

## EXPERIMENTAL STUDY

# miR-328 prevents renal fibrogenesis by directly targeting TGF- $\beta$ 2

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**ABSTRACT**

MicroRNAs have been shown to be crucial in the post-transcriptional regulation of various cellular biological functions. They also play important roles in various human diseases. In renal fibrosis, aberrant miRNA expression is observed. However, little is known about the roles of miR-328 in renal fibrogenesis and its regulating mechanisms. In the present study, we investigated the miR-328 expression profile in TGF- $\beta$ 1-induced renal fibrogenesis cell model using qRT-PCR. The results showed that TGF- $\beta$ 1 could induce EMT expression and fibrogenesis and significantly down-regulated the expression of miR-328. Through bioinformatical analysis, we identified that TGF- $\beta$ 2 was a direct target gene of miR-328. Luciferase reporter assay was conducted to further confirm this finding. The results also demonstrated that miR-328 mimics transfection could significantly upregulate miR-328 expression. Furthermore, upregulation of miR-328 could further repress the expression of TGF- $\beta$ 2 and ECM proteins. In conclusion, this study demonstrated that miR-328 could prevent renal fibrogenesis by directly targeting TGF- $\beta$ 2. Our findings suggested that elevated renal miR-328 levels might be a novel therapeutic strategy for treating renal fibrosis (Fig. 4, Ref. 36). Text in PDF [www.elis.sk](http://www.elis.sk).

KEY WORDS: renal fibrosis, miRNA, miR-328, TGF- $\beta$ , ECM, EMT.

**Introduction**

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs of 19–23 nucleotides that regulate almost all biological cell functions, including proliferation, differentiation, metabolism, and apoptosis via interference of target gene expression at posttranslational level (1, 2). As expressed in a tissue-specific manner, miRNAs are essential for maintaining the normal physiological function of organs and are involved in the pathological pathways in many diseases. Aberrant miRNA expression is observed in numerous human diseases, such as: diabetes, hypercholesterolemia, cancer and tissue fibrosis (3). Therefore, identifying and correcting specific miRNA expression maybe a novel therapeutic strategy for diseases.

Fibrosis, closely related to approximately 45 % of all deaths, has been gradually recognized to be a crucial predictor of disease progression (4, 5). Renal fibrosis, irrespective of its etiology, is a common end stage of almost all chronic kidney diseases. Leukocyte infiltration, tubular cell apoptosis and necrosis, tubulointerstitial fibroblast proliferation, and extracellular matrix (ECM) deposition characterize the injured kidney (6–8). ECM is a hallmark of renal fibrogenesis as continuous deposition of ECM results in fibrous scars and distorts the fine architecture of kidney tissues, leading to the collapse of renal parenchyma and the alteration of

kidney physiological function. Activation of the matrix-producing effector cells is generally regarded as the central event in renal fibrogenesis due to its direct relevance to matrix accumulation (9). Therefore, identification of key molecular pathway leading to fibrogenic cells activation is one of the crucial issues to deeply understand and to treat renal fibrosis better.

Although literature has reported tons of different fibrogenic factors including cytokines and hormonal, metabolic, and hemodynamic factors, transforming growth factor- $\beta$  (TGF- $\beta$ ) is widely accepted as the key pro-fibrotic mediator in renal fibrosis (10). It plays a critical role not only in synthesis and degradation of ECM, but also in the response of cells to ECM mediated through integrin receptors; Moreover, specific components of the ECM, in return, can both deliver TGF- $\beta$  and regulate its activity. Three distinct isoforms of TGF- $\beta$  have been identified in all mammalian species, termed TGF- $\beta$  1, 2, and 3, of which TGF- $\beta$ 1 is the most abundant isoform and is well known for its profibrotic activity during renal fibrosis (6, 10–12).

