

Upregulation of miRNA-1228-3p alleviates TGF- β -induced fibrosis in renal tubular epithelial cells

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Summary. Background. Chronic kidney disease (CKD) has become a major public health issue, which can lead to renal fibrosis regardless of the initial injury. It has been previously reported that miRNA-1228-3p was correlate with the progression of kidney fibrosis. However, the mechanism by which miRNA-1228-3p regulates renal fibrosis remains unclear. Methods. Renal tubular epithelial cells (HK-2) were treated with TGF- β 1 (10 ng/ml) in an *in vitro* model of renal fibrosis. Gene and protein expressions in HK-2 cells were measured by Western-blot and RT-qPCR, respectively. The relation between miRNA-1228-3p and its target gene was investigated by dual luciferase report analysis. Results. Upregulation of miRNA-1228-3p significantly inhibited TGF- β 1-induced fibrosis of HK-2 cells *in vitro* by targeting GDF11. In addition, miRNA-1228-3p exhibited anti-fibrosis effect through inhibition of the smad2/sm4 signaling pathway. Conclusion. Upregulation of miRNA-1228-3p markedly inhibited the progression of renal fibrosis *in vitro*, indicating that miRNA-1228-3p may serve as a potential novel target for the treatment of renal fibrosis.

Key words: miRNA-1228-3p, Renal fibrosis, TGF- β 1 signaling pathway

Introduction

It has been reported that hypertension and diabetes mellitus are histopathologically characterized by interstitial inflammation, tubular atrophy and fibrosis (Nogueira et al., 2017). Renal fibrosis has been regarded as an aberration of tissue healing process, in which there is progression rather than improvement of scar formation (Nogueira et al., 2017). Most patients with chronic kidney disease (CKD) eventually develop to renal fibrosis, followed by end-stage renal disease (ESRD) (Lv et al., 2018). In that situation, transplantation is the only effective therapeutic strategy (Sun et al., 2016). Therefore, any novel effective strategy that inhibits the progression of renal fibrosis is of great significance.

It has been confirmed that TGF- β 1 can promote fibronectin and collagen production by transcriptional activation of the relevant genes (Jin et al., 2019). In addition, previous studies have indicated that TGF- β 1 plays an important role in the progression of renal fibrosis (Loboda et al., 2016; Ma et al., 2018).

MicroRNAs (miRNAs) are endogenous noncoding small RNAs which are abundant in the body. Upregulation or downregulation of miRNA has been related with the progression of multiple diseases in recent studies (Pu et al., 2019). It has been previously reported that miR-21 played an inhibitory effect during the occurrence of renal fibrosis (Loboda et al., 2016). Moreover, Cao et al. indicated that miR-192 downregulation can inhibit the progression of renal fibrosis (Cao et al., 2019). Meanwhile, miR-1228-3p has been confirmed to be correlated with the progression of kidney fibrosis (Zhao et al., 2018a; Conserva et al., 2019). However, the mechanism by which miRNA-

1228-3p regulates renal fibrosis remains unclear. Therefore, we aimed to explore the role of miRNA-1228-3p during the progression of renal fibrosis in the present study.

Materials and methods

Cell culture

Human renal tubular epithelial cell lines (HK-2, ATCC, Manassas, VA, USA) were used to examine the effects of miRNA-1228-3p on renal fibrosis *in vitro*. The cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 1% penicillin (Thermo Fisher Scientific) and 1% streptomycin (Thermo Fisher Scientific) in a humidified incubator with 5% CO₂ at 37°C. To establish *in vitro* renal fibrosis model, HK-2 cells were treated with 10 ng/ml TGF- β 1 (Pepro Tech, Rocky Hill, NJ, USA) for 72 h.

Quantitative real time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from HK-2 cell lines using TRIzol reagent (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using the reverse transcription kit (TaKaRa, Ver.3.0) according to the manufacturer's protocol. RT-qPCRs were performed in triplicate under the following protocol: 2 minutes at 94°C, followed by 35 cycles (30 s at 94°C and 45 s at 55°C). The primer for miRNA-1228-3p, GAPDH and U6 were obtained from GenePharma (Shanghai, China). Smad2: forward, 5'-CTATGCCTCTGGACGCACAAC-3' and reverse, 5'-CCCATCAGGCAACTCGTAACTC-3'; GAPDH: forward, 5'-CATCATCCCTGCCTCTACTGG-3' and reverse, 5'-GTGGGTGTCGCTGTTGAAGTC-3'; miRNA-1228-3p: forward, 5'-GTCCACCGCAAATGCTTCTA-3' and reverse 5'-CCATCAGTCCCGTCTTGAAAC-3'. U6: forward, 5'-CTCGCTTCGGCAGCACAT-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'. The relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method by the formula: $2^{-(\text{sample } \Delta Ct - \text{control } \Delta Ct)}$, where ΔCt is the difference between the amplification fluorescent thresholds of the gene of interest and the internal reference gene (U6 or GAPDH) used for normalization.

Cell transfection

HK-2 cells were transfected with miRNA-1228-3p agomir or negative control (agomir-ctrl) according to the previous reference (Rippa et al., 2011). MiRNA-1228-3p agomir and agomir-ctrl RNAs were purchased from GenePharma (Shanghai, China).

