < 0.01$).

**Effects of LY294002 on EMT molecular markers**

RT-PCR and Western blot analyses of the kidney tissue extracts also showed that α-SMA was expressed in the obstructed kidney tissue, but the correlation with obstruction time was not obvious. LY294002 treatment significantly...
inhibited the increase in α-SMA expression in a dose-dependent manner (Fig. 3a, c, f). E-cadherin levels in mice treated with LY294002 were increased, along with the concentration of LY294002 (Fig. 3b, c, g). Consistent with the Western blot results, the same tendencies were observed in the IHC assays (Fig. 2a, b). Animal experiments showed that LY294002 can ameliorate tubulointerstitial fibrosis after UUO.

To explore the effect of LY294002 on the tubular epithelial cells transformed into myofibroblasts induced by Ang II, we examined the changes in cell phenotype molecules expressed by NRK-52E cells after Ang II stimulation. By RT-PCR, the mRNA expression levels of E-cadherin, and α-SMA were significantly different between the experimental group and the control group. Ang II inhibited E-cadherin expression in a time- and dose-dependent manner. In contrast, α-SMA showed a time- and concentration-dependent increase (Fig. 4a–f). Simultaneous incubation of LY294002 with AngII obviously reduced the increase in α-SMA expression and restored the loss of E-cadherin expression, leading to significantly attenuated EMT of the cells (Fig. 4k, l). Taken together, these data clearly indicated that LY294002-treated cells showed significant repression of EMT in TECs.

**Effects of LY294002 on the PI3K/AKT signaling pathway**

Using Western blot analyses of proteins, the expression of the signaling pathway in the non-obstructed kidney was observed to be low. We found a significant increase in the protein levels of p-PI3K and p-AKT in CD-1 mice after UUO. Treatment with LY294002 (30 mg/kg/day) markedly reduced the phosphorylation of PI3K and AKT after UUO (Fig. 3d, e, h, i).

The p-PI3K, PI3K, p-AKT and AKT levels were determined in the Ang II group and control group by Western blot. AngII induced an increase in the phosphorylation rate of PI3K and AKT in NRK52E cells compared to control group (Fig. 4g, h, i, j). The ability of AngII to increase PI3K-induced phosphorylation of AKT in NRK52E cells was largely abolished by the addition of LY294002 (Fig. 4m, n).

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**Fig. 3** Expression of α-SMA, E-cad and PI3K/AKT in kidney tissue. 
(a, b) The expression of α-SMA and E-cad as assessed by RT-PCR; (e, f, g) Western blot revealed a similar change in α-SMA and E-cad protein expression; (d, h) The expression of p-PI3K as assessed by Western blot; (e, i) The expression of p-AKT as assessed by Western blot. RT-PCR and Western blot showed LY294002 attenuated the de novo expression of α-SMA on day 7 and 14 after UUO. Mice treated with LY294002 had increased levels of E-cad, along with the concentration of LY294002 (a, b, e, f, g). LY294002 reduced the phosphorylation of PI3K and AKT (d, e, h, i). (***p<0.01; ****p<0.001)
The expression of miR-29b in vivo and in vitro

The results showed that the levels of miR-29b in the UUO group were significantly reduced compared with those in the Sham group on days 7 and days 14. However, there was no statistically significant difference between the expression levels at 7 and 14 days (Fig. 5a).

In an effort to identify the involvement of miR-29b in EMT, we treated NRK-52E cells with Ang II (0, 10, 100 and 1,000 nmol/l) for 24 h. Treatment NRK-52E cells with Ang II (100 nmol/l) for 0, 12, 24 and 48 h, resulted in a reduction in E-cad expression and increase in α-SMA expression as assessed by RT-PCR (p < 0.05). Western blot revealed a similar change in α-SMA and E-cad protein expression after NRK-52E cells treated with Ang II in doses 0, 10, 100 and 1000 nmol/l for 24 h. After treatment with Ang II (100 nmol/l) for 0, 12, 24 and 48 h, α-SMA protein was significantly increased by Western blot, whereas E-cad protein expression was significantly decreased (p < 0.05).