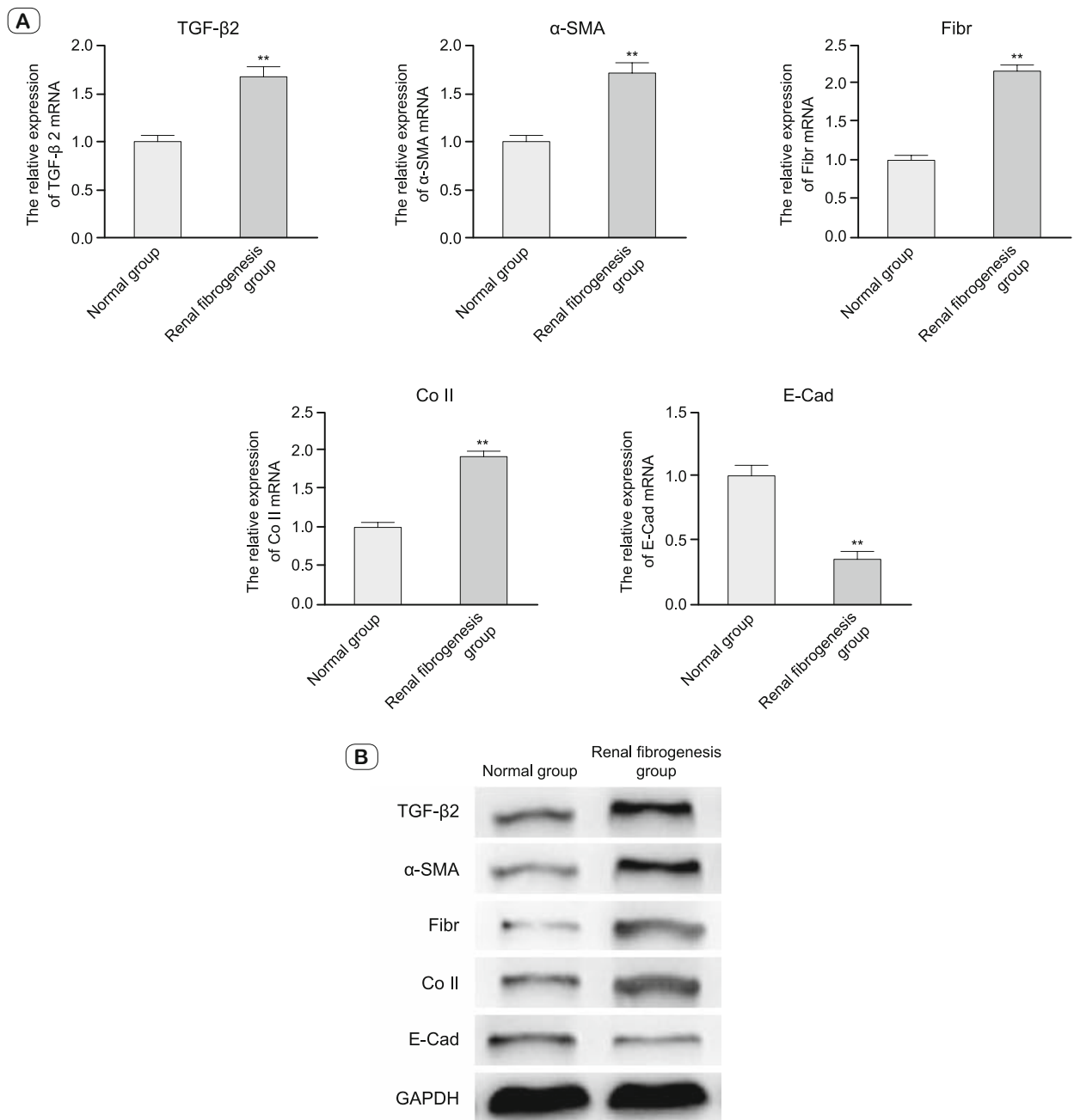
In kidneys, miRNAs have been associated with renal development, homeostasis, and physiological functions. Emerging evidences demonstrated the relationship between TGF- $\beta$  signaling and miRNAs expression during renal diseases. TGF- $\beta$  regulates expression of several microRNAs, such as miR-21, miR-192, miR-196, miR-200, miR-433, and miR-29 (2, 13). For example, Jiao et al identified that miR-196a and miR-196b are predominantly expressed in the kidney and play an inhibitory role in the progress of renal fibrosis (13). In addition, miR-29 has the ability to inhibit TGF- $\beta$ -mediated deposition of ECM, thus protecting kidney from fibrosis (14, 15). However, the function of miRNA-328 and its direct target in renal fibrogenesis still remains elusive.