For Smad2 overexpression, HK-2 cells were plated into 60 mm plates at 4×10^5 cells/well and cultured

overnight. Then, supernatants with pcDNA3.1 vector carrying the Smad2 gene were added directly to cells (at 50-60% of confluence) for 24 h. Next, cells were replated on selection medium containing puromycin (2.5 μ g/ml) for another 3 days. The overexpression of Smad2 was verified by using RT-qPCR and Western blot. pcDNA3.1 vector was purchased from GenePharma.

Western-blot detection

Total protein was isolated from tissue or cell lysates by using RIPA buffer, and quantified by BCA protein assay kit (Beyotime, Shanghai, China). Proteins were resolved on 10% SDS-PAGE, and transferred to PVDF (Bio-Rad) membranes. After blocking with 5% skim milk, the membranes were incubated with primary antibodies at 4°C overnight, then incubated with secondary anti-rabbit antibody (Abcam; 1:5000) at room temperature for 1 h. Then, the membranes were scanned using an Odyssey Imaging System and analyzed with Odyssey v2.0 software (LICOR Biosciences, Lincoln, NE, USA). Primary antibodies used in this study were as follows: anti-Smad2 (Abcam; 1:1000), anti-Smad4 (Abcam; 1:1000), anti-Collagen I (Abcam; 1:1000), anti-Fibronectin (Abcam; 1:1000), anti-Vimentin (Abcam; 1:1000) and anti-GAPDH (Abcam; 1:1000). GAPDH was used as an internal control.

Luciferase reporter assay

MiRNA-1228-3p targeted gene predication was performed using 2 publicly available programs (miRDB and TargetScan). The results were selected for further analysis. Then, the wild type (WT) or mutate type (MT) of Smad2 3'-UTR was built based on pmiRGLO vector (Promega, Fitchburg, WI, USA) and named as WT-Smad2 or MT-Smad2. Cells were co-transfected with synthetic miRNAs and wt or mt-Smad2 using Lipofectamine 2000 (Thermo Fisher Scientific). After 48 h of transfection, luciferase activity was measured using dual-luciferase reporter assay system (Promega).

Statistical analysis

Statistical analysis was performed by using GraphPad Prism software (version 7, La Jolla, CA, USA). The data were presented as mean \pm standard deviation (SD) of at least three independent experiments. One-way ANOVA followed by Tukey's test was performed to analyze differences among groups. P value less than 0.05 was considered as a significant difference.

Results

In vitro model of renal fibrosis was successfully established

To create an *in vitro* model of renal fibrosis, HK-2 cells were treated with different concentrations of TGF-

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β 1 (2, 5 or 10 ng/ml). As indicated in Fig. 1A-D, TGF- β 1 increased the expressions of collagen I, Fibronectin and Vimentin in HK-2 cells in a dose-dependent manner. Collagen I, Fibronectin and Vimentin are key regulators during fibrosis (Villa et al., 2020; Zhao et al., 2020), and this result suggested that an *in vitro* model of renal fibrosis was successfully established. Next, to detect the gene expression, RT-qPCR was performed. As we expected, TGF- β 1 significantly decreased the expression of miR-1228-3p in HK-2 cells in a dose-dependent manner (Fig. 1E). Since HK-2 cells were more sensitive to 10 ng/ml TGF- β 1, 10 ng/ml TGF- β 1 was selected for use in following experiments.

miR-1228-3p agomir significantly suppressed the progression of renal fibrosis *in vitro*

In order to detect the efficiency of cell transfection, RT-qPCR was used. As shown in Fig. 2A, the expression

of miR-1228-3p was notably upregulated in HK-2 cells when transfected with miR-1228-3p agomir. This result confirmed that miR-1228-3p was stably transfected into HK-2 cells. In addition, the results of western blot revealed that the expressions of collagen I, Fibronectin and Vimentin in HK-2 cells were significantly increased by TGF- β 1, which were partially rescued in the presence of miR-1228-3p agomir (Fig. 2B-E). All these results indicated that overexpression of miR-1228-3p significantly suppressed the progression of renal fibrosis *in vitro*.

miR-1228-3p directly targeted Smad2 in HK-2 cells

To explore the mechanism by which miR-1228-3p mediated the progression of renal fibrosis, targetscan (http://www.targetscan.org/vert_71/) and dual luciferase report assay were used. As demonstrated in Fig. 3A,B, Smad2 might be the direct target of miR-1228-3p.

