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Microvesicles containing microRNA-216a secreted by tubular epithelial cells participate in renal interstitial fibrosis through activating PTEN/AKT pathway

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Abstract. – OBJECTIVE: The aim of this study was to elucidate the role of microRNA-216a in microvesicles (MVs) during the process of renal interstitial fibrosis and to investigate its underlying mechanism.

MATERIALS AND METHODS: Unilateral ureteral occlusion (UUO) model was first established in mice, and kidney tissues and urine in the obscured kidney were collected. NRK-52E cells were induced with 5 ng/mL transforming growth factor-β1 (TGF-β1) for constructing the renal interstitial fibrosis model *in vitro*. Subsequently, the expression levels of E-cadherin, α-smooth muscle actin (α-SMA) and fibronectin (FN) in NRK-52E cells induced with or without TGF-β1 were determined, respectively. The culture medium was collected from NRK-52E cells of the control group (without TGF-β1 induction) and the TGF-β1 group (TGF-β1 induction), and MVs were observed. Afterward, NRK-52E cells were treated with MVs isolated from the control group or the TGF-β1 group, followed by detecting the expressions of E-cadherin, α-SMA and FN. Meanwhile, the expression levels of CD63, microRNA-216a, PTEN and p-AKT were determined as well. The microRNA-216 level in kidney tissues and urine of UUO mice were determined. Furthermore, the expressions of PTEN and p-AKT in mouse kidney tissues were accessed by Western blot and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RESULTS: TGF-β1 induction in NRK-52E cells gradually downregulated E-cadherin, whereas upregulated α-SMA and FN with the prolongation of induction time. MVs isolated from the culture medium of the TGF-β1 group downregulated E-cadherin, and upregulated FN and α-SMA. The expression levels of CD63 and microRNA-216a were markedly higher in the TGF-β1 group compared with the control group. Downregulated PTEN and upregulated p-AKT were observed in TGF-β1-induced cells at both mRNA and protein levels. Besides, microRNA-216a expression in mouse kidney tissues and urine from obscured kidney was remarkably increased with the prolongation of UUO. Consistent with those in NRK-52E cells, the protein level of PTEN was significantly decreased, whereas p-AKT was markedly increased with the prolongation of UUO.

CONCLUSIONS: MVs containing microRNA-216a secreted by injured proximal tubular epithelial cells participate in renal interstitial fibrosis by activating the PTEN/AKT pathway.

Key Words:
MicroRNA-216a, Microvesicle, Renal intestinal fibrosis, PTEN, AKT.

Introduction

Previous studies have shown that renal interstitial fibrosis is the final pathological result of kidney diseases[1-3]. It is an irreversible process from local pathological lesions to the whole body. However, current therapeutic methods for renal interstitial fibrosis, including inflammation control, immunosuppression, pre-renal ischemia relief and renin-angiotensin-aldosterone system blockage, can't effectively reverse the process of chronic kidney injury. Therefore, exploring possible fibrotic mechanisms through an effective fibrosis model is important for improving the therapeutic efficacy of chronic kidney diseases. Currently, the unilateral ureteral occlusion (UUO) model is the most commonly used animal model for investigating renal interstitial fibrosis[4]. In this study, we constructed a UUO mouse model *in vivo* for subsequent researches.