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In this study, we investigated the miR-328 expression in TGF-β1-induced renal fibrogenesis cell model using *qRT-PCR*. The results showed that miR-328 was remarkably declined in renal fibrogenesis cells. Additionally, through bioinformatical analysis,

we identified the potential targets of miR-328 and further confirmation was conducted by Luciferase reporter assay. After miR-328 mimics transfection, we found that up-regulation of miR-328 could alter the expression profile of fibrogenesis-related proteins.



**Fig. 1.** TGF-β1 induced EMT in HK-2 cells. (A) The mRNA expression levels of ECM proteins and EMT markers determined by qRT-PCR after exposure to TGF-β1 (10 ng/ml) for 3 days; (B) The expression levels of ECM proteins and EMT markers determined by western blot after exposure to TGF-β1 (10 ng/ml) for 3 days. \*\* p < 0.01 vs Normal group. TGF-β, transforming growth factor-β; α-SMA, α-Smooth Muscle Actin; Fibr, Fibrillarin; Co II, cytochrome oxidase subunit II; E-Cad, E-Cadherin.

## Materials and methods

### Reagents

Recombinant Human Transforming Growth Factor- $\beta$ 1 (TGF- $\beta$ 1) was purchased from R&D systems (Minneapolis, MN, USA) and dissolved in 0.22 mm filtered distilled water to a concentration of 50 mg/ml as stock solution. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco™ (USA). All other chemicals of reagent grade were obtained from Sigma (St. Louis, MO, USA). The primary antibody for TGF- $\beta$ 2 was obtained from ABCAM (US). The primary antibody for Fibrillar (Firi),  $\alpha$ -Smooth Muscle Actin ( $\alpha$ -SMA), E-Cadherin (E-Cad) were from Beyotime Biotechnology (Nanjing, China). The primary antibody for cytochrome oxidase subunit II (COX II) and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell culture

The human renal proximal tubular epithelial (HK-2) cells were obtained from the American Tissue Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% FBS and 25 mmol/l of glucose in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. For experimental treatments, FBS was reduced to 2%. To establish renal fibrogenesis cell model, HK-2 cells were cultured in the presence of TGF- $\beta$ 1 (10 ng/ml) in three consecutive days (16).

### RNA isolation and quantitative RT-PCR assays

Total RNA from cultured cells were extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For miR-328 quantification, miRNA in total RNA were reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA), and then applied in TaqMan MicroRNA Assays with specific primers of rat miR-328 according to the manufacturer's instructions (Life Technologies). The real-time PCR analysis was performed on StepOne™ Real-Time PCR System (Applied Biosystems Life Technologies, Foster City, CA, USA). Specific primers for U6 were used to normalize the amount of sample added. Relative amounts of the miR-328 were quantitated using the comparative Ct method. All quantifications were performed on triplicate samples of three separate experiments.

### Transfection of miRNA

HK-2 cells ( $3 \times 10^4$  cells per well) were seeded into twelve-well culture plates and grew overnight. The microRNAs specific to miRNA-328 and the negative control (NC) were designed and synthesized by Shanghai GenePharma, Co. Ltd., (Shanghai, China). Transfection of HK-2 cells with miRNA mimics was optimized utilizing lipofectamine 2000 (Invitrogen, CA, USA) according to the recommendations of the manufacturer. The experiments were divided into four groups: non-transfected HK-2 cells (blank control), non-transfected HK-2 cells treated with TGF- $\beta$ 1 (TGF- $\beta$ 1), negative control treated with TGF- $\beta$ 1 (NC+TGF- $\beta$ 1) and miRNA-328 mimics transfected cells treated with TGF- $\beta$ 1 (miR-328 mimics + TGF- $\beta$ 1).