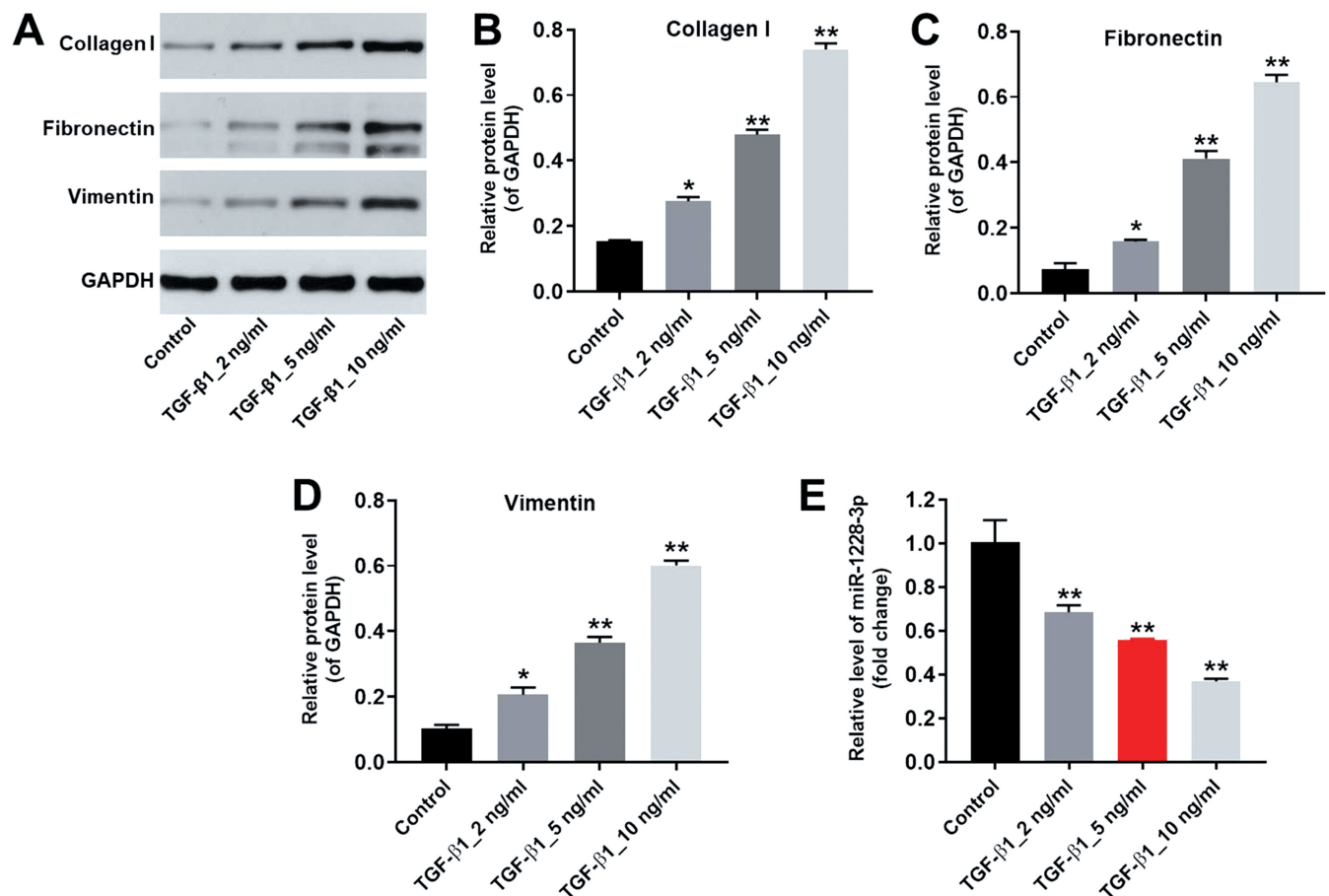


Fig. 1. An *in vitro* model of renal fibrosis was successfully established. HK-2 cells were treated with 2, 5 or 10 ng/ml TGF- β 1 for 72 h. **A.** The protein expressions of collagen I, Fibronectin and Vimentin in HK-2 cells were detected by Western blot. **B.** The relative expression of collagen I was quantified by normalizing to GAPDH. **C.** The relative expression of Fibronectin was quantified by normalizing to GAPDH. **D.** The relative expression of Vimentin was quantified by normalizing to GAPDH. **E.** The expression of miR-1228-3p in HK-2 cells was detected by RT-qPCR. *P<0.05, **P<0.01 compared to control.

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Moreover, RT-qPCR and western blot further confirmed that expression of Smad2 in HK-2 cells was notably decreased by overexpression of miR-1228-3p (Fig. 3C-E). In summary, miR-1228-3p directly targeted Smad2 in HK-2 cells.

Smad2 pcDNA3.1 was successfully transfected into HK-2 cells

For the purpose of investigating the efficiency of Smad2 overexpression, fluorescence, RT-qPCR and

western blot were performed. As revealed in Fig. 4A-D, the expression of Smad2 in HK-2 cells was obviously upregulated in the presence of Smad2 pcDNA3.1. These data indicated that Smad2 was successfully overexpressed in HK-2 cells.

MiR-1228-3p agomir suppressed the progression of renal fibrosis through inactivation of TGF- β 1 signaling pathway

