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Microvesicles containing microRNA-21 secreted by proximal tubular epithelial cells are involved in renal interstitial fibrosis by activating AKT pathway

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Abstract. – OBJECTIVE: To investigate the role of microvesicles containing microRNA-21 in renal interstitial fibrosis and its possible mechanism.

MATERIALS AND METHODS: The renal interstitial fibrosis model was established by unilateral ureteral obstruction, and proximal tubule epithelial cell line (NRK52E) was used for cell model. The phenotype changes of the microvesicles containing microRNA-21 secreted by tubule cells during fibrosis were detected, and possible mechanisms responsible for the process were also analyzed.

RESULTS: During the process of renal interstitial fibrosis, microRNA-21 level in the microvesicles secreted by tubule cells was increased. The microRNA-21 released from the damaged renal tubules inhibited the expression of PTEN and activated the AKT-mTOR signaling pathway, thereby exacerbating the renal interstitial fibrosis.

CONCLUSIONS: MicroRNA-21 secreted by injured proximal tubule epithelial cells participated in renal interstitial fibrosis by activating the PTEN/AKT signaling pathway.

Key Words:
microRNA-21, Microvesicles, Renal interstitial fibrosis, AKT.

Introduction

Chronic kidney disease describes the gradual loss of kidney function. Unfortunately, as the disease progresses, most of the chronic kidney diseases will progress to renal interstitial fibrosis[1,2]. The invasion of tubulointerstitial lesions from the local to the whole is an irreversible process. Also, some current therapies, such as immunosuppression, removal of anterior renal obstruction, blocking renin-angiotensin-aldosterone system, and reducing inflammation, cannot effectively reverse the process of chronic kidney injury. Therefore, it is of great urgency for researchers to investigate the underlying mechanisms in pathogenesis of renal interstitial fibrosis. Currently, the most commonly used animal model of renal interstitial fibrosis is the unilateral ureteral obstruction (UUO)[3]. The major features of UUO include obstruction time prolonged. Blood flow of obstructed vessels decreased, thus gradually inhibiting pressure growth caused by urinary tract obstruction. However, renal interstitial fibrosis still persists. We speculated that, when the external force is damaged by the renal tubule, lesions of the renal tubules also produced some reactions that aggravated further renal damage. In recent years, microvesicles as a way of cell-cell communication have been studied more frequently[4]. Microvesicles are secreted by almost all mammalian cells. These microvesicles contain proteins, lipids, and nucleic acids, which can be transmitted to target cells through ligand-receptors. Researches have shown that microvesicles were also involved in the immune escape of tumor cells[5]. Moreover, microvesicles secreted by hypoxic injured tubular epithelial cells was demonstrated to regulate activation of fibroblasts and interstitial fibrosis[6]. Another *in vivo* study[7] indicated that microvesicles in the urine from mice with polycystic kidney disease were closely related to the development of polycystic kidney disease. Therefore, we hypothesized that in the process of renal interstitial fibrosis, the microvesicles secreted by epithelial cells of the renal tubules participated in the damage of the renal tissues, and were involved in the development of renal interstitial fibrosis.

MicroRNAs are a class of endogenous non-coding small RNAs[8]. MicroRNAs are widely found

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in eukaryotes and regulate about 60% of human genes[9,10], which determine the fate of various cellular process. Studies[11,12] have shown that the decreased expression of miRNA-200 family was associated with tubular epithelial cell trans-differentiation. Therefore, miRNA may play a regulatory role in renal development and renal homeostasis disease. In this paper, mouse renal interstitial fibrosis model was established by UUO. TGF-β1-induced proximal tubular epithelial cell phenotype changes were studied to explore whether the injured tubular transmitted information to other cells through the microvesicles. The results of our work will provide a new therapeutic target for the treatment of renal interstitial fibrosis.

Materials and Methods

Animal Model

Male CD-1 mice weighing 18-20 g were purchased from Shanghai Experimental Animal Center (Shanghai, China). Mice were maintained according to the experimental animal feeding standards. Mice were randomly divided into 4 groups (6 in each group), including sham operation group, 1-day UUO group, 3-day UUO group and 7-day UUO group. UUO surgery was performed according to a previous report[3]. Mice were sacrificed at 1, 3, 7 days after the operation. Then, kidneys and urine in obstructed renal pelvis were collected. This study was approved by the Animal Ethics Committee of Wenzhou Medical University Animal Center.