### Luciferase reporter assay

To determine the effect of miR-328 on TGF- $\beta$ 2 3'-UTR and the functional binding sites in the 3'UTR of TGF- $\beta$ 2, the full-length 3'UTR fragments of TGF- $\beta$ 2 gene and its mutant of the miR-328 binding sites were subcloned into a pmirGlo Dual-luciferase miRNA target Expression Vector (Promega, Madison, WI, USA) to form TGF- $\beta$ 2-3'UTR-Wt and TGF- $\beta$ 2-3'UTR-Mut (GenePharma), respectively. HK-2 cells seeded in 24-well plates were co-transfected with TGF- $\beta$ 2-3'UTR-Wt or TGF- $\beta$ 2-3'UTR-Mut and the indicated miRNAs. Luciferase activity assays were performed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocols. The results represent 3 independent experiments that were each performed in triplicate.

### Western blot

Western Blot was carried out using standard procedures. Cells were lysed in RIPA lysis buffer (Beyotime, Nanjing, China) containing protease inhibitors and phosphatase inhibitors. Protein concentrations were determined using a BCA Protein Assay kit (Beyotime). Equal amounts of protein were separated with 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Each membrane was then blocked with 5% skimmed milk for 2 h and then incubated with antibodies against TGF- $\beta$ 2,  $\alpha$ -SMA, Fibr, Co II, E-cad or GAPDH at 4 °C overnight. The membranes were then washed and incubated in the presence of horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. Immunoreactive bands were detected by ChemiDoc XRS+ System (Bio-Rad Laboratories) using the ECL plus kit (Beyotime, China). GAPDH was used as an internal control for all the experiments. The statistical data from at least 3 experiments was graphed to provide additional validation of results.

### Statistical analyses

All data were presented as the mean  $\pm$  standard deviation (SD) and analyzed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences were analyzed using the Student's t-test or one-way ANOVA.  $p < 0.05$  was considered statistically significant.

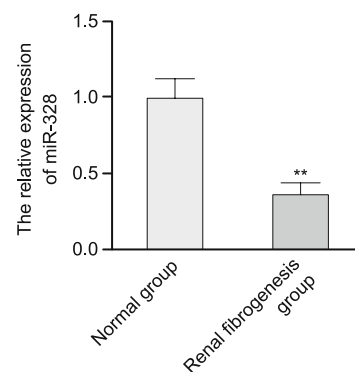
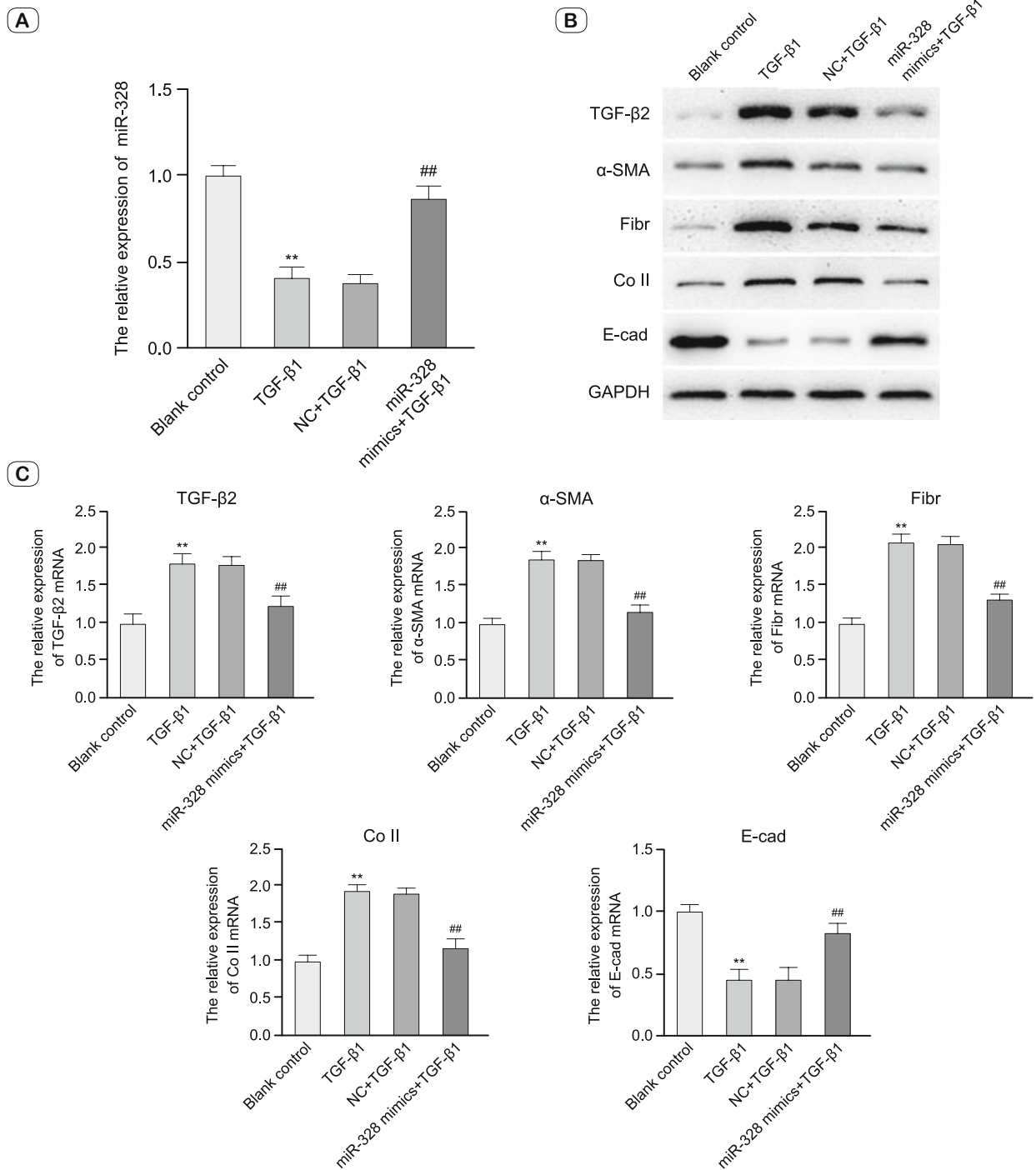