To further verify the mechanism by which miR-

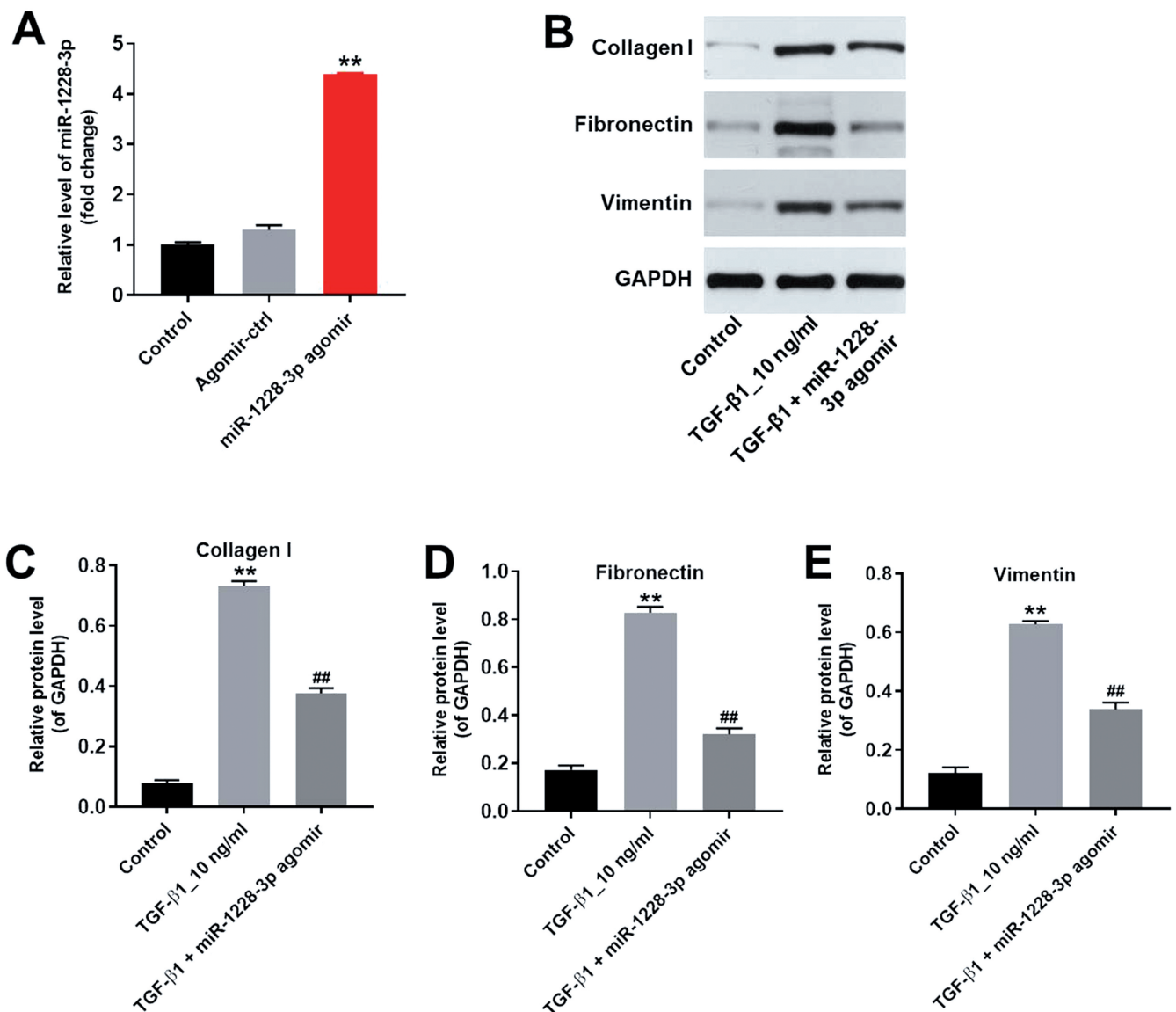


Fig. 2. Overexpression of miR-1228-3p significantly suppressed the progression of renal fibrosis *in vitro*. HK-2 cells were NC or miR-1228-3p agomir for 24 h. **A.** The expression of miR-1228-3p in HK-2 cells was detected by RT-qPCR. **B.** The expressions of collagen I, Fibronectin and Vimentin in HK-2 cells were detected by Western blot. **C.** The relative expression of collagen I was quantified by normalizing to GAPDH. **D.** The relative expression of Fibronectin was quantified by normalizing to GAPDH. **E.** The relative expression of Vimentin was quantified by normalizing to GAPDH. **P<0.01 compared to control. ##P<0.01 compared to 10 ng/ml TGF- β 1 group.

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1228-3p modulated the progression of renal fibrosis, Western blot was performed. As revealed in Fig. 5A-E, the expressions of collagen I, Fibronectin, Vimentin and p-Smad2 in HK-2 cells were significantly increased by TGF- β 1, which were partially reversed by miR-1228-3p agomir. However, the inhibitory effect of miR-1228-3p agomir on these proteins was notably reversed by Smad2 overexpression (Fig. 5A-E). Meanwhile, the expression of Smad2 in TGF- β 1-treated HK-2 cells was significantly decreased by miR-1228-3p agomir, which

was partially rescued in the presence of Smad2 overexpression (Fig. 5F).

In addition, TGF- β 1-induced a decrease of Smad4 expression in cytoplasm of HK-2 cells was significantly reversed by miR-1228-3p agomir, while the effect of miR-1228-3p agomir was partially rescued in the presence of Smad2 overexpression. In contrast, expression of Smad4 in nucleus of HK-2 cells was significantly increased by TGF- β 1, while this phenomenon was reversed in the presence of miR-1228-

