Abstract. Function and potential mechanism of microvesicles (MVs) containing microRNA-34a in renal interstitial fibrosis were investigated. A rat model of renal interstitial fibrosis was established by unilateral ureteral ligation (UUO). Rat proximal tubular epithelial cell line (NRK-52E) was used to explore the effect of MVs containing microRNA-34a on tubular epithelial cells during fibrosis, which were secreted by tubulointerstitial fibroblasts. Regardless of the UUO renal interstitial fibrosis model, or the TGF-β1-treated renal tubular epithelial cells, microRNA-34a was increased in the MVs secreted by tubulointerstitial fibroblasts. miR-34a could be transmitted through the damaged tubule basement membrane to proximal tubular epithelial cells, where it induced apoptosis of renal tubular epithelial cells by inhibiting the expression of Bcl-2, further aggravating renal interstitial fibrosis. MicroRNA-34a secreted by damaged renal interstitial fibroblasts can promote renal tubular epithelial cell apoptosis and participate in renal interstitial fibrosis by inhibiting Bcl-2.

Introduction

The final common pathological result of chronic kidney disease (CKD) is renal interstitial fibrosis (1,2), which is characterized by tubular atrophy and accumulation of extracellular matrix (3). Apoptosis of tubular epithelial cells is one of the causes of tubular atrophy and interstitial fibrosis (4-7). Hypoxia, oxidative stress, and TGF-β1 treatment are all leading causes of apoptosis in the model of unilateral ureteral obstruction (UUO)-induced renal interstitial fibrosis (8,9), which can promote inflammatory reactions and increase synthesis of extracellular matrix, eventually leading to renal interstitial fibrosis. Factors that can reduce apoptosis such as hepatocyte growth factor (HGF) and bone morphogenetic protein-7 (BMP-7) can delay renal interstitial fibrosis (6,10,11). However, the molecular mechanism underlying apoptosis of renal tubular epithelial cells remains unknown.

MiRNAs are endogenous non-coding small RNAs that not only regulate the expression of target genes directly, but also regulate the expression of target genes in other cells through the delivery by microvesicles (MVs). In recent years, MVs have attracted wide attention as the most important link between cells (19). MiRNAs found in serum derived from tumor cell MVs have been shown to be associated with tumor metastasis and apoptosis (20-23).

In this study, UUO was used as a renal interstitial fibrosis model and TGF-β1-treatment was used as an attempt to investigate the role of MVs containing miR-34a in regulating renal tubular epithelial cell apoptosis. We found that miR-34a was mainly distributed in renal tubular interstitial cells and that its expression was significantly increased in obstructed renal tissues after obstruction. TGF-β1 treatment upregulated miR-34a expression in mesenchymal fibroblasts. MiR-34a derived from mesenchymal fibroblast MVs can be transmitted to proximal tubular epithelial cells through the damaged tubular basement membrane and induced apoptosis through inhibition of Bcl-2 expression. This study provides a new molecular mechanism for microRNA-mediated apoptosis of renal tubular epithelial cells and a new theoretical basis for the pathogenesis of renal interstitial fibrosis.
Materials and methods

**Mice.** Male CD-1 mice weighing 18-20 g were purchased from the Nanjing University Laboratory, and were raised according to the experimental animal feeding standard. The mice were randomly divided into 4 groups, which were sham-operated group, UUO groups for 1, 3, and 7 days (n=6 each group). Mice were sacrificed on 1, 3 and 7 days after operation, respectively and the kidney tissues were obtained. This study was approved by The Animal Care and Use Review Committee of Wujiang Hospital Affiliated to Nantong University (Suzhou, China).

**Cell culture and treatment.** The rat proximal tubular epithelial cells (NRK-52E) and the renal interstitial fibroblast cells (NRK-49F) were planted on a petri dish and cultured at 37°C, 5% CO2 environment. The culture medium was DMEM/F12 (12400-024), containing 10% fetal bovine serum (FBS; 16000-044) and 1% penicillin-streptomycin (15140-122) (all from Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). When cells grew to 80% confluency, the culture medium was replaced with serum-free medium for 16 h. After 16 h, the serum-free medium was replaced again, and TGFB1 (240B; R&D Systems, Inc., Minneapolis, MN, USA) was added to stimulate the cells. Cells and supernatants were collected at different times. The control group was collected in the same way.