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The intercellular signal transmission depends on various molecular mediators, among which microvesicles (MVs) have attracted great attention in recent years[5,6]. MVs are widely used as a signal transmission method. Meanwhile, MVs are abundantly expressed in various mammalian cells. MVs transmit cellular information, such as proteins, lipids and nucleic acids to target cells in a ligand-dependent way. Studies have shown that MVs are involved in numerous pathophysiological processes, including the immune escape of tumor cells and fibroblast activation in tissue fibrosis[7,8]. Hogan et al[9] have pointed out that MVs in the urine of polycystic kidney mice are closely related to the occurrence and development of the polycystic kidney. Therefore, we speculated that MVs secreted by renal tubular epithelial cells might participate in the development of renal interstitial fibrosis under the stimulation of external damage. MicroRNAs are widely present in eukaryotes, which regulate approximately 60% of human gene expressions[10-12]. Recently, microRNAs are found to be crucial in kidney growth and disease status[13,14]. For example, microRNA-200 downregulation is related to the trans-differentiation of renal tubular epithelial cells. Another study has demonstrated that microRNA-216a is involved in the occurrence and development of diabetic nephropathy. Since PTEN is the target gene of microRNA-216a, we therefore speculated whether the PTEN/AKT pathway regulated the development of renal interstitial fibrosis.

In this work, a mouse UUO model and transforming growth factor (TGF)-β1-induced renal tubular epithelial cells were selected as *in vivo* and *in vitro* objects, respectively. We aimed to elucidate the expression changes of microRNA-216a in MVs secreted from impaired renal tubules, as well as subsequent pathological lesions after information transmission. Our study might provide a novel therapeutic target for renal interstitial fibrosis.

Materials and Methods

Experimental Animals

Male CD-1 mice with 18-20 g in weight were obtained from Model Animal Research Center of Shandong University. All mice were housed in a standard SPF environment with a room temperature of 23±2°C and relative humidity of 55±10%. Normal diet was provided. Mice were randomly assigned into 4 groups, including the sham group, the Day 1 group, the Day 3 group and the Day 7 group, with 4 mice in each group. They were sacrificed at the postoperative 1st, 3rd, and 7th day, respectively. Meanwhile, kidney tissues and urine in the obscured kidney were collected. This study was approved by the Animal Ethics Committee of Traditional Chinese Medicine Hospital of Rizhao Animal Center.

Cell Culture

Rat renal tubular epithelial cell line NRK-52E was cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained in a 5% CO_2 incubator at 37°C. When the confluence was up to 80%, the serum-free medium was replaced for overnight culture, and then was replaced again at 16 hours later. Cells were induced with 5 ng/mL TGF-β1 for the following experiments.

MVs Isolation and Observation

MVs were isolated from cell supernatant by ultracentrifugation. Briefly, the cell culture was collected and centrifuged at 4°C, 300 g/min for 5 min, 1,200 g/min for 20 min, and 10,000 g/min for 30 min sequentially. The suspension was harvested for centrifugation again at 110,000 g/min for 60 min, and the precipitate was MVs. MVs were re-suspended in phosphate-buffered saline (PBS) and observed under a transmission electron microscope.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) reagent to extract total RNA in renal tissues. Reverse transcription was performed according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). 1 μL of cDNA was collected for qRT-PCR using the SYBR Green method. CT value was recorded by ABI7300 system (Applied Biosystems, Foster City, CA, USA), and relative gene expression was calculated by the $2^{\Delta CT}$ method. Primer sequences used in this study were as follows: E-cadherin, F: 5'- CTTTAGTTTTGGGGAGGGGTT-3', R: 5'-ACTACAACTCCAAAAACCCATAACTAA-3'; microRNA-216a, F: 5'-GTGTAAACATCCGGGTCGACTG-3', R: 5'-GTCGTGGAGTCATTGCGTG-3'; FN, F: 5'-TGCTGGGACTTCCTACGTCG-3', R: 5'-CGTTTGAGTTGCCACCGTAAG-3'; α-SMA,

F: 5'-TGGGAGAACTGTGCA CGGAG-3', R: 5'-TCATTGGTAACCCGGGTGG-3'; PTEN, F: 5'-CTAATGGTGGACCGCAAC AAC-3', R: 5'-TCC ACT TCC AACCCAGGTCC-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Total protein was extracted, and the concentration of the extracted protein was determined. Subsequently, the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes. After blocking with 5% skim milk for 1 hour, the membranes were incubated with specific primary antibody overnight at 4°C. After washing with 1×Tris-Buffered Saline-and Tween 20 (TBST) 5 times, the membranes were incubated with the corresponding secondary antibody at room temperature for 2 h. After washing with 1×TBST for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Immunofluorescence