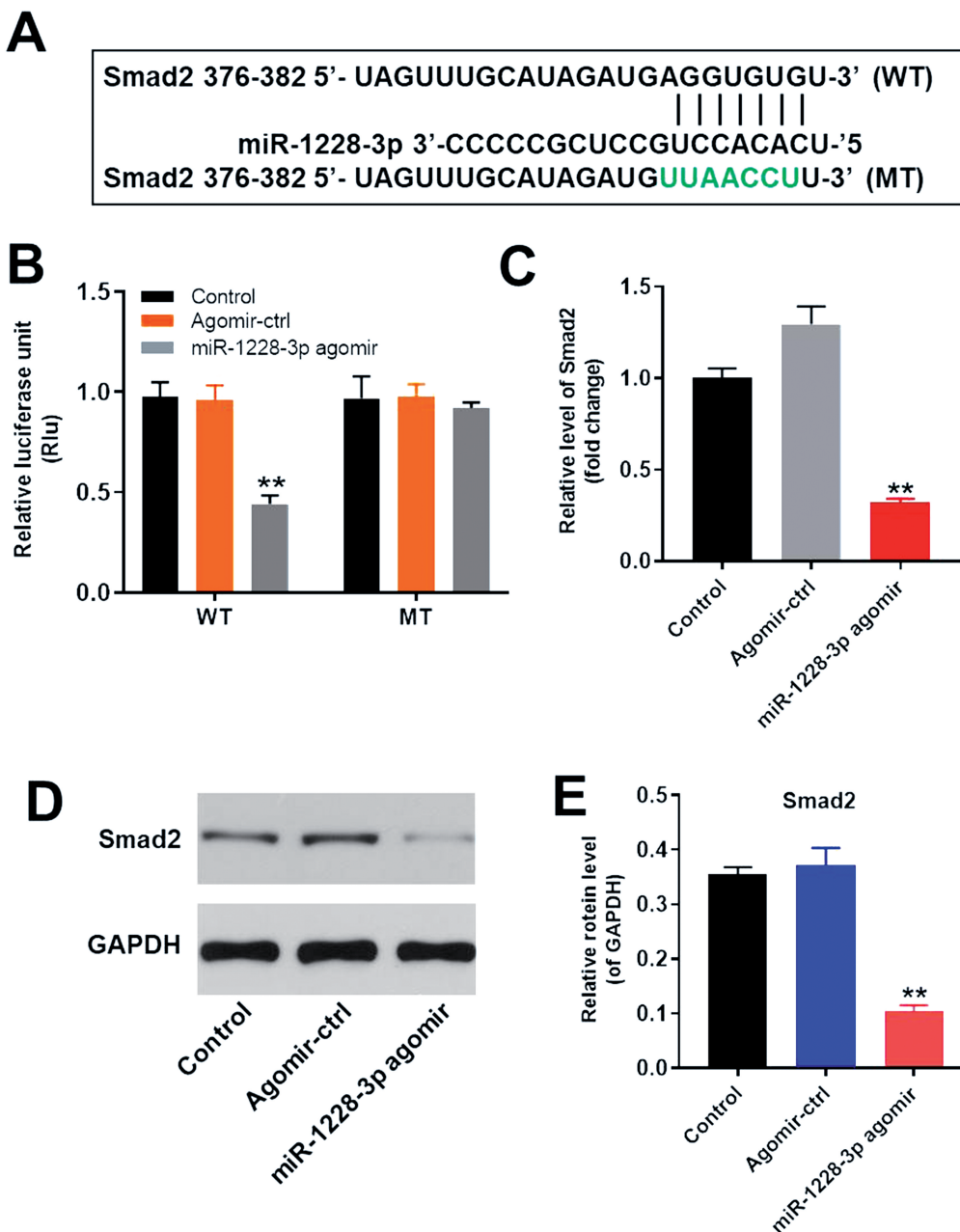


Fig. 3. MiR-1228-3p directly targeted Smad2. **A.** Gene structure of Smad2 at the position of 376-382 indicated the predicted target site of miR-1228-3p in its 3'-UTR, with a sequence of UUAACCU. **B.** The luciferase activity was measured in HK-2 cells following co-transfecting with WT/MT Smad2 3'-UTR plasmid and miR-1228-3p with the dual luciferase reporter assay. **C.** The expression of Smad2 in HK-2 cells was detected by q-PCR. **D.** The protein expression of Smad2 in HK-2 cells was detected by Western blot. **E.** The relative expression of Smad2 was quantified by normalizing to GAPDH. **P<0.01 compared to control.

3p agomir (Fig. 6A). Moreover, HK-2 cells cultured with TGF- β 1 exhibited a fibroblast phenotype, while this phenomenon was reversed by miR-1228-3p agomir (Fig. 6B). Altogether, overexpression of miR-1228-3p suppressed the progression of renal fibrosis through inactivation of TGF- β 1 signaling pathway.

Discussion

Previous studies have reported that miRNA was involved in progression of renal fibrosis, and dysregulation of miRNAs plays a critical role during fibrosis (Fierro-Fernandez et al., 2020; Su et al., 2020). In this study, we found that the progression of renal fibrosis was significantly suppressed by miR-1228-3p

agomir. A previous study found that miR-1228-3p was significantly downregulated in renal fibrosis (Conserva et al., 2019). Our data were consistent with this previous research, suggesting that miR-1228-3p can act as an inhibitor in renal fibrosis.