Cell Culture and Treatment

Rat proximal tubular epithelial cell line (NRK-52E) was seeded on a cell dish and incubated in an atmosphere at 5% CO_2 and 37°C, with Dulbecco's modified Eagle medium (DMEM)/F12 (FBS, 12400-024, Life Technology, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS, 16000-044, Life Technology, Gaithersburg, MD, USA), 1% penicillin-streptomycin (15140-122, Life Technology, Gaithersburg, MD, USA). When cells confluency reached 80%, the growth medium was replaced with serum-free medium for another 16h incubation, and then repeated. After that, cells were stimulated with 5 ng/ml TGFβ1 (240B, R&D System, Minneapolis, MN, USA). After TGFβ1 stimulation, cells and cell supernatants from both experimental and control groups were collected at different time points.

Extraction and Observation of Microvesicles

Microvesicles in the cell supernatant were extracted through ultracentrifugation. Briefly, the cell supernatant was collected and centrifuged at 4°C, 300 g for 5 min, 1200 g for 20 min, 10,000 g for 30 min. After ultracentrifugation, supernatant was collected and centrifuged at 4°C, 110,000 g for 60 min The precipitate was collected as microvesicles, which were resuspended with PBS. mRNAs in microvesicles were extracted with TRIzol (10296-028, Life Technology, Gaithersburg, MD, USA). And the microvesicles were observed by transmission electron microscopy following instruction[13].

RT-PCR

Real-time PCR for detecting miRNA was based on a previous study[14]. In brief, mRNAs from tissues or cells were extracted with TRIzol. cDNA library was synthesized with miScriptRT II Kit (15596-026, Life Technology, Gaithersburg, MD, USA) and stored at -20°C. cDNAs were then amplified using the miScript SYBR Green PCR Kit (28073, Qiagen, Hilden, Germany). Each sample was performed in triplicates, and the CT value was read using ABI7300. The expression of the target gene was calculated by the relative quantification method.

Western Blotting

Tissues and cell samples were processed as follows. Tissues were ground on ice and cells were treated with lysate. Samples were then centrifuged at 16,000 g for 30 min to collect the supernatant. Bicinchoninic acid (BCA) protein detection kit was utilized for detecting protein concentration. Then 4X sodium dodecyl sulphate (SDS) sample buffer and deionized water were used to adjust each sample to 10-20 ug/well. The immunoblotting method was based on the previous report3. The primary antibodies used were fibronectin (FN, cat: 3648, Sigma-Aldrich, St. Louis, MO, USA), α-smooth muscle actin antibody (α-SMA, cat: 5691, Sigma-Aldrich, St. Louis, MO, USA), E-cadherin (cat: ab1416, Abcam, Cambridge, MA, USA), actin (sc1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA), PTEN antibody (9188, Cell Signaling, Danvers, MA, USA), phosphorylated AKT (4060, Cell Signaling, Danvers, MA, USA) and AKT (4691, Cell Signaling, Danvers, MA, USA).

Immunofluorescence

Coverslip was pre-placed in a 24-well plate. Cells were washed three times with PBS at room