**Microvesicle extraction.** We used ultracentrifugation to extract the MVs in the cell supernatant. Cell supernatants were centrifuged at 300 x g for 10 min at 4°C, then 1,200 x g for 30 min. The supernatant was collected, and then centrifuged at 4°C for 110,000 x g for 60 min. Finally, the precipitate was the MVs. The MVs were resuspended with appropriate phosphate-buffered saline (PBS). TRIZol reagent (10926-028; Life Technologies; Thermo Fisher Scientific, Inc.) was used to extract mRNA of MVs.

**miRNA detection by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR).** mRNA was extracted from tissues or cells using TRIZol, and the cDNA was synthesized using miScript RT II kit (15596-026; Life Technologies; Thermo Fisher Scientific, Inc.), and was stored at -20°C.

**Statistical analysis.** Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 16.1 statistical software (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard error. One-way ANOVA (post-hoc LSD or SNK) was used for comparison between multiple experimental groups. The experimental data were compared between the two groups using Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

**Increased expression of miR-34a in obstructed renal tissue and MVs.** A large amount of cell apoptosis occurred in a UUO-induced renal interstitial fibrosis model. We first detected the expression of miR-34a in renal tissue, and found that miR-34a expression began to increase on the first day after surgery, and had a time-dependent elevation (Fig. 1A). At the same time, the mRNA expression of miR-34a in renal tissue was also significantly increased after treatment; it was also the same time, the mRNA expression of miR-34a in renal tissue was also significantly increased after treatment; it was also
of miR-34a in the MVs of the obstructed kidney was significantly increased (Fig. 1C). CD63 is a marker protein of MVs. CD63 protein expression represents the content of MVs. The results showed that compared with the sham-operated renal tissue, the expression of CD63 was increased in obstructed kidney tissue (Fig. 1D), suggesting that in the UUO renal interstitial fibrosis model, the secretion of MVs in the kidney was increased. Thus, these data demonstrated that in the UUO-induced renal interstitial fibrosis model, MVs containing microRNA-34a were increased.

miR-34a is increased in TGF-β1-treated fibroblasts and aggravated renal interstitial fibrosis. To clarify the expression of miR-34a in the renal tissue after obstruction, we treated NRK-52E and NRK-49F cells with TGF-β1 to observe the expression of miR-34a. Firstly, we stimulated cells with different concentrations of TGF-β1 for 24 h and extracted MVs from cell culture fluid, then we found that miR-34a expression was significantly increased in fibroblasts compared to the control group. The cells treated with TGF-β1 at a concentration of 5 ng/ml had the highest miR-34a expression. However, miR-34a expression in tubule epithelial cells did not change significantly (Fig. 2A). We then treated both cell lines with 5 ng/ml TGF-β1 for different time-points to detect the expression of miR-34a in MVs from cell culture fluid. A similar result was found that the expression of miR-34a was significantly increased in fibroblasts compared to the control group. The cells treated with TGF-β1 at a concentration of 5 ng/ml had the highest miR-34a expression. However, miR-34a expression in tubule epithelial cells did not change significantly (Fig. 2A). We then treated both cell lines with 5 ng/ml TGF-β1 for different time-points to detect the expression of miR-34a in MVs from cell culture fluid. A similar result was found that the expression of miR-34a was significantly increased in fibroblasts and was time-dependent, whereas the expression of miR-34a was slightly increased in tubule epithelial cells (Fig. 2B). The MVs collected from the culture fluid of NRK-49F cells were treated with TGF-β1 at a concentration of 5 ng/ml, then stimulated NRK-52E cells with MVs at a concentration of 200 nmol/l for different times, then we found that the expression of α-SMA and FN were increased and were time-dependent (Fig. 2C). Similar results were found in the mRNA expression of α-SMA and FN which were increased in experimental groups (Fig. 2D and E).