Slides were pre-placed in the 24-well plates. Cells were washed with PBS three times and fixed with pre-cooled methanol/acetone (1:1) at -20°C for 20 min. Subsequently, the cells were incubated with the blocking buffer containing 0.1% TritonX-100 and 2% bovine serum albumin (BSA) for 40 min at room temperature. Then the cells were incubated with primary and secondary antibodies. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in the dark, and the slides were captured using a Nikon Eclipse 80i microscope (Tokyo, Japan).

Immunohistochemistry

Paraffin-embedded kidney tissues were sliced into 3 μm of thickness. Kidney slices were deparaffinized, hydrated in ethyl alcohol and blocked in blocking solution for 30 min. After incubation with primary antibody at 4°C overnight and secondary antibody at room temperature for 1 h, immunohistochemistry results were captured using Nikon Eclipse 80i microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). The comparison of measurement data was conducted using *t*-test. The comparison of differences among each group was conducted using one-way ANOVA, followed by post-hoc analysis. $p<0.05$ was considered statistically significant.

Results

TGF-β1 Induced Changes in Renal Tubular Epithelial Phenotype

Reactive oxygen species, inflammatory factors and chemokines produced during the renal tissue damage process lead to oxidative stress and further aggravate the damage of renal tubular epithelial cells[15-19]. TGF-β1 induction can markedly change phenotypes of renal tubular epithelial cells. Here in this study, NRK-52E cells were induced with 5 ng/ml TGF-β1 for different time points. Western blot results elucidated that the protein expression of E-cadherin gradually downregulated, whereas α-SMA and fibronectin (FN) were upregulated in a time-dependent manner (Figure 1A). The mRNA levels of E-cadherin, α-SMA and FN showed similar trends (Figure 1B-1D). Results of immunofluorescence were consistent with qRT-PCR results. After TGF-β1 induction in NRK-52E cells, the positive expression of E-cadherin at the cell junction was significantly decreased. However, intracellular filamentous α-SMA and extracellular FN deposition were markedly increased (Figure 1E-1J). The above data indicated that the TGF-β1 induction could lead to changes in the phenotype of renal tubular epithelial cells.

TGF-β1 Stimulated MVs Secretion from Renal Tubular Epithelial Cells and Aggravated Tubular Phenotypic Changes

It has been reported that during renal injury, abundant MVs secreted from renal tubular epithelial cells promote fibroblast activation and further aggravate renal interstitial fibrosis[8]. Therefore, we collected the culture medium from the TGF-β1 group after TGF-β1 induction. After the collection of the precipitate containing MVs by ultracentrifugation, MVs were observed under a transmission electron microscope (Figure 2A). However, MVs were absent in the culture medium without TGF-β1 induction (control group). To verify whether MVs produced after TGF-β1 induction could lead to renal interstitial fibrosis by transmitting some certain substances, NRK-52E

