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miR-152 regulates TGF-β1-induced epithelial-mesenchymal transition by targeting HPIP in tubular epithelial cells

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Abstract. Renal fibrosis is a common pathological feature of chronic kidney diseases, and their development and progression are influenced by epigenetic modifications including aberrant microRNA (miRNA or miR) expression. miRNAs have been demonstrated to modulate the aggressiveness of various cancers and have emerged as possible therapeutic agents for the management of renal fibrosis. Transforming growth factor β1 (TGF-β1)-induced epithelial-mesenchymal transition (EMT) of tubular epithelial cells serves a role in the initiation and progression of renal fibrosis. Furthermore, recent results indicated that the progression of EMT is reversible. The present study aimed to clarify the role of miR-152 in EMT of the tubular epithelial cell line HK-2, stimulated by TGF-β1, using *in vitro* transfection with a miR-152 mimic and to further investigate the underlying mechanism of miR-152 activity. In the present study, miR-152 expression was significantly reduced in TGF-β1-treated HK-2 cells, accompanied by an increased expression of hematopoietic pre-B-cell leukemia transcription factor (PBX)-interacting protein (HPIP). Additionally, miR-152 overexpression inhibited TGF-β1-induced EMT and suppressed HPIP expression by directly targeting the 3' untranslated region of HPIP in HK-2 cells. Furthermore, upregulation of HPIP reversed miR-152-mediated inhibitory effects on the EMT. Collectively, the results suggest that downregulation of miR-152 initiates the dedifferentiation of renal tubules and progression of renal fibrosis, which may provide important targets for prevention strategies of renal fibrosis.

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Abbreviations: EMT, epithelial-mesenchymal transition; miRNA, microRNA; TGF-β1, transforming growth factor β1; HPIP, hematopoietic pre-B-cell leukemia transcription factor (PBX)-interacting protein

Key words: chronic kidney disease, renal fibrosis, microRNA, epithelial-mesenchymal transition, hematopoietic pre-B-cell leukemia transcription factor (PBX)-interacting protein

Introduction

Chronic kidney disease (CKD) is a global healthcare concern that causes significant morbidity and mortality in the human population (1). CKD patients, even with similar etiologies, frequently exhibit different susceptibility and severity of renal fibrosis, leading to different clinical outcomes (2). Epithelial-mesenchymal transition (EMT) contributes to the pathogenesis of renal fibrosis, which is characterized by the loss of intercellular contacts caused by downregulation of E-cadherin, *de novo* expression of α-smooth muscle actin (αSMA) and accumulation of collagen (3). Pro-fibrotic growth factors, especially transforming growth factor β1 (TGF-β1) is considered to be the most important factor contributing to EMT during the fibrogenic phase of fibrosis (4,5). Regulation of EMT may be a promising target for the prevention of the progression of renal fibrosis.

MicroRNAs (miRNAs or miRs) are non-coding, single-stranded RNA molecules that can regulate target mRNAs predominantly by binding to the 3' untranslated region (UTR) at the post-transcriptional level (6). Aberrant expression of miRNAs is associated with the initiation and progression of several pathological processes, including autoimmune diseases, cancer and cardiovascular disease (7-9). In addition, previous studies have revealed a role for miRNAs in kidney injury and repair, providing novel insights into the mechanism underlying EMT in renal fibrosis (10). It has been demonstrated that miR-205 and other members of the miR-200 family can inhibit the TGF-β-induced EMT by downregulating zinc finger E-box-binding homeobox 1 and 2, two major transcriptional repressors of E-cadherin (11,12). Chen *et al* (13) suggested that miR-328-mediated transient upregulation of CD44 triggers pressure-induced EMT in renal fibrosis. Expression of miR-192 was upregulated by stimulation of mouse mesangial cells with TGF-β1, and miR-192 is responsible for increased collagen II in diabetic kidney glomeruli (14). The above results suggest important roles of miRNAs in renal fibrosis and EMT of tubular epithelial cells. A recent study demonstrated that TGF-β1 inhibits the expression of miR-152/30a, therfore enhancing DNA methyltransferase 1/3a and contributing to the promotion of pro-fibrotic protein expression and renal fibrosis (15). However, the association between miR-152 and TGF-β1-induced tubular epithelial cell EMT remains to be elucidated.

Table I. Primer sequences for reverse transcription quantitative polymerase chain reaction.

Target	Forward primer 5'-3'	Reverse primer 5'-3'
Has-miR-152	GTCGTATCCAGTGCGTGTCGTGGA	GTCGGCAATTGCACTGGATACGACAGTCGG
α-SMA	GCGCAGGTTCTGTGATACACT	TGGTGTCGTGGAGTCG
E-cadherin	GTGTTGCCCCTGAAGAGCAT	GGGTGTCGAGGGAAAAATAGG
Collagen I	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

miRNA, microRNA; αSMA, α-smooth muscle actin.