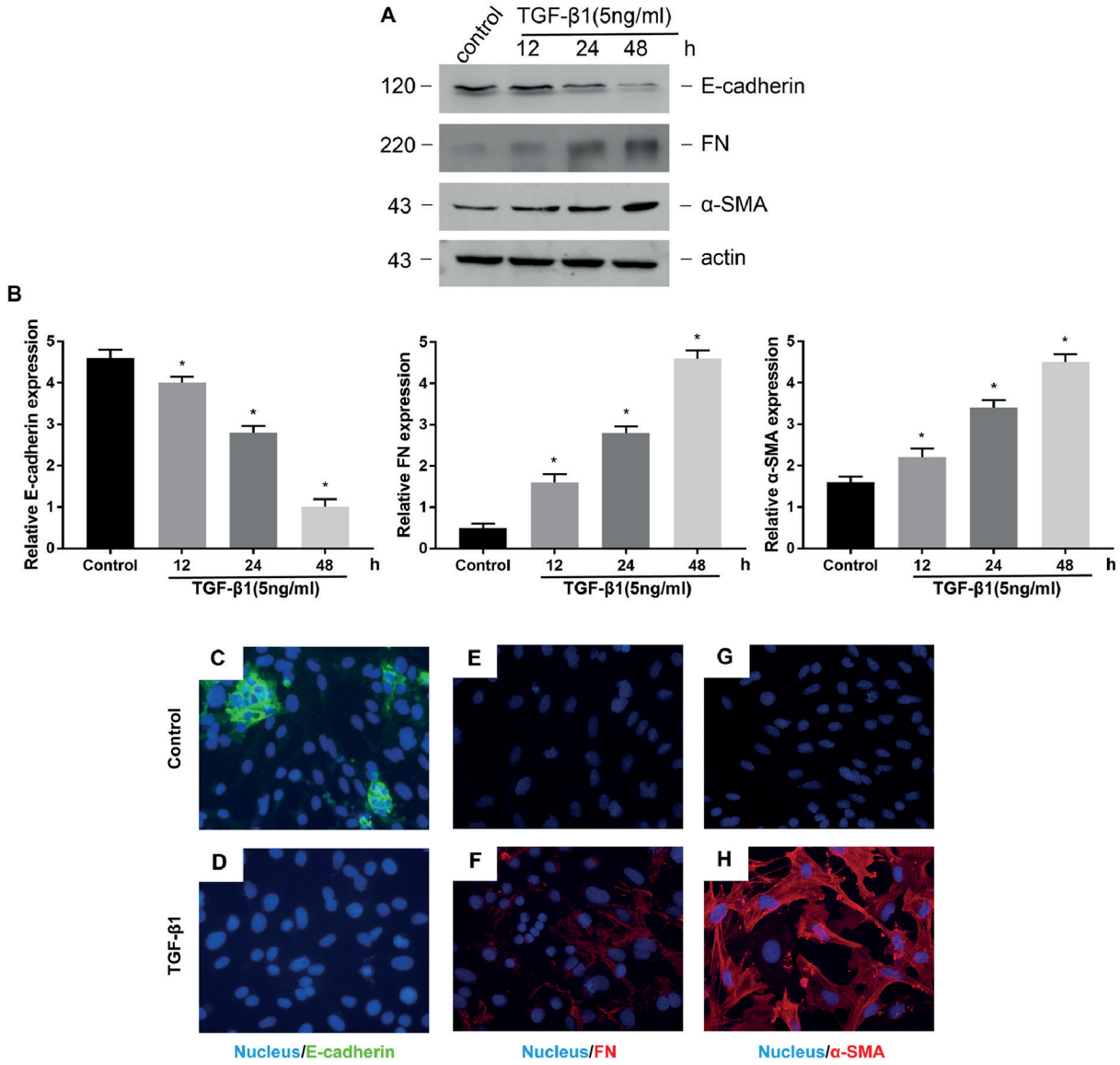

Figure 1. TGF-β1 changes the phenotype of renal tubular epithelial cells. ***(A)*** Immunoblotting results of E-cadherin, FN and α-SMA in NRK-52E cells stimulated with 5 ng/ml TGF-β1 at different time points. ***(B)*** Protein semi-quantitative results of E-cadherin, FN and α-SMA in NRK-52E cells stimulated with 5 ng/mL TGF-β1 at different time points. $^{*}p<0.05$. ***(C-H)*** Immunofluorescence results of E-cadherin, FN and α-SMA in NRK-52E cells stimulated with 5 ng/mL TGF-β1 for 48 h. ***C, D, E*** were control groups, ***F, G, H*** were experimental groups.

temperature and, then, pre-cooled with methanol/acetone (1:1) for 20 min at -20°C. Cells were then blocked by solution containing 0.1% TritonX -100 and 2% bovine serum albumin (BSA) for 40 min at room temperature. After blocking, cells were treated with the primary antibody for incubation overnight at 4°C. Cells were then incubated with the corresponding secondary antibody for 1 h at room temperature. Finally, 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. And Nikon Eclipse 80i Fluorescence Microscope (Nikon, Tokyo, Japan) was used to obtain images.