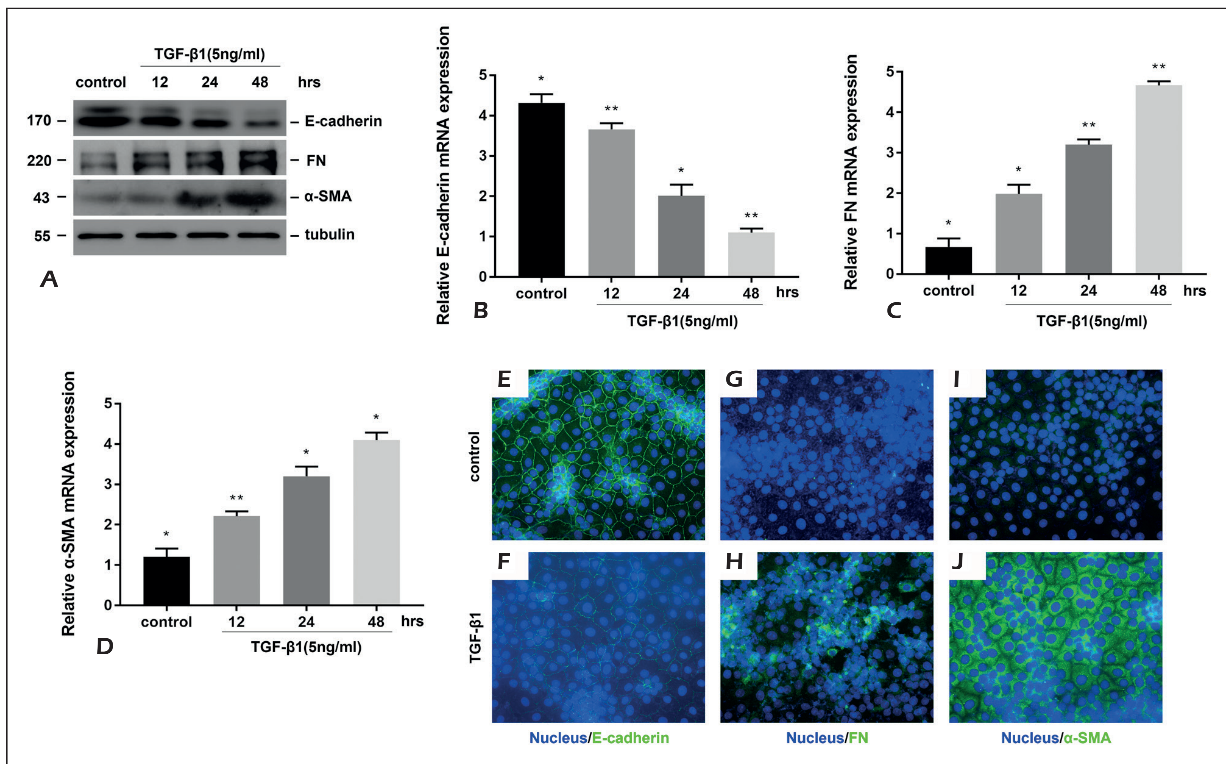

Figure 1. TGF-β1 induced changes in renal tubular epithelial phenotype. ***A,*** Protein expressions of E-cadherin, FN and α-SMA in NRK-52E cells after induction of 5 ng/mL TGF-β1 for different time points. ***B-D,*** The mRNA expressions of E-cadherin, FN and α-SMA in NRK-52E cells after induction of 5 ng/mL TGF-β1 for different time points. ***E-J,*** Results of immunofluorescence of E-cadherin, FN and α-SMA in NRK-52E cells after induction of 5 ng/mL TGF-β1 for different time points (***E-G,*** the control group; ***H-J,*** the experimental group). $*p<0.05$ was statistically significant.

cells were treated with MVs isolated from the culture medium of the TGF-β1 group or the control group for 48 h. Western blot results indicated that downregulated E-cadherin, and upregulated FN and α-SMA were observed in NRK-52E cells treated with MVs isolated from the TGF-β1 group (Figure 2B). However, no significant differences in the protein levels of E-cadherin, FN and α-SMA were found in those treated with MVs of the control group. Similar trends were identically obtained at the mRNA levels (Figure 2C-2E). Hence, we believed that TGF-β1-induced MVs produced by renal tubular cells could aggravate the changes in renal tubular epithelial cell phenotype.