It has been verified that miRNAs exert their biological functions through mediation of their target genes (Giallombardo et al., 2016; Hide and Komohara, 2020). In this study, luciferase reporter assay indicated that Smad2 was considered as a direct target of miR-1228-3p in renal fibrosis. Smad2 is the downstream protein of the TGF- β 1 signaling pathway (Cai et al., 2020; Kaminska and Cyranowski, 2020). Zhou et al. have found that miR-10a suppressed the progression of renal fibrosis via downregulation of Smad2 (Zhou et

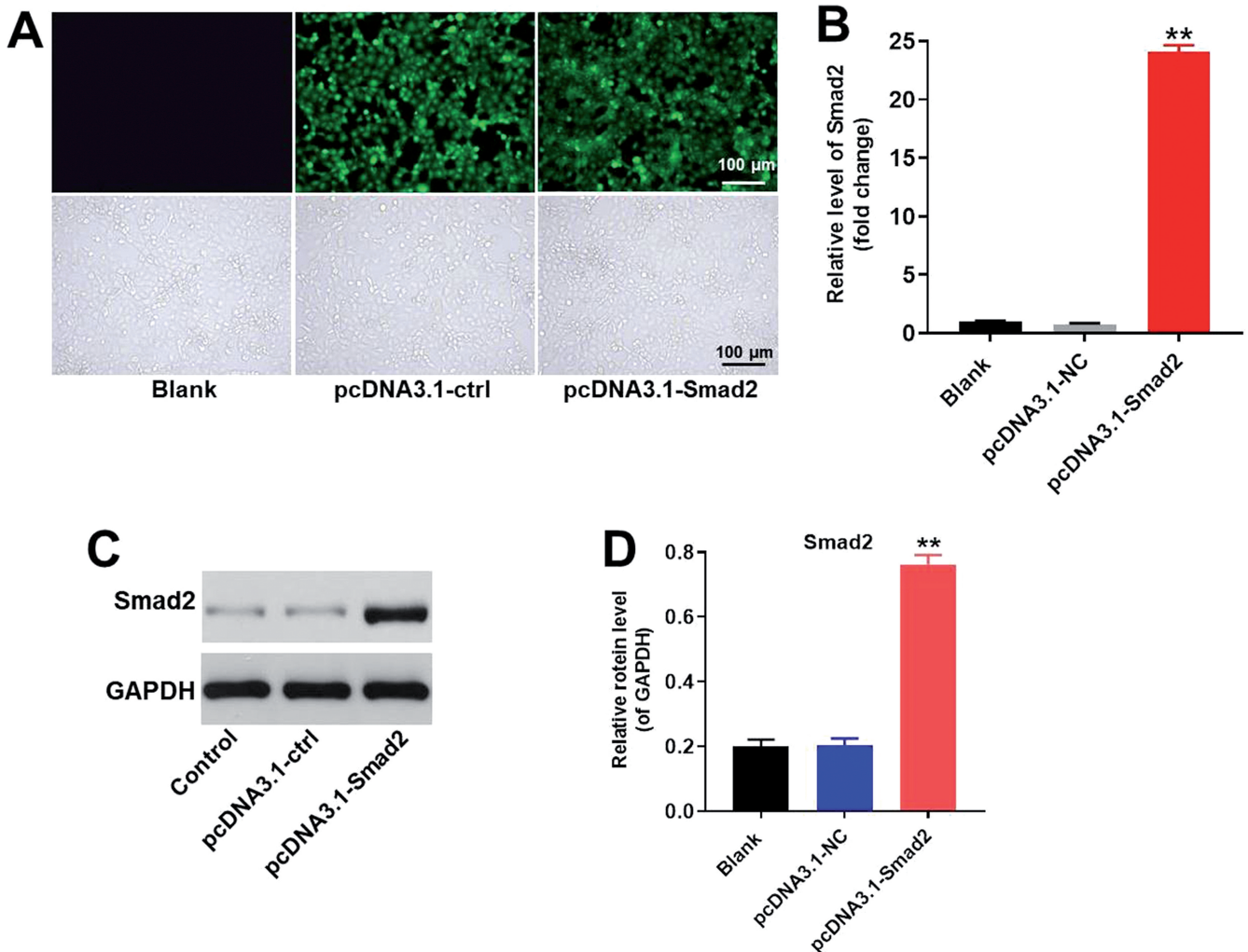


Fig. 4. Smad2 pcDNA3.1 was stably transfected into HK-2 cells. HK-2 cells were treated with pcDNA3.1-control or pcDNA-Smad2 for 24 h. **A.** The efficiency of Smad2 overexpression was observed under a microscope. **B.** The expression of Smad2 in HK-2 cells was measured by RT-qPCR. **C.** The protein expression of Smad2 in HK-2 cells was measured by Western blot. **D.** The relative expression of Smad2 was quantified by normalizing to GAPDH. **P<0.01 compared to control.