Fig. 2. miR-328 expression is downregulated in TGF- $\beta$ 1-induced renal fibrogenesis. \*\*  $p < 0.01$  vs Normal group. miR-328, microRNA-328.



**Fig. 3.** miR-328 suppresses the expression of ECM proteins and EMT markers induced by TGF- $\beta$ . (A) Relative miR-328 expression in HK-cells after transfection; (B) The expression levels of ECM proteins and EMT markers determined by western blot after transfection; (C) The mRNA expression levels of ECM proteins and EMT markers determined by qRT-PCR after transfection. \*\*  $p < 0.01$  vs Blank Control. #  $p < 0.01$  vs NC+TGF- $\beta$ . NC, Normal control; TGF- $\beta$ , transforming growth factor- $\beta$ ;  $\alpha$ -SMA,  $\alpha$ -Smooth Muscle Actin; Firi, Fibrillarlin; Co II, cytochrome oxidase subunit II; E-Cad, E-Cadherin.

## Results

### TGF- $\beta$ 1 induces EMT.

Epithelial-to-mesenchymal transition (EMT) is a major contributor to the pathogenesis of renal fibrosis, which is characterized by the loss of epithelial characteristics and increased mesenchymal phenotype (17, 18). To explore whether TGF- $\beta$ 1 induces EMT in HK-2 cells, the expression patterns of TGF- $\beta$ 2 and EMT markers were detected by qRT-PCR and western blot. As shown in Figure 1, exposure of HK-2 cells to TGF- $\beta$ 1 (10 ng/ml) for 3 days reduced the epithelial marker E-cad and induced TGF- $\beta$ 2,  $\alpha$ -SMA, Fibr and Co II both in mRNA and protein level. These results suggested that TGF- $\beta$ 1 induced EMT in HK-2 cells.

### miR-328 expression is downregulated in TGF- $\beta$ 1-induced Renal Fibrogenesis.

To investigate the role of miR-328 in renal fibrogenesis, we first compared the expression level between normal HK-2 cells and TGF- $\beta$ 1-treated HK-2 cells through qRT-PCR. As shown in Figure 2, miR-328 was transiently downregulated in HK-2 cells after treatment by TGF- $\beta$ 1. The results suggested that an aberrant expression of miR-328 existed in renal fibrogenesis.