MVs Secreted by Renal Tubular Epithelial Cells During Renal Interstitial Fibrosis Contained MicroRNA-216a

MVs produced by cells are important mediators for transmitting signal factors such as proteins, lipids, mRNA and miRNAs[20]. Tubular phenotype changes resulted from substances in MVs, not MVs themselves. Recent studies have confirmed the crucial interaction between microRNAs and the development of chronic kidney diseases, including microRNA-216a. Therefore, we detected the expression level of microRNA-216a in MVs during phenotypic changes in tubular epithelial cells. NRK-52E cells were induced with 5 ng/ml TGF-β1 for different time points, followed by the collection of cryoprecipitate in cell culture. As a hallmark for MVs, the protein expression of CD63 was markedly higher in the TGF-β1 group than that of the control group (Figure 3A). Consistently, the mRNA level of microRNA-216a also significantly increased in the TGF-β1 group in a dose-dependent manner (Figure 3B). Furthermore, we collected mouse urine from the obstructed side of the renal pelvis for isolation and cryoprecipitation. Results showed that microRNA-216a expression in mouse kidney tissues was remarkably increased with the prolongation of UUO (Figure 3C). Similar results were obtained in the mRNA level of microRNA-216a in mouse urine (Figure 3D). The above data indicated that microRNA-216a level in MVs secreted by tubule