miR-34a induces apoptosis of proximal tubular epithelial cells. To directly verify whether miR-34a promotes apoptosis of proximal tubular epithelial cells, we transfected miR-34a mimic and inhibitor to observe apoptosis to achieve miR-34a overexpression and knockdown. First, we tested the transfection efficiency. The results showed that transfection of miR-34a inhibitor significantly downregulated the expression of miR-34a in NRK-52E cells (Fig. 3A), while miR-34a mimic transfection significantly increased the expression of miR-34a (Fig. 3B). We used TUNEL staining to compare the apoptosis of proximal tubular epithelial cells after transfection with 80 nmol/l negative control, miR-34a mimic and miR-34a inhibitor. The results showed that the number of apoptotic cells in the mimic group was significantly increased (Fig. 3C), while the number of apoptosis in the inhibitor transfected group was decreased. The same result was obtained by counting the number of cells (Fig. 3D).

Caspase-3 is a marker of apoptosis. Therefore, we observed the effect of miR-34a on apoptosis of renal tubular epithelial cells by observing the expression of caspase-3. The results showed that when miR34a was upregulated in NRK-52E cells, the expression of caspase-3 protein was significantly increased as well, whereas after downregulating miR-34a, the expression of caspase-3 protein was decreased (Fig. 3E). The mRNA levels of caspase-3 in each group were found to be consistent with the protein results. These results suggested that miR-34a can promote renal tubular epithelial cell apoptosis.

miR-34a participates in renal interstitial fibrosis by inhibiting apoptosis of target gene Bcl-2. Bioinformatics studies have
Figure 2. miR-34a was increased in TGF-β1-treated fibroblasts and aggravated renal interstitial fibrosis. (A) The miR-34a level in MVs of cell culture fluid in each group (*P<0.05, **P<0.01). (B) The miR-34a level in MVs of cell culture fluid in each group (*P<0.05, **P<0.01). (C) The protein expression of FN, α-SMA in NRK-52E cells of each group. (D) The relative α-SMA mRNA in each group (*P<0.05, **P<0.01). (E) The relative FN mRNA in each group (*P<0.05, **P<0.01). MVs, microvesicles; FN, fibronectin.

Figure 3. miR-34a induces apoptosis of proximal tubular epithelial cells. (A) The relative miR-34a mRNA in NRK-52E cells treated with miR-34a inhibitors in each group (*P<0.05, **P<0.01). (B) The relative miR-34a mRNA in NRK-52E cells treated with miR-34a mimics in each group (*P<0.05, **P<0.01). (C) The result of TUNEL staining in NRK-52E cells. (D) The percentage of NRK-52E cells that were stained positive (*P<0.05, **P<0.01). (E) The protein expression of p-caspase-3, and caspase-3 in NRK-52E cells in each group.
shown that Bcl-2 is the target protein of miR-34a. Previous studies have confirmed that miR-34a regulates the expression of Bcl-2 in tumor cells (17,18,24). We then hypothesize whether miR-34a regulates apoptosis through Bcl-2. We found that transfection with miR-34a mimic inhibited Bcl-2 protein expression, whereas transfection with miR-34a inhibitor increased Bcl-2 protein expression (Fig. 4A). Similarly, at the gene level, Bcl-2 mRNA levels decreased when miR-34a was upregulated, whereas Bcl-2 mRNA levels increased when miR-34a was downregulated (Fig. 4B). Later, we treated NRK-49F and NRK-52E cells with TGF-β1 and found that Bcl-2 protein levels and mRNA levels were all reduced (Fig. 4C and D).
Downregulated Bcl-2 induces apoptosis of proximal tubular epithelial cells. Previously, we found that upregulation of miR-34a or TGF-β1 treatment of NRK-49F micro-vessels can inhibit the expression of Bcl-2 in proximal tubular epithelial cells and induce apoptosis. Then we transfected Bcl-2-specific plasmids to downregulate the expression of Bcl-2 in NRK-52E cells to observe whether apoptosis and fibrosis were further aggravated. We first observed the transfection efficiency of different concentrations of plasmids. We selected a concentration of 80 nmol/l. After treatment of NRK-52E 24 h, we found that downregulation of Bcl-2 increased the expression of p-caspase-3 and promoted interstitial fibrosis (Fig. 5B). Similar results were found in mRNA level analysis (Fig. 5C and D). The data show that the downregulation of Bcl-2 can promote apoptosis of proximal tubular epithelial cells and aggravate renal interstitial fibrosis.