Ang II-induced NRK-52E cells and nontreated primary NRK-52E cells control cells from the same passages. The results showed that the miR-29b levels were relatively lower in NRK-52E cells incubated with Ang II than in control cells (Fig. 5b).
Overexpression of miR-29b suppressed phenotypic genes involved in EMT and inhibited PI3K and p-AKT expression

NRK-52E cells were transfected with double-stranded RNA oligonucleotides containing the mature miR-29b sequence (miR-29b mimic), control miRNAs (con-miR-mimic), miR-29b inhibitor or con-miR-inhibitor. The results showed that miR-29b overexpression significantly reversed the increase in α-SMA, and increased the protein expression of E-cadherin (Fig. 6a, b). In contrast, low expression of miR-29b by transfection of the miR-29b inhibitor resulted in the increased expression of α-SMA and decreased expression of E-cadherin (Fig. 6a and b). Finally, to further assess the effect of miR-29b on PI3K/AKT, the levels of PI3K, and p-AKT were detected by Western blot analysis after transfection of miR-29b-3p. We found that Ang II-treated cells transfected with miR-29b mimic showed significant inhibition of the AngII-induced expression of PI3K and phosphorylation of AKT compared to con-miR-mimic-treated and untreated cells (Fig. 6c–f). Conversely, this effect was slightly reversed in the presence of the miR-29b inhibitor, indicating a particular role of miR-29b in decreasing AngII-induced AKT phosphorylation without altering total AKT levels (Fig. 6c–f).

miR-29b inhibits PI3K and AKT expression by directly binding to their 3’-UTRs

We searched for the targets of miR-29b using computational prediction analysis in several miRNA target databases, including TargetScan, Scan6.2, miRanda and miRDB. Then, we determined that the p85-β regulatory subunit of PI3K (PI3KR2, PI3K regulatory subunit 2) contains a putative binding site for miR-29b in the 3′-UTR (Fig. 6g). To confirm whether miR-29b directly binds to the 3′-UTR of PIK3R2 and causes translational suppression, we constructed pMIR reporter plasmids encoding a firefly luciferase transcript with either the wild-type or mutant 3′-UTR of PIK3R2 (wt-PIK3R2 and mut-PIK3R2). We tested their respective luciferase reporter activities in NRK-52E cells after cotransfection with miR-29b mimic or con-miR-mimic. The results indicated that the miR-29b mimic suppressed the luciferase activity of the reporter containing the wild-type 3′-UTR of PIK3R2 (Fig. 6h), suggesting that PIK3R2 is the target of miR-29b.

Discussion

The crucial roles of miRNAs in regulating human physiologic and pathologic processes have recently been recognized [19]. Although evidence implying the potential for miRNAs as biomarkers and novel targets for treatment in kidney disease has been reported [20–23], the effects of known renal protection treatments on miRNA expression, or their potential role as protective agents against renal fibrosis, has yet to be fully confirmed [24]. Overexpression of miR-29 suppresses and inhibition of miR-29 promotes the expression of fibrotic markers in mouse embryonic fibroblasts and TECs, suggesting an antifibrotic effect of miR-29 [25, 26]. The results of our present study indicated that compared with that in normal kidney tissue, the expression level of miR-29b in mouse renal fibrotic tissue was reduced. Compared with those in quiescent cells, miR-29b levels in NRK-52E cells treated with AngII were similarly downregulated. These results indicate that miR-29b may play a role in renal fibrogenesis and that its inhibition may be involved in EMT in renal tubular epithelial cells.