Immunohistochemistry

Paraffin-embedded kidney tissues were cut into slices with 3 mm, paraffinized and rehydrated using xylene, ethanol and purified water. Primary antibodies were used for incubation overnight, followed by incubation with secondary antibodies

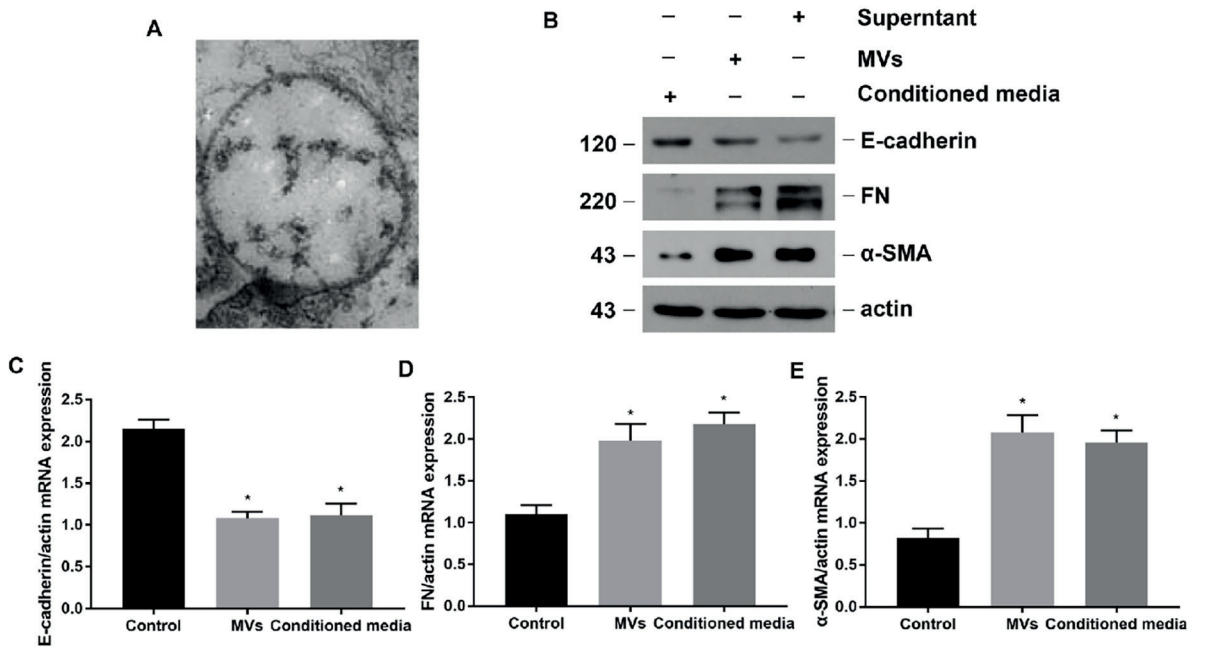

Figure 2. TGF-β1 stimulates tubular epithelial cells to secrete microvesicles and aggravates tubular cell phenotype changes. ***(A)*** Transmission electron microscopy image of cryoprecipitate obtained from culture medium after ultracentrifugation, scale bar = 100 nm. ***(B)*** Western blot results of E-cadherin, FN and α-SMA in NRK-52E cells treated with culture medium from the experimental group, supernatant in the experimental group after ultracentrifugation, and microvesicles in precipitation. ***(C-E)*** The mRNA levels of E-cadherin, FN and α-SMA in NRK-52E cells treated with culture medium from the experimental group, supernatant in the experimental group after ultracentrifugation, and microvesicles in precipitation. $^{*}p<0.05$.

for 1 h at room temperature. Then slices were sealed, and Nikon Eclipse 80i microscope was used for taking pictures.