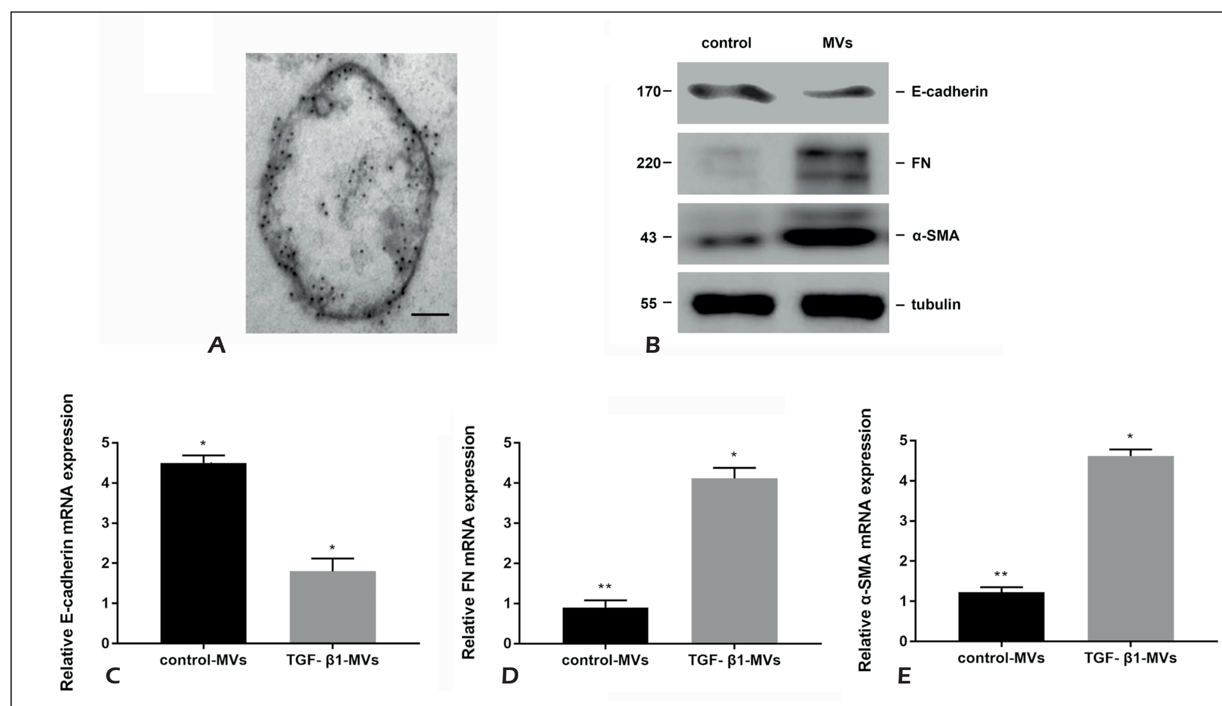

Figure 2. TGF-β1 stimulated MVs secretion from renal tubular epithelial cells and aggravated tubular phenotypic changes. ***A,*** Cryoprecipitation after ultracentrifugation of culture medium under an electron microscope (scale bar = 100 nm) (Magnification x 20000). ***B,*** Protein expressions of E-cadherin, FN and α-SMA in NRK-52E cells after treatment with MVs isolated from the TGF-β1 group or the control group. ***C-E,*** The mRNA expressions of E-cadherin, FN and α-SMA in NRK-52E cells after treatment with MVs isolated from the TGF-β1 group or the control group. $^*p<0.05$ was statistically significant.

epithelial cells was significantly increased during renal interstitial fibrosis.

MicroRNA-216a Participated in Renal Interstitial Fibrosis by Activating the PTEN/AKT Pathway

The above results revealed that microRNA-216a was highly expressed in MVs secreted by tubular epithelial cells and aggravated renal interstitial fibrosis. Subsequently, we explored the possible mechanism of microRNA-216a in promoting renal interstitial fibrosis. Through literature review, PTEN is the target gene of microRNA-216a[20-22]. The AKT pathway is enhanced by de-phosphorylation of creatine inositol triphosphate (PIP3) to generate PIP2. Therefore, we detected the expressions of PTEN and p-AKT in NRK-52E cells after TGF-β1 induction. Western blot demonstrated that downregulated PTEN and upregulated p-AKT were observed in TGF-β1-induced cells (Figure 4A and 4B). QRT-PCR obtained similar results at the mRNA levels of PTEN and p-AKT (Figure 4C and 4D). Moreover, the expressions of PTEN and p-AKT in mouse kidney tissues were determined as well. With the prolongation of UUO, the protein level of PTEN was significantly decreased, whereas p-AKT was markedly increased (Figure 4E). This indicated that microRNA-216a was involved in renal interstitial fibrosis by regulating the AKT pathway.

Discussion

The incidence of chronic kidney diseases increases year by year. As the final pathological result of chronic kidney diseases caused by various reasons, the pathogenesis of renal interstitial fibrosis is complex and remains unclear. Renal tubular epithelial cells and interstitial fibroblasts are the two most important renal cells involved in renal interstitial fibrosis[23]. Meanwhile, these two types of cells are widely studied in various *in vivo* and *in vitro* experiments. In this study, we selected renal tubular epithelial cells as experimental subjects *in vitro*.

MVs can be produced by almost all types of cells in the body. Studies have shown that MVs contain important cellular information and are