Based on the results of the previous studies, the present study investigated the expression of miR-152 in the TGF-β1-treated tubular epithelial HK-2 cell line and examined whether miR-152 modification could ameliorate TGF-β1-induced EMT. The results of the present study demonstrated that miR-152 expression is significantly reduced in HK-2 cells following stimulation with TGF-β1 and that enhanced expression of miR-152 prevents EMT induced by TGF-β1, possibly via negative regulation of hematopoietic pre-B-cell leukemia transcription factor (PBX)-interacting protein (HPIP). The results of the present study further support the role of miR-152 in TGF-β1-induced EMT, suggesting that it may be an effective therapeutic target for the treatment of renal fibrosis.

Materials and methods

Cell culture and treatment. The human kidney proximal tubule cell line (HK-2) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in keratinocyte serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were incubated at 37˚C in a 5% CO_2 atmosphere. TGF-β1 was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and used at a working concentration of 10 ng/ml, as previously described (16-18). miR-152 mimic and control RNA mimic (miR-NC) were obtained from GeneCopoeia Inc. (Rockville, MD, USA). Cells were plated on 6-well plates and grown to 60% confluency and then transfected with 35 nM miR-152 mimic or miR-NC using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A rescue experiment was performed by HPIP overexpression using the HPIP ORF expression clone (GeneCopoecia, Inc.) and pcDNA3.1 empty vector was used as a negative control.

Plasmid construction and 3' UTR target assay. Potential targets of miR-152 were predicted using miRbase (www.mirbase.org), miTarget (cbit.snu.ac.kr/~miTarget), and TargetScanS (www.targetscan.org/vert_71), and luciferase assay was performed to determine whether miR-152 targeted the 3'-UTR of HPIP. Luciferase reporter vectors were constructed using the 3' UTR of the HPIP gene, which was amplified in a polymerase chain reaction (PCR), using the following primers: Forward, 5'-CTGAGCACGTCGCAATCTCTACTCACCAGA-3' and reverse, 5'-GATAACGTCTTGAGCGATCTCTGTATCCTT-3'; extracted from the HK-2 cell genomic DNA and inserted into the luciferase coding region in the psiCHECK™-2 vector (Promega Corporation, Madison, WI, USA). A mutant vector with the HPIP 3'UTR was identical to the wild-type sequences, apart from the seed region, which was generated using the QuikChange™ Site-Directed Mutagenesis kit (Biocompare Inc., South San Francisco, CA, USA) and served as a negative control. The primers used to amplify the mutant HPIP 3'-UTR were as follows: Forward, 5'-GAGTTCCGCATGCACCCTATACTCAGACAC-3' and reverse, 5'-GCAGTTAACTGTTCGTCAGACTCGTATTCT-3'. *Renilla* luciferase, encoded by the vector, served as an internal control. HK-2 cells were seeded in 6-well plates overnight prior to transfection. The following day, each luciferase reporter construct, including the miR-152 mimic or miR-NC, was co-transfected into HK-2 cells using Lipofectamine 2000. Following 24 h incubation, cells were collected, and firefly and *Renilla* luciferase activities were determined using a dual-luciferase reporter assay system (Promega Corporation). All experiments were performed in triplicate and repeated three times.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from cultured cells, according to the manufacturer's protocol. For mRNA detection, cDNA synthesis was performed using the PrimeScript RT-PCR kit (Takara Bio, Inc., Otsu, Japan) in a reaction system of 20 µl, at 16˚C (30 min), 45˚C (30 min), and 85˚C (5 min). RT-qPCR was performed using GoTaq qPCR Master Mix (Promega Corporation) using the ABI PRISM 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. GAPDH served as an internal control. The following thermocycling conditions were used for the PCR: Initial denaturation at 94˚C for 5 min; 40 cycles of 95˚C for 15 sec, 65˚C for 30 sec and 72˚C for 30 sec; and a final extension at 72˚C for 5 min. Expression of mature miR-152 was determined with the Bulge-Loop™ miRNA qRT-PCR Primer Set (Guanghzou RiboBio Co., Ltd., Guanghzou, China). U6 RNA was used as an internal control. All sequences used are presented in Table I. The fold-change in gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (19). Each sample was detected in triplicate.