### miR-328 suppresses the expression of ECM proteins and EMT markers induced by TGF- $\beta$ .

To further explore whether miR-328 plays a role in TGF- $\beta$ 1-induced EMT, we transfected the miR-328 mimics or miR-328 control into TGF- $\beta$ 1 treated HK-2 cells. As shown in Figure 3A, compared to cells transfected with miR-328 control (NC+TGF- $\beta$ 1), miR-328 levels significantly increased in miR-328 mimics trans-

ected cells (miR-328 mimics+TGF- $\beta$ 1). Then the expression of TGF- $\beta$ 2,  $\alpha$ -SMA, Fibr, Co II and E-cad was detected by western blot. After exposure to TGF- $\beta$ 1 for 3 days, transfection with miR-328 mimics suppressed the TGF- $\beta$ 1-induced EMT (Figs 3B–C). That is, both the mRNA and protein expression levels of TGF- $\beta$ 2,  $\alpha$ -SMA, Fibr and Co II were significantly decreased in miR-328 mimics transfected cells (miR-328 mimics+TGF- $\beta$ 1). Simultaneously, the level of E-cad was remarkably increased.

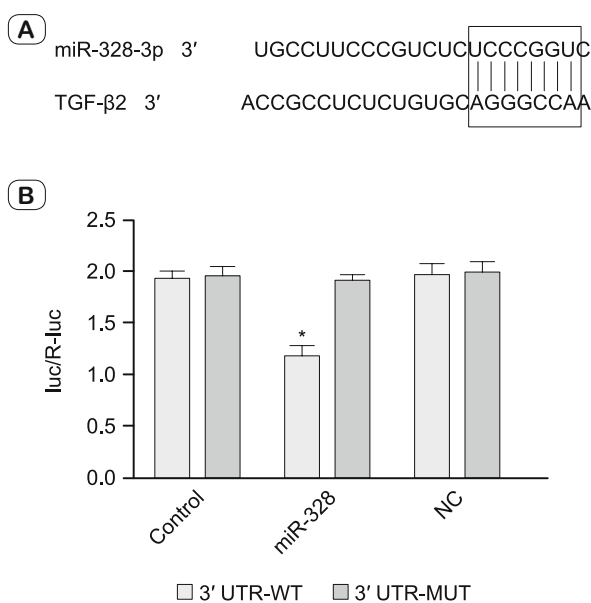
### miR-328 directly targets TGF- $\beta$ 2

Through bioinformatics analysis, we found that TGF- $\beta$ 2 was a potential target of miR-328. A highly complementary sequence of miR-328 was found in TGF- $\beta$ 2 3'-UTR (Fig. 4A). To further confirm the direct repression of TGF- $\beta$ 2 expression by miR-328, we integrated fragments of the TGF- $\beta$ 2 3'UTR with the complementary sequence of miR-328 into a dual-luciferase reporter system. The luciferase results indicated that overexpression of miR-328 in HK-2 cells co-transfected with TGF- $\beta$ 2 3'-UTR-WT led to a reduction of luciferase, while there was no reduction of luciferase in the HK-2 cells co-transfected with TGF- $\beta$ 2 3'-UTR-MUT (Fig. 4B). The results suggested that TGF- $\beta$ 2 was a direct target of miR-328.

## Discussion

Since the first microRNA lin-4, which negatively regulates lin-14, was identified in 1993, vast literature regarding the role of miRNAs in mammalian organogenesis and pathogenesis has been reported (19). There are currently over 20000 miRNAs that have been identified in a wide range of species including metazoa, mycetozoa, viridiplantae, and viruses, of which at least over 1000, to date, are found in humans (20–22). It is estimated that more than 60 % of human protein-coding genes is targeted by miRNAs at the miRNA recognition elements of their 3' untranslated region, leading to cleavage of target mRNAs and/or inhibition of their translation (19, 23). So, it is essential to identify the mRNA targets for exploring the significant change in the level of a certain miRNA during health and disease.