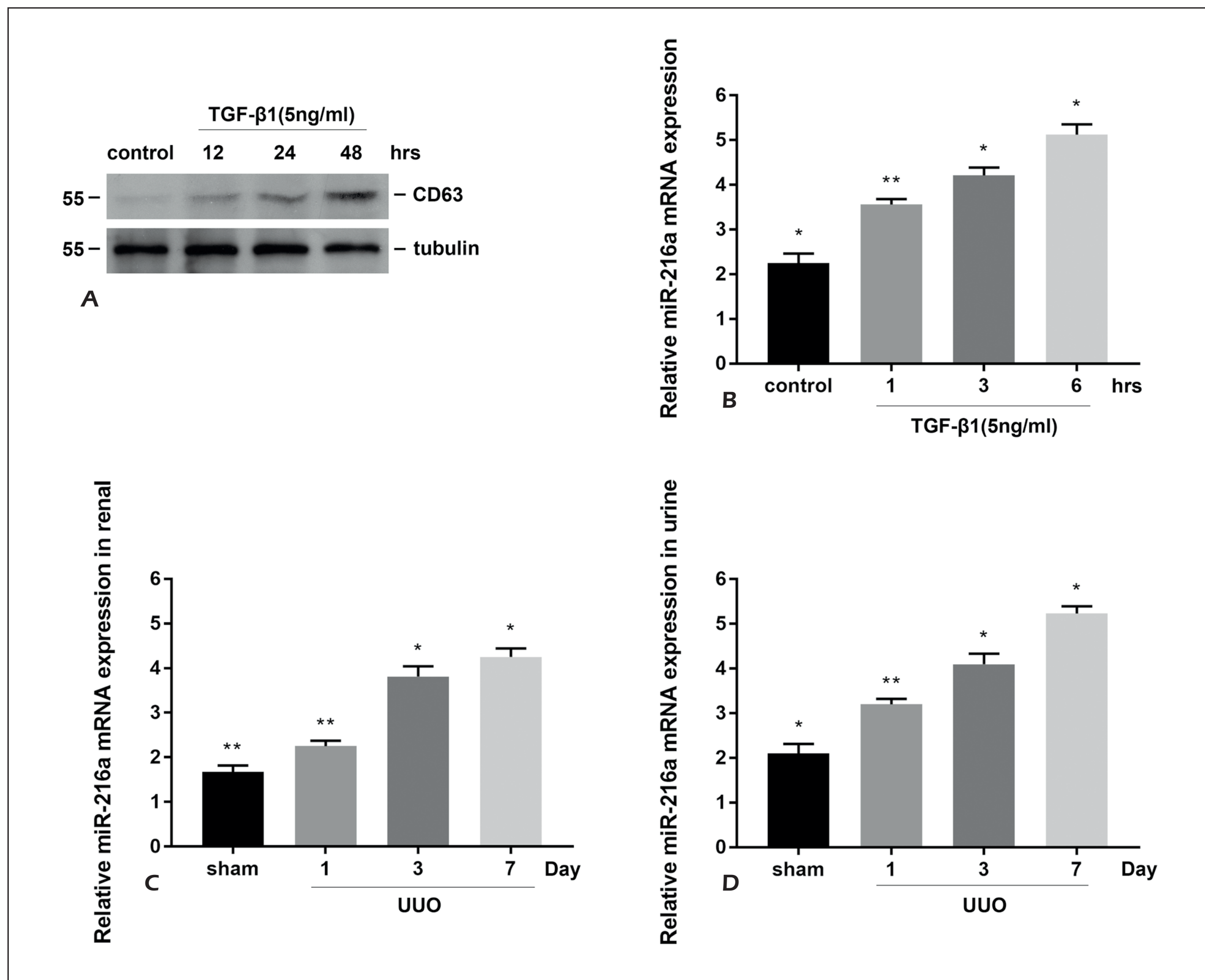

Figure 3. MVs secreted by renal tubular epithelial cells during renal interstitial fibrosis contained microRNA-216a. ***A,*** Protein expression of CD63 in NRK-52E cells after induction of 5 ng/mL TGF-β1 for different time points. ***B,*** MicroRNA-216a expression in NRK-52E cells after induction of 5 ng/mL TGF-β1 for different time points. ***C,*** MicroRNA-216a expression in kidney tissues of mice in the sham group, the Day 1 group, the Day 3 group and the Day 7 group. ***D,*** MicroRNA-216a expression in mouse urine isolated from obscured kidney in the sham group, the Day 1 group, the Day 3 group and the Day 7 group. *$p<0.05$ was statistically significant.

significant mediators of cell-cell signals. Therefore, we believed that MVs played a crucial role in the development of chronic kidney diseases. Here, we used TGF-β1 to induce trans-differentiation of renal tubular epithelial cells. Meanwhile, a UUO mouse model was used as research subjects. During renal interstitial fibrosis, renal microtubules secreted MVs increased significantly. They contained a variety of possible pathogenic factors that could be involved in the pathogenic process. Previous studies have elucidated the non-negligible effect of microRNAs on MVs. Our findings demonstrated that microRNA-216a was markedly upregulated in MVs secreted from renal tubular cells during renal interstitial fibrosis. Meanwhile, as the target gene of microRNA-216a, the expression level of PTEN was downregulated while p-AKT was upregulated. It is suggested that microRNA-216a participates in renal interstitial fibrosis by regulating the PTEN/AKT pathway, which may provide a possible explanation for the pathogenesis of renal interstitial fibrosis.