Western blotting. Cells were lysed using cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of

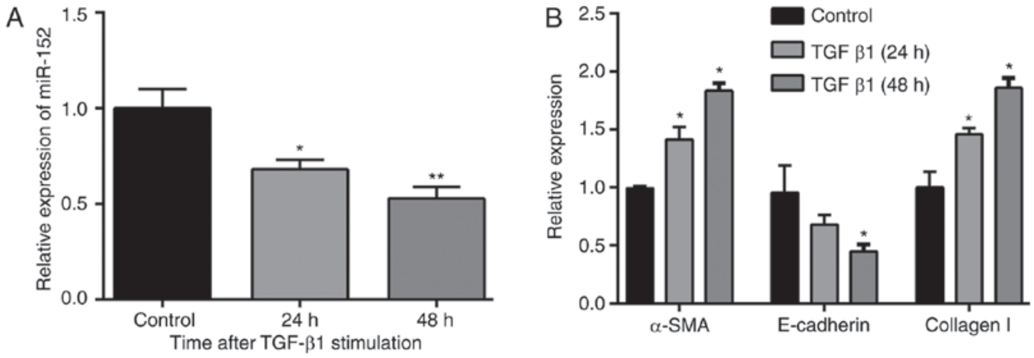

Figure 1. TGF-β1-induced miR-152 downregulation and alterations in gene expression of EMT-factors in HK-2 cells. (A) miR-152 expression in HK-2 cells following stimulation with TGF-β1 detected by reverse transcription-quantitative polymerase chain reaction. (B) Effects of TGF-β1 on the expression of EMT-associated genes. Data are presented as the mean ± standard deviation *P<0.05 and **P<0.01 vs. the control group. TGF-β1, transforming growth factor β1; miR-152, microRNA-152; EMT, epithelial-mesenchymal transition.

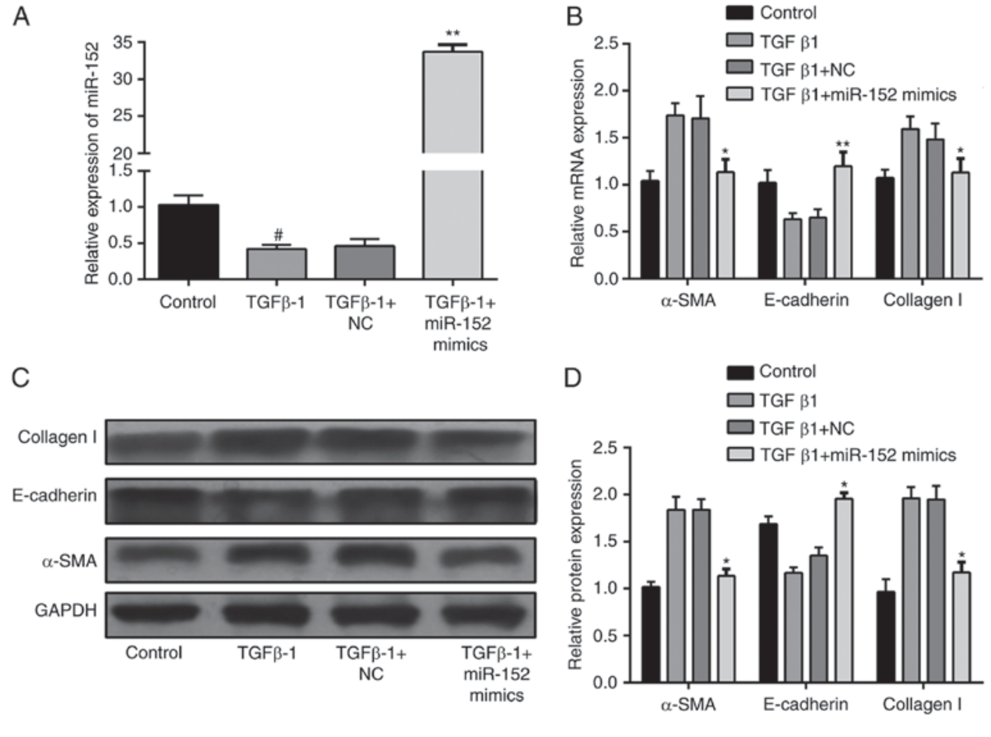

Figure 2. Overexpression of miR-152 inhibits TGF-β1-induced EMT in HK-2 cells. (A) HK-2 cells were transiently transfected with the miR-152 mimic or corresponding negative control plasmid. (B) Relative expression of EMT-associated genes, E-cadherin, αSMA and collagen I, analyzed by reverse transcription-quantitative polymerase chain reaction. Alterations in the expression of EMT-associated proteins in HK-2 cells transfected with miR-152 or miR-NC, measured by (C) western blotting and (D) quantified. Data are presented as the mean ± standard deviation. $^{\#}$P<0.05 vs. the control group; *P<0.05 and **P<0.01 vs. the TGF-β1+NC group. miR-152, microRNA-152; EMT, epithelial-mesenchymal transition; TGF-β1, transforming growth factor β1; NC, negative control.