Statistical Analysis

SPSS 20.0 (SPSS IBM, Armonk, NY USA) was used for statistical analysis. All measurement data were expressed as mean ± SEM. One-way ANOVA (with LSD or SNK as its post-hoc test) was used for comparison among multiple groups. Experimental data between the two groups were compared using Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

Results

TGF-β1 Changes Tubular Epithelial Phenotype

Tubular epithelial cells, as innate cells of the kidney, are of great importance to the physiology of the kidneys. Studies[15,16] have reported that during renal injury, reactive oxygen species and oxidative stress promoted renal tubular epithelial cell injury. Additionally, TGF-β1 had an important pathogenic effect on renal injury. Therefore, we used rat renal tubular epithelial cell line (NRK-52E) as the research object and stimulated the cells with 5 ng/mL TGF-β1 at different time points. After collecting the cells, we found that the expression of E-cadherin decreased, while expressions of SMA and FN gradually increased in a time-dependent manner (Figure 1A). Semi-quantitative analysis of the protein indicated the similar results (Figure 1B). Immunofluorescence also showed decreased E-cadherin, and increased intracellular a-SMA and extracellular FN deposition after TGF-β1 stimulation (Figure 1C-H). These data indicated that TGF-β1 can cause changes of tubular epithelial cell phenotypes.

TGF-β1 Stimulates Tubular Epithelial Cells to Secrete Microvesicles and Aggravates the Phenotype of Tubular Cells

In recent years, studies of microvesicles in chronic kidney disease have become more and more prevalent. Research has shown that renal tubular epithelial cells promoted the activation of fibroblasts and exacerbated renal interstitial

fibrosis by secreting microvesicles[6]. Therefore, we collected culture medium from TGF-β1-treated cells. After ultracentrifugation, the pellet containing microvesicles was collected and observed by electron microscopy. Results confirmed the presence of microvesicles in culture medium from the experimental group (Figure 2A). To verify whether the renal interstitial fibrosis resulted from TGF-β1-induced microvesicles, culture medium from the experimental group, supernatant in the experimental group after ultracentrifugation, and microvesicles in precipitation were used to treat NRK-52E cells, respectively. The results of mRNA measurements showed that the expression of E-cadherin decreased. The expressions of FN and α-SMA increased after treatment with 50 uL of culture medium from experimental group or microvesicles in precipitation for 48 h. However, there was no effect of the supernatant after ultracentrifugation on the expression of E-cadherin (Figure 2B). Western blot results of E-cadherin, FN, and α-SMA indicated similar findings (Figure 2C, D, E). Therefore, we believed that microvesicles produced by tubular cells with TGF-β1 induction could induce the changes of tubular epithelial phenotype.

Microvesicles Secreted by Injured Tubular Epithelial Cells Contain miRNA-21

Microvesicles produced by cells are important mediators of signal transduction[4]. However, the executor of biological process consists of substances in microvesicles, including protein, lipid, mRNA, and miRNA. In recent years, many investigation have shown that miRNAs are associated with the development of chronic kidney disease. For example, miRNA-93 was found to be involved in the development of diabetic nephropathy. Previous experiments illustrated that microRNA-21 was of great importance in chronic kidney disease. We, therefore, examined the miRNA-21 expression in microvesicles secreted during phenotype changes of renal tubular epithelial cells induced by TGF-β1. The cells were stimulated with TGF-β1 at a concentration of 5 ng/mL for different time points, and then the supernatant and cryoprecipitate were collected by ultracentrifugation to detect the miRNA-21 expression in the microvesicles in the cryoprecipitate. Results showed that miRNA-21 expression in the experimental group was significantly higher than that in the control group in a time-dependent manner (Figure 3A). Similarly, in the UUO mouse model of renal interstitial fibrosis, we collected urine in the renal pelvis of the UUO mouse, and we separated the urine supernatant and cryoprecipitate. Not surprisingly, we found that the miRNA-21 expression was significantly increased also in a time-dependent manner (Figure 3B). Similar results were observed for the relative value of miRNA-21 in urine (Figure 3C). The above data showed that during the process of renal interstitial fibrosis, miRNA21 expression in microvesicles secreted by tubule cells was significantly increased.