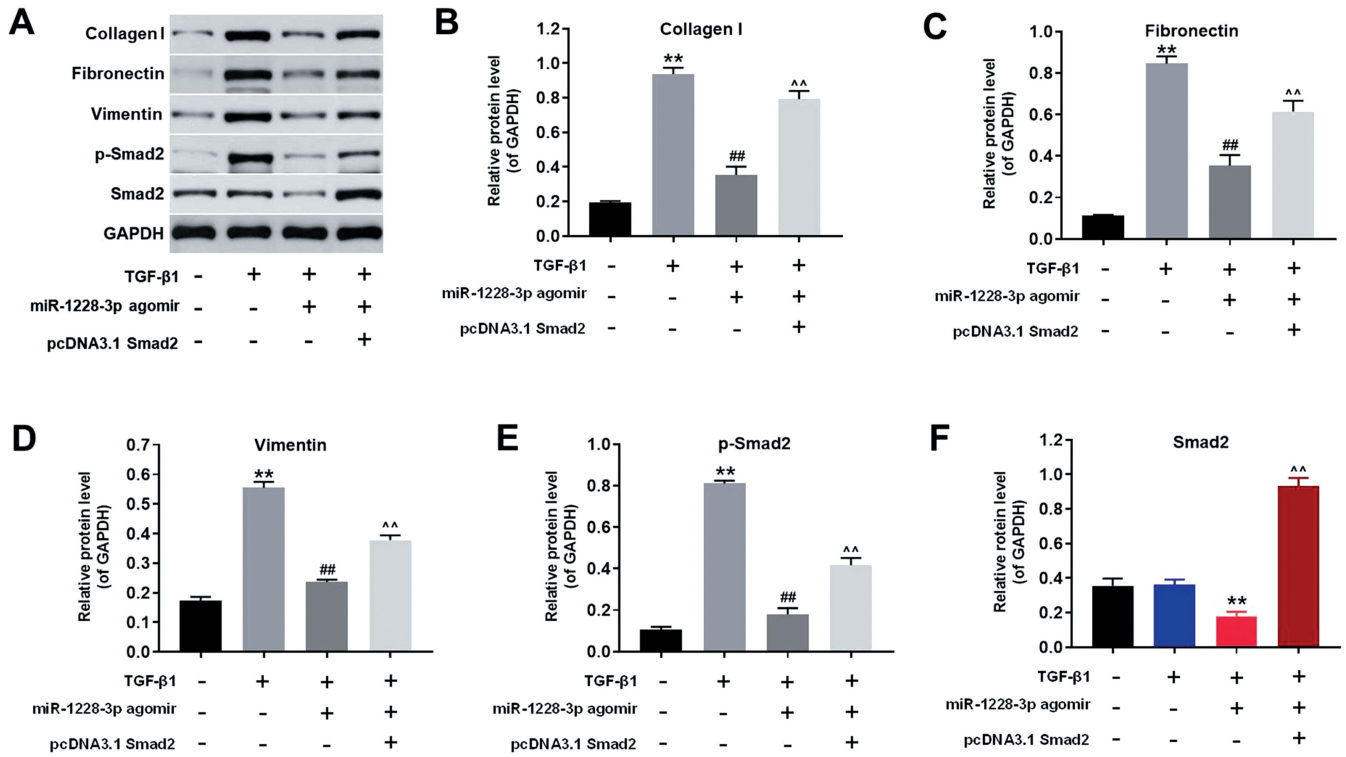
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Fig. 5. Overexpression of miR-1228-3p suppressed the progression of renal fibrosis through inactivation of TGF- β 1 signaling pathway. **A.** The expressions of collagen I, Fibronectin, Vimentin, Smad2 and p-Smad2 in HK-2 cells were detected by Western blot. **B.** The relative expression of collagen I was quantified by normalizing to GAPDH. **C.** The relative expression of Fibronectin was quantified by normalizing to GAPDH. **D.** The relative expression of Vimentin was quantified by normalizing to GAPDH. **E.** The relative expression of Vimentin was quantified by normalizing to GAPDH. **F.** The expression of Smad2 in HK-2 cells was detected by RT-qPCR. ** $P < 0.01$ compared to control. ## $P < 0.01$ compared to 10 ng/ml TGF- β 1 group. ^^ $P < 0.01$ compared to TGF- β 1 + miR-1228-3p agomir.