Discussion
Renal interstitial fibrosis is a common pathological result of CKD, in which apoptosis of renal tubular epithelial cells plays an important role. However, the molecular mechanism is still not clear. Apoptosis, a programmed cell death, is a common form of cell death (25). Many studies have shown that there is abundant apoptosis of cells in various fibrotic organs, suggesting that apoptosis is likely to induce and promote the occurrence and development of organ tissue fibrosis (26). Renal interstitial fibrosis is characterized by tubular atrophy and extracellular accumulation of tubulointerstitial cells (3). Apoptosis of renal tubular epithelial cells is one of the causes of renal tubular atrophy and tubulointerstitial fibrosis (5-7). Previous studies have found evidence of apoptosis in fibrotic kidney tissue by detecting changes in cellular DNA, mitochondria, cell membranes, and cell morphology.

Recent studies have found that miR-34s can regulate tumor cell apoptosis. Among them, the transcriptional expression of miR-34a alone is most widely distributed in various tissues. Bcl-2 is an important anti-apoptosis gene in caspase cell apoptosis pathway. Studies have shown that Bcl-2 expression was inhibited after miR-34s transfection into colon cancer cells. After silencing of miR-34 expression, the expression of Bcl-2 was increased and the anti-apoptotic ability of cells was enhanced (18). Therefore, we hypothesize that miR-34a may also have a role in apoptosis of renal tubular epithelial cells.

To investigate whether miR-34a is involved in the regulation of renal tubular epithelial cell apoptosis, we first examined the expression of miR-34a in the UUO renal interstitial fibrosis model. As a result, we found that the expression of miR-34a as well as the MVs was significantly increased in the renal tissue. Afterwards, we found that MVs containing miR-34a also increased significantly. Subsequently, we used in vitro experiments to detect the distribution of miR-34a. We used TGF-β1 to treat NRK-49F and NRK-52E cells to alter their phenotypes, producing extracellular matrix and promoting fibrosis. We found that miR-34a was only significantly increased in interstitial fibroblasts, suggesting that miR-34a is mainly distributed in interstitial fibroblasts but not in tubule epithelial cells. Studies have confirmed that TGF-β1 can be transmitted from injured epithelial cells to fibroblasts and induce renal interstitial fibrosis (27). Therefore, we hypothesize that in the obstruction model, the tubule basement membrane was broken and lost its integrity, allowing miR-34a to pass from the mesenchymal fibroblasts to the renal tubular epithelial cells, inducing apoptosis of the renal tubular epithelial cells and promoting renal interstitial fibrosis.

In conclusion, this study shows that in the process of renal interstitial fibrosis, interstitial fibroblasts can secrete miR-34a-containing MVs and transmit signals to renal tubular epithelial cells through the ruptured basement membrane. miR-34a can inhibit the target protein Bcl-2, activate caspase apoptosis pathway, induce apoptosis of renal tubular epithelial cells, and promote renal interstitial fibrosis. MVs containing miRNAs serve as an important molecular platform for mediating cells, helping to further understand the molecular mechanisms of renal tubular epithelial cell apoptosis and tubulointerstitial fibrosis. Our identification of miR-34a in renal interstitial fibrosis suggests miR-34a mimics or inhibitors as potential therapeutics for treating renal interstitial fibrosis.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
HL and KL designed the study and performed the experiments, YuX, QZ and HX established the animal models, HX and YaX collected the data, HL and YuX analyzed the data, HL and KL prepared the manuscript. All authors read and approved the final study.

Ethics approval and consent to participate
This study was approved by The Animal Care and Use Review Committee of Wujiang Hospital Affiliated to Nantong University (Suzhou, China).

Patient consent for publication
No patients participated in this study.