MiRNA-21 Activates the AKT Signaling Pathway

Our experimental data showed that during renal interstitial fibrosis, miRNA-21 expression

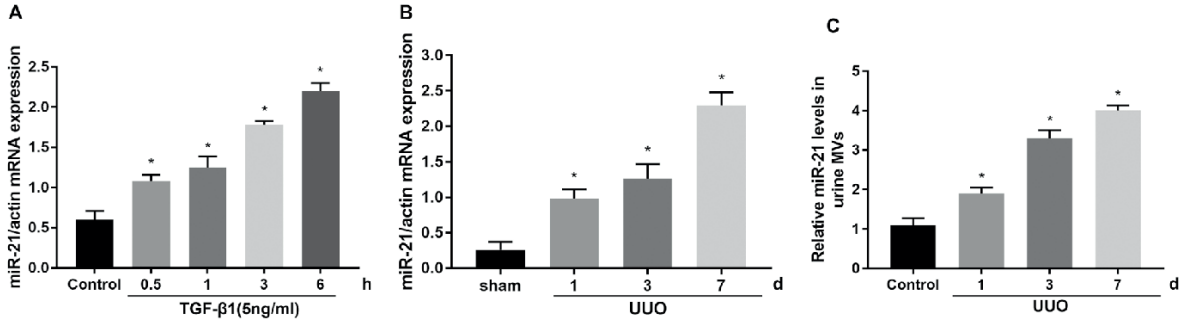

Figure 3. MicroRNAs secreted by damaged tubular epithelial cells contain miRNA21. ***(A)*** The mRNA level of miRNA in cells from each group after stimulating the cells with TGF-β1 at the concentration of 5 ng/mL for different time points, $^{*}p<0.05$. ***(B)*** The mRNA level of miRNA in the kidney of mice with different UUO operation time, $^{*}p<0.05$. ***(C)*** The relative level of miRNA21 in the urine of the renal pelvis in different UUO operation time, $*p<0.05$.