Therefore, the antifibrotic effect of miR-29b in vivo is at least partly due to attenuated EMT of renal tubular epithelial cells, decreased collagen production and increased matrix degradation, thereby preventing the development of fibrosis. To clarify the role of endogenous transactivation of miR-29b and to understand the functional consequences and molecular basis in renal fibrosis, we investigated its direct regulation of NRK-52E biology, a principal mechanism implicated in the antifibrotic effect of miR-29b. NRK-52E cells were
transfected with miR-29b mimics or miR-29b inhibitor and treated with Ang II for 24 h following transfection. Western blot analysis showed that miR-29b overexpression down-regulated the PI3K/AKT signaling pathway and inhibited AngII-induced α-SMA up-regulation and E-cadherin down-regulation, which is consistent with the experimental results in mice. Conversely, we inhibited of miR-29b by transfecting NRK-52E cells with a miR-29b inhibitor. These results indicate that miR-29b negatively regulates fibrosis by targeting EMT in tubular epithelial cells.

After confirming that miR-29b is an important antifibrotic through inhibition of EMT, we explored downstream effector molecules that may be involved in its function. In this study, it was shown that AKT phosphorylation increased in the fibrotic kidney by day 7 and that the expression of p-AKT continued to increase until day 14. This apparent and sustained increase in the level of p-AKT indicates that this protein plays an important role in regulating the activation of renal interstitial fibroblasts and the progression of renal fibrosis after UUO. The data also clearly indicated that administration of LY294002 partially decreased UUO injury-induced AKT phosphorylation and renal fibrosis. In addition, treatment of NRK-52E cells with Ang II resulted in the obvious upregulation of the PI3K/AKT signaling pathway, as reported by Yano et al. [27], who showed that Ang II can activate the PI3K/AKT signaling pathway by binding to AT1R. The PI3K/AKT signaling pathway plays pivotal roles in the occurrence and development of renal fibrosis by regulating the degradation of extracellular matrix and EMT of renal tubular
epithelial cells. It is worth noting that a single miRNA can simultaneously regulate multiple target genes. Among the predicted miRNA target genes, we first revealed that PIK3R2 acts as a key effector of miR-29b in renal fibrosis. Through luciferase activity detection, we confirmed the direct interaction of miR-29b with the 3′-UTR of PIK3R2 and the resulting negative regulation PIK3R2 in NRK-52E cells. In addition, miR-29b significantly reduced PIK3R2 protein expression in NRK-52E cells, suggesting that miR-29b has translational inhibitory effects on PIK3R2. PIK3R2 is a regulatory subunit of phosphatidylinositol 3-kinase (PI3K). Activated PI3K causes activation of its key downstream kinase AKT, which induces EMT in renal tubular epithelial cells. These results indicated a possible mechanism by which miR-29b negatively regulates the PI3K/AKT signaling pathway to prevent renal fibrosis through direct interaction with PIK3R2.

Mammalian target of rapamycin (mTOR), which is the downstream to PI3K/AKT, is an important serine/threonine kinase. At present, mTOR inhibitors are clinical used in immunosuppression and cancer. In recent years, more and more studies have found that mTOR signal pathway is closely related to EMT. Rapamycin, a specific mTOR signaling inhibitor, exerts an anti-fibrosis effect in many tissue fibrosis diseases [28].

In conclusion, regardless of the nature of the initial renal injury, renal interstitial fibrosis is the common, terminal pathway leading to end-stage renal failure. Our data provide novel evidence that TECs undergoing EMT play crucial roles in the progression of tubulointerstitial fibrosis in CKD. miR-29b prevents renal fibrosis by attenuating EMT of renal tubular epithelial cells through targeting the PI3K/AKT pathway. There are many articles related to renal fibrosis conducted in human renal tubule cells, although our research carried out in mice, which is interspecific different from human research. These results of our study suggest that specific PI3K/AKT signal inhibitors or increasing miR-29b levels through gene therapy may be helpful in the treatment of human renal interstitial fibrosis in the future.

Funding  This work was supported by the Joint Fund of Health Commission of Hubei Province (Grant no. WJ2019H024).

Declarations

Conflict interest  None.

Ethical approval  All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals published by the NIH. Mice were housed under Specific pathogen-free (SPF) conditions in the Experimental Animal Center of Wuhan University.

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