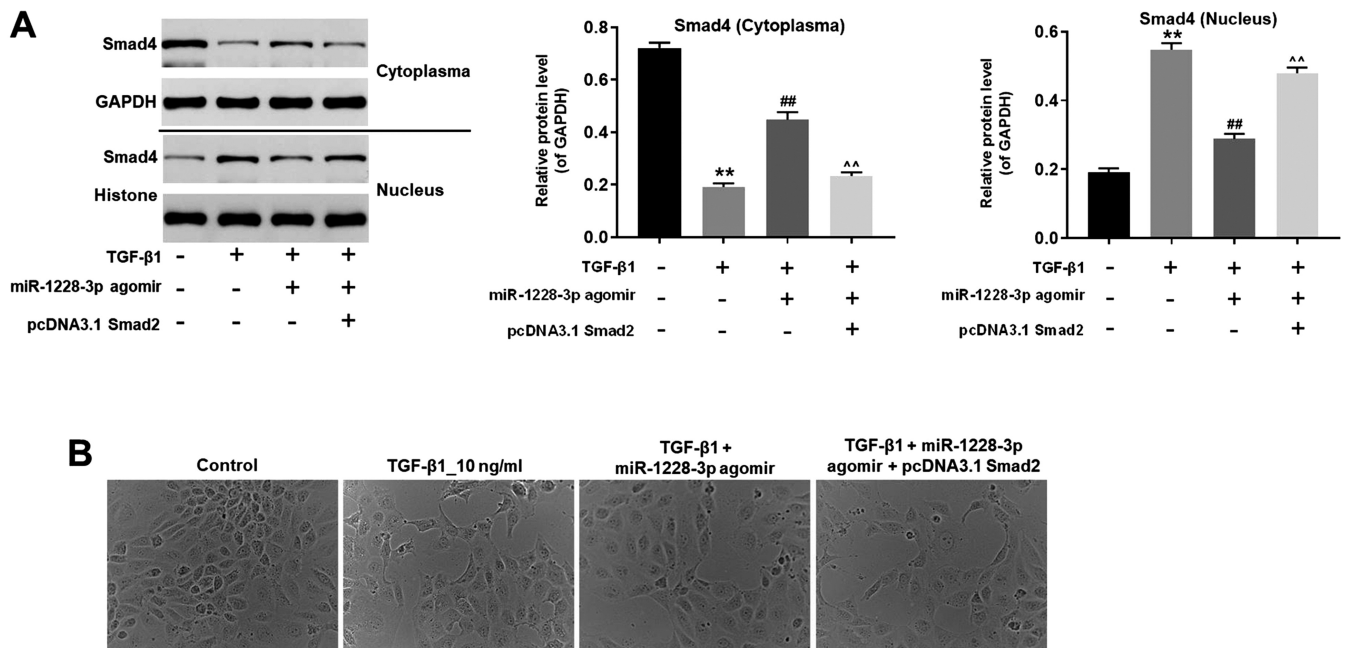


Fig. 6. Overexpression of miR-1228-3p suppressed the morphological changes of HK2 cells through inactivation of TGF- β 1 signaling pathway. **A.** The expression of Smad4 in cytoplasm or nucleus of HK-2 cells was detected by Western blot. The relative expression of Smad4 was quantified by normalizing to GAPDH. **B.** The morphological changes of HK2 cells were observed by microscope. ** $P < 0.01$ compared to control. ## $P < 0.01$ compared to 10 ng/ml TGF- β 1 group. ^^ $P < 0.01$ compared to TGF- β 1 + miR-1228-3p agomir.

al., 2020). Furthermore, Smad2 has been confirmed to play a key role during the occurrence of fibrosis (Zheng et al., 2020). In our study, upregulation of miR-1228-3p significantly decreased the protein level of Smad2. Our findings were consistent with the previous studies. Collectively, our data revealed that miR-1228-3p exhibited an anti-fibrotic effect on HK-2 cells by targeting Smad2. Since the direct target of miR-1228-3p in renal fibrosis remains unclear, this finding supplemented the biological function of miR-1228-3p.

TGF- β 1 signaling plays a key role in fibrosis (Vander Ark et al., 2018; Gewin, 2020). It was persistently upregulated in many types of fibrosis (Morikawa et al., 2016; Daniels et al., 2020). It has been reported that TGF- β 1 can activate Smad2 (Cai et al., 2020; Kaminska and Cyranowski, 2020). Moreover, multiple studies have found that Smad4 loss on its own does not initiate tumor formation, but can promote fibrosis initiated by other genes, such as KRAS activation in pancreatic duct adenocarcinoma and APC inactivation in renal diseases (Zhao et al., 2018b). In the current study, we found that upregulation of miR-1228-3p downregulated the expression of p-Smad2 in TGF- β 1-induced HK-2 cells. In addition, miR-1228-3p agomir also increased the expression of Smad4 in cytoplasm of HK-2 cells in the presence of TGF- β 1. Based on these data, the mechanism underlying the anti-fibrosis effect of miR-1228-3p upregulation *in vitro* was associated with the inactivation of TGF- β 1 signaling pathways. According to Liu K et al, Lnc-LFAR1 attenuated the proliferation and invasion of TGF- β 1-induced HK-2 cells through mediation of TGF- β 1/Smad 2/4 signaling (Chen et al., 2019). Our results were consistent with this previous study. Besides, we also found that the expression of Vimentin was notably upregulated in TGF- β 1-treated HK-2 cells. Vimentin plays an important role in EMT process (Saitoh, 2018). Li et al. has revealed that activation of TGF- β 1 signaling can enhance the EMT process of renal fibrosis (Li et al., 2020). Consistently, our data further suggested that miR-1228-3p can play an inhibitory role in TGF- β 1/EMT axis. According to Shi et al. (2020), miR-27a can induce renal fibrosis via activation of Wnt/ β -Catenin signaling. Sfrp1 (secreted frizzled-related protein 1) is known to be a promoter in Wnt/ β -Catenin signaling (Islam et al., 2020). Thus, different functions between Smad2 and Sfrp1 may be due to different mechanisms between miR-1228-3p and miR-27a in renal fibrosis. Frankly speaking, this study only focused on the effect of miR-1228-3p on TGF- β 1 so far. Since bFGF/PI3K/ESRP1 signaling pathways are involved in the fibrotic process, (Weng et al., 2020) further studies are needed to explore the role of miR-1228-3p on the bFGF/PI3K/ESRP1 pathway.

In conclusion, miR-1228-3p agomir can suppress the progression of renal fibrosis via mediation of TGF- β 1/Smad2/Smad4 axis. Thus, miR-1228-3p can serve as a new target for treatment of renal fibrosis.

Acknowledgements. Not applicable.

Conflicts of Interest. The authors declare no competing interests in this study.

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