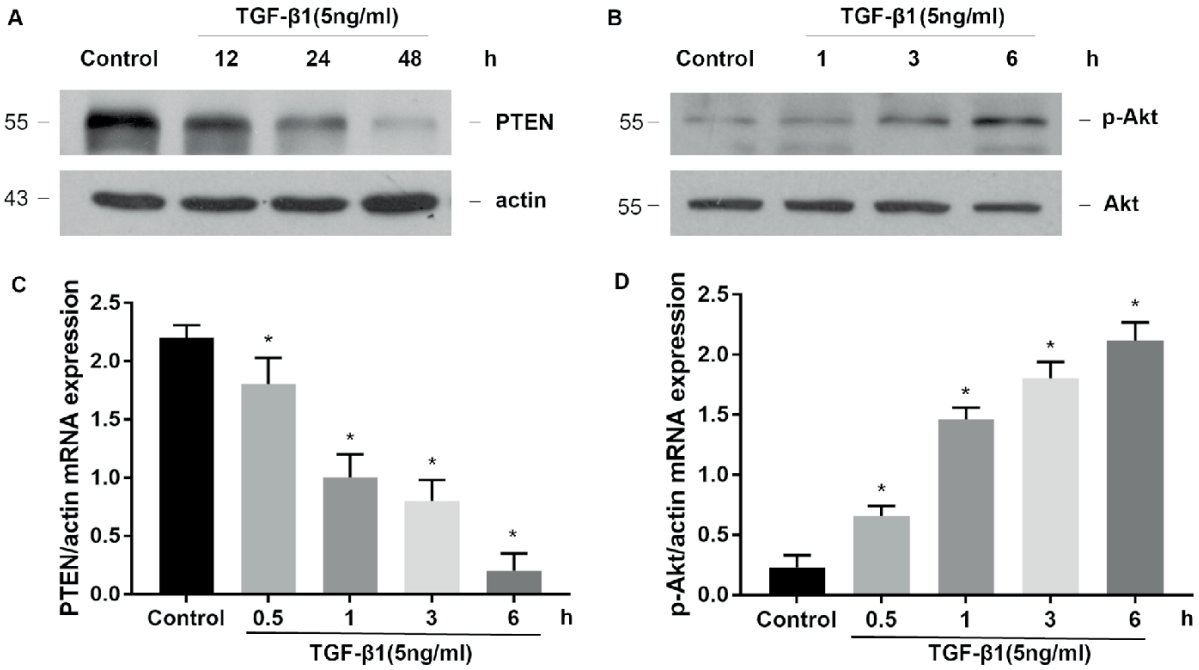

Figure 4. MicroRNA21 activates AKT signaling pathway. ***(A)*** Western blot results of PTEN in each group after 5 ng/mL TGF-β1 stimulation of NRK-52E for different time points. ***(B)*** Western blot results of p-AKT in each group after 5 ng/mL TGF-β1 stimulation of NRK-52E for different time points. ***(C)*** The mRNA level of PTEN in each group after 5 ng/ml TGF-β1 stimulation of NRK-52E for different time points, $^{*}p<0.05$. ***(D)*** The mRNA level of p-AKT in each group after 5 ng/mL TGF-β1 stimulation of NRK-52E for different time points, $^{*}p<0.05$. ***(E)*** Western blot results of PTEN and AKT in renal tissues of each UUO mouse.

was significantly increased in microvesicles secreted by tubular epithelial cells, thus aggravating renal damage. Next, we aimed to explore the mechanism of miRNA-21 in promoting renal interstitial fibrosis. Previous studies[17-19] have shown that phosphatidylserine and tensin homolog (PTEN) were the target proteins of miRNA21. AKT signaling was enhanced by dephosphorylating creatine triphosphate (PIP3) to PIP2. Therefore, we examined the effect of NRK-52E on the expression of TGF-β1 in different time points. Western blot showed that the expression of PTEN in experimental group was decreased and the expression of p-AKT was significantly increased (Figure 4A, B). Detection of mRNA expression obtained similar results (Figure 4C, D). Also, we detected renal tissues in UUO mice and found that expression of PTEN was decreased and p-AKT was increased as the surgery time increased (Figure 4E). All these data indicated that miRNA-21 was involved in renal interstitial fibrosis through AKT signaling pathway.

Discussion

According to a large number of studies, the final pathological result of chronic kidney disease is caused by various causes. This complex pathophysiological process has become a hot research topic in recent years. The pathogenesis of renal interstitial fibrosis has not been clarified yet. Tubular epithelial cells and interstitial fibroblasts, the two major innate renal cells, were demonstrated to be involved in renal interstitial fibrosis[20]. Tubules have an important role in the kidney, containing the largest number of renal tubular epithelial cells in the renal interstitium. As a consequence, during injury phenotype changes, tubular cells play an important role in the occurrence and development of chronic kidney diseases.

Tubular epithelial cells are involved in pathophysiological processes in various forms. Among them, the transmission of intercellular signal molecules is of great significance, including endocrine, paracrine, autocrine and chemical synapses. In recent years, the signal transduction

of microvesicles has been widely studied[4]. It is found that a majority of cell types produce microvesicles, which contain important cellular information and are important mediators of cell-to-cell signaling. The biological effects of microvesicles in the tumor cells have frequently been analyzed, but little was known in the study of kidney disease. Studies have shown that in acute kidney injury, microvesicles secreted by mesenchymal cells could reduce renal damage and promote repair[21]. So in our work, we further explored the effect of microvesicles on chronic kidney disease. TGF-β1-induced tubular epithelial cell transdifferentiation and UUO mouse model were constructed for the study. Our results found that in the process of renal interstitial fibrosis, microvesicles secreted by tubular epithelial cells were involved in the development of chronic kidney disease, which contained a variety of signaling molecules that may be involved in the pathogenesis. Based on previous studies, we also examined if there were any particular miRNAs in microvesicles that are involved in mediating the pathogenesis of chronic kidney disease. Notably, we found that miRNA-21 expression in microvesicles secreted by tubular cells was significantly increased, which further affected PTEN/AKT signaling pathway. This finding may provide a possible explanation for the pathogenesis of renal interstitial fibrosis.

Conclusions

During renal interstitial fibrosis, miRNA-21 expression was significantly increased in microvesicles secreted by renal tubular epithelial cells and, which was demonstrated involved in the development of chronic kidney disease by altering the PTEN/AKT signaling pathway. Our findings may provide novel therapeutic targets and research directions in clinical practice.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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