Conclusions

We observed that MVs containing microRNA-216a secreted by injured proximal tubule epithelial cells participate in renal interstitial fibrosis by activating the PTEN/AKT pathway. Our study provides novel direction and therapeutic targets for the treatment of chronic kidney diseases.

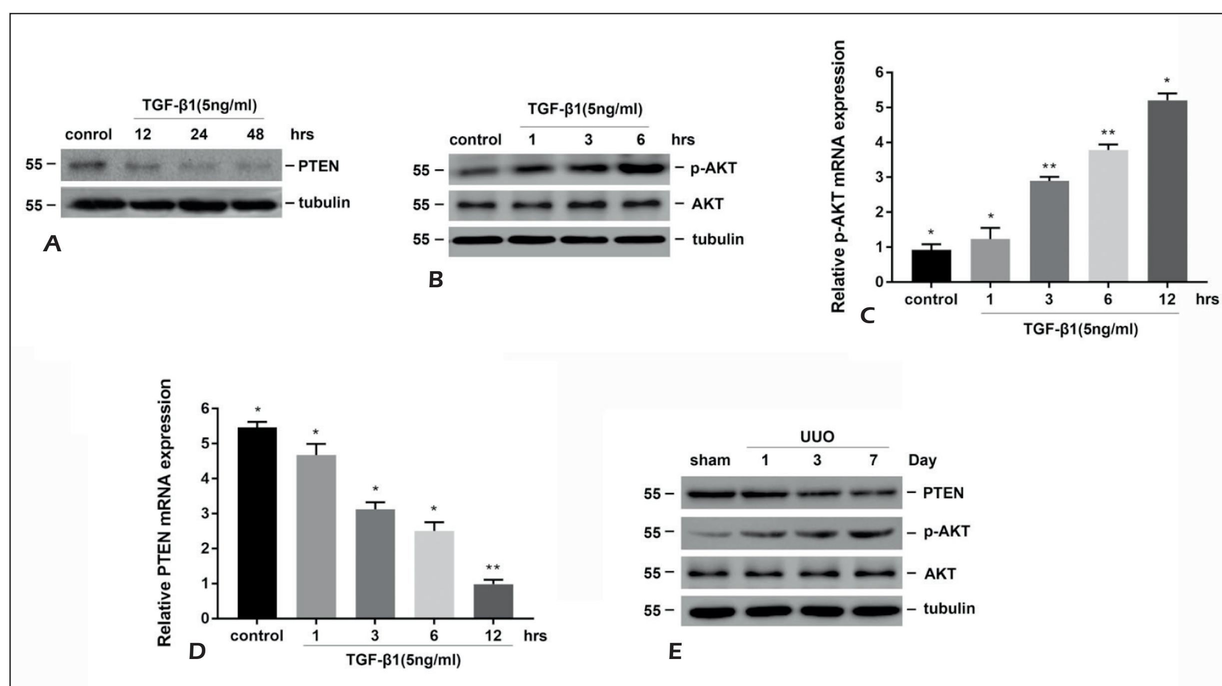

Figure 4. MicroRNA-216a participated in renal interstitial fibrosis by activating the PTEN/AKT pathway. ***A-B,*** Protein expressions of PTEN and p-AKT in NRK-52E cells after induction of 5 ng/mL TGF-β1 for different time points. ***C-D,*** The mRNA levels of PTEN and p-AKT in NRK-52E cells after induction of 5 ng/mL TGF-β1 for different time points. ***E,*** Protein expressions of PTEN and p-AKT in kidney tissues of mice in the sham group, the Day 1 group, the Day 3 group and the Day 7 group. *$p<0.05$ was statistically significant.

Conflict of Interests

The authors declared there is no conflict of interest.

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