Competing interests
The authors declare that they have no competing interests.

References
1. Liu Y: New insights into epithelial-mesenchymal transition in kidney fibrosis. J Am Soc Nephrol 21: 212-222, 2010.
2. Zeisberg M and Neilson EG: Mechanisms of tubulointerstitial fibrosis. J Am Soc Nephrol 21: 1819-1834, 2010.
3. Bohle A, Christ H, Grund KE and Mackensen S: The role of the ..... 1979.
4. Johnson A and DiPietro LA: Apoptosis and angiogenesis: An evolving mechanism for fibrosis. FASEB J 27: 3893-3901, 2013.
5. Docherty NG, O’Sullivan OE, Healy DA, Fitzpatrick JM and Watson RW: Evidence that inhibition of tubular cell apoptosis protects against renal damage and development of fibrosis following ureteric obstruction. Am J Physiol Renal Physiol 290: F4-F13, 2006.
6. Liu Y: Hepatocyte growth factor in kidney fibrosis: Therapeutic potential and mechanisms of action. Am J Physiol Renal Physiol 287: F7-F16, 2004.
7. Zhang G, Oldroyd SD, Huang LH, Yang B, Li Y, Ye R and El Nahas AM: Role of apoptosis and Bcl-2/Bax in the development of tubulo-interstitial fibrosis during experimental obstructive nephropathy, Exp Nephrol 9: 71-80, 2001.
8. Liu CF, Liu H, Fang Y, Jiang SH, Zhu JM and Ding XQ: Kapamycin reduces renal hypoxia, interstitial inflammation and fibrosis in a rat model of unilateral ureteral obstruction. Clin Invest Med 37: E142, 2014.
9. Miyajima A, Chen J, Lawrence C, Ledbetter S, Soslow RA, Stern J, Jha S, Pigato J, Lemer ML, Popps PA, et al: Antibody to transforming growth factor-beta ameliorates tubular apoptosis in unilateral ureteral obstruction. Kidney Int 58: 2301-2313, 2000.
10. Gao X, Ma E, Ayane N, Takai T, Oshima K, Hattori M, Ueki T, Fujimoto J and Tanizawa T: Hepatocyte growth factor gene therapy retards the progression of chronic obstructive nephropathy. Kidney Int 62: 1238-1248, 2002.
11. Morrissey J, Hruska K, Guo G, Wang S, Chen Q and Klahr S: Bone morphogenetic protein-7 improves renal fibrosis and accelerates the return of renal function. J Am Soc Nephrol 13 (Suppl 1): S14-S21, 2002.
12. Fu JH, Yang S, Nan CJ, Zhou CC, Lu DQ, Li S and Mu HQ: MiR-182 affects renal cancer cell proliferation, apoptosis, and invasion by regulating PI3K/AKT/mTOR signaling pathway. Eur Rev Med Pharmacol Sci 22: 351-357, 2018.
13. Harvey SJ, Jarad G, Cunningham J, Goldberg S, Schermer B, Harfe BD, McManus MT, Benzing T and Miner JH: Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. J Am Soc Nephrol 19: 2150-2158, 2008.
14. Ho JJ and Marsden PA: Dicer cuts the kidney. J Am Soc Nephrol 19: 2043-2046, 2008.
15. Wang Y and Lee CG: MicroRNA and cancer - focus on apoptosis. J Cell Mol Med 13: 12-23, 2009.
16. Hermeking H: The miR-34 family in cancer and apoptosis. Cell Death Differ 17: 193-199, 2010.
17. Qi R, An H, Yu Y, Zhang M, Liu S, Xu H, Guo Z, Cheng T and Cao X: Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. Cancer Res 63: 8323-8329, 2003.
18. Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, Zhai Y, Giordano TJ, Qin ZS, Moore BB, et al: p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol 17: 1298-1307, 2007.
19. Rajczak J, Wysockzynski M, Hayek F, Janowska-Wiezorek A and Rajczak MZ: Membrane-derived microvesicles: Important and underappreciated mediators of cell-to-cell communication. Leukemia 20: 1487-1495, 2006.