Kidneys are vital organs, which are responsible for the regulation of fluid, water, electrolytes, arterial blood pressure, and the elimination of toxins like the waste products of metabolic activity (24). Although the biochemistry and physiology of kidney function has been thoroughly understood for a very long period of time, it was only in recent years that the involvement of miRNAs in kidney regulation has become apparent. Nowadays, it is clear that miRNAs form a vital part of the regulatory cascade in renal development, maintenance of renal function, and progression of kidney diseases (25–26). Renal fibrosis is a common response to many chronic kidney diseases with the feature of excessive deposition of ECM. Increasing evidence showed that miRNAs regulated fibrosis in kidney. Three miRNA families, miR-21, miR-200, and miR-29 have been shown to modulate renal fibrosis by promoting fibroblast proliferation, inhibiting EMT, and preventing deposition and remodeling of ECM (3). In addition, Alison et al. provided experimental evidence in the form of reciprocal expression at the protein level for a large number of predicted miRNA-



**Fig. 4.** TGF- $\beta$ 2 is a direct target of miR-328. (A) miR-328 and its targeting site in TGF- $\beta$ 2 3'UTR. (B) Luciferase reporter assay in HK-2 cells transfected with reporter vectors containing wildtype or mutant TGF- $\beta$ 2 3'UTR. \*  $p < 0.05$  vs Control. NC, negative control.

target pairs and discovered a novel role of miR-382 and SOD2 in the loss of epithelial characteristics induced by TGF-β1 (27). Ryuji et al showed miR-34c attenuated epithelial-mesenchymal transition and kidney fibrosis of mice with ureteral obstruction (28). Daniela et al. pointed out that microRNA-324-3p promoted renal fibrosis and was a target of angiotensin-converting enzyme inhibition (29). The related literature reported that miR-378 was capable of reducing mesangial hypertrophy and kidney tubular fibrosis via MAPK signaling. Furthermore, miR-132 could reduce renal fibrosis by selectively inhibiting myofibroblast proliferation and miR-196a/b could mitigate renal fibrosis by targeting TGF-β receptor 2 (13, 30–31).

miR-328 is now reported to have several functions against different biological processes. It may be considered as an oncogene in human invasive breast carcinoma (32) and a possible biomarker for estimating breast cancer resistance protein function in the human intestines (33). In addition, it may be a biomarker for recurrence of early gastric cancer after endoscopic submucosal dissection by modulating CD44 (34). However, the role of miR-328 in renal fibrogenesis needs to be elucidated. And the target genes of miR-328 are important to understand the molecular pathway of miR-328. Here, in this study, we discovered that aberrant expression of miR-328 exists in renal fibrogenesis. Through bioinformatical analysis, we found that TGF-β2 was a potential target of miR-328 and what is more, luciferase reporter assay confirmed this result. Based on these results, we hypothesized that miR-328 could attenuate EMT induced by TGF-β in renal fibrogenesis.

TGF-β acts directly to stimulate the synthesis of most matrix molecules including fibronectin, collagen (35). EMT can be caused by the changes in integrin-mediated adhesion between cells and matrix. Invasiveness of EMT-derived fibroblasts is induced via breakdown of the tubular basement membrane, which prevents contacts between tubular epithelial cells and interstitial matrix components. E-cadherin is a well-characterized adhesion receptor, playing an essential role in maintaining the structural integrity of renal epithelia and its polarization. The loss of epithelial adhesion is followed by re-organization of the actin cytoskeleton into stress fibers, containing de novo-expressed αSMA (36). To further explore the underlying molecular mechanisms of our hypothesis, we up-regulated miR-328 expression level in HK-2 cells and found that overexpression miR-328 significantly suppressed the expression of ECM proteins and EMT markers both in gene and protein levels.

In conclusion, we presented the first evidence that miR-328 was involved in the development of TGF-β-induced EMT and could prevent renal fibrogenesis by directly targeting TGF-β2 in tubular epithelial cells. These findings suggested that elevating renal miR-328 levels might be a novel therapeutic strategy for treating renal fibrosis.

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