Biotechnology, Haimen, China) and the protein concentrations were determined using the Bicinchoninic Acid protein assay kit (Beyotime Institute of Biotechnology). Subsequently, 30 μg protein was separated by 10-12% sodium dodecyl sulfate polyacrylamide gels, transferred onto polyvinylidene fluoride membranes. After blocked with 5% non-fat milk powder at room temperature for 1 h, membranes were probed with primary antibodies: anti-αSMA (1:5,000, cat no. EPR5308), anti-E-cadherin (1:1,000, cat no. BS1098) (both from Bioworld Technology, Inc., St. Louis Park, MN, USA), anti-collagen I (1:2,000, cat no. ab34710), anti-HPIP (1:500, cat no. ab197260) and anti-GAPDH (1:1,000, ab8245) (all from Abcam, Cambridge, MA, USA), overnight at 4˚C. The membranes were then incubated with goat anti-rabbit IgG H&L antibodies (1:5,000, cat no. ab6721; Abcam) for 2 h at room temperature. Results were visualized using the enhanced chemiluminescence detection reagent (Beyotime Institute of Biotechnology) and quantified by ImageJ software (version 6.0; the National Institute of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. SPSS (version 20.0; IBM Corp., Armonk, NY, USA) was used to perform statistical analyses using a two-tailed Student's t-test or one-way analysis of variance followed by

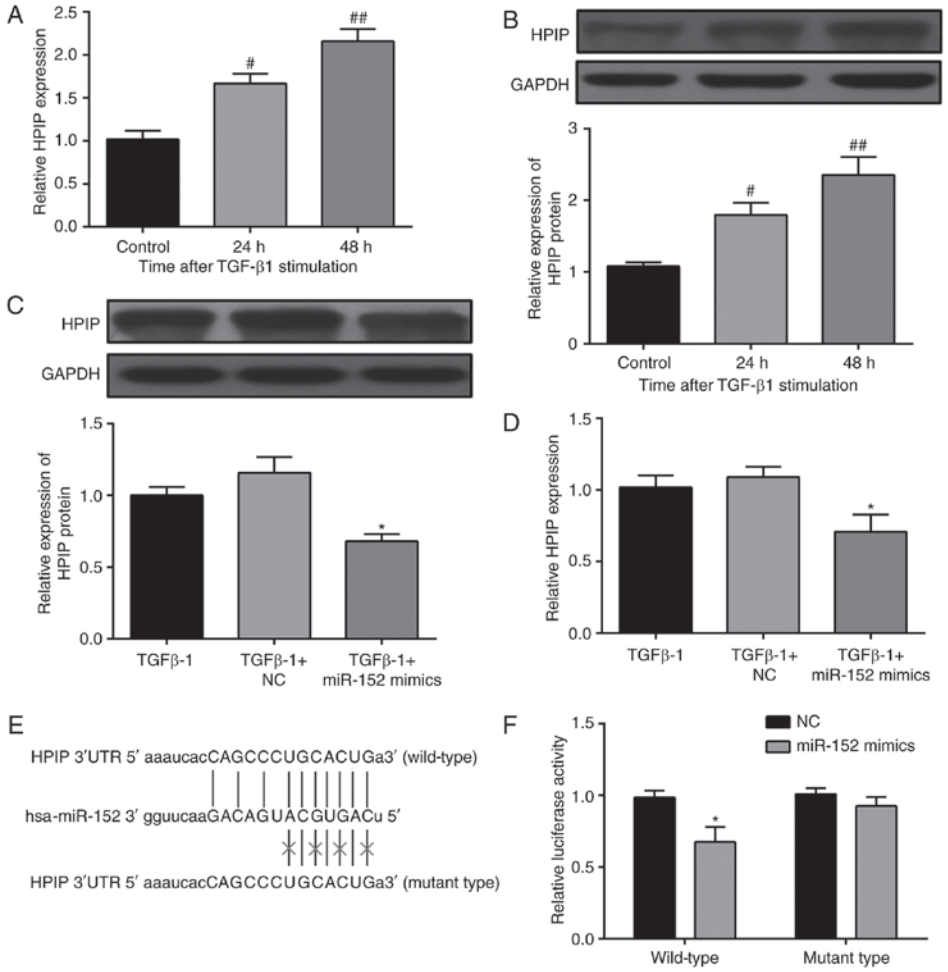

Figure 3. HPIP is a direct target gene of miR-152. (A) RT-qPCR and (B) western blot analysis of HPIP expression in HK-2 cells treated with 10 ng/ml TGF-β1. (C) Western blot and (D) RT-qPCR analysis of HPIP expression in HK-2 cells transfected with the miR-152 mimic or miR-NC from at least three independent experiments. (E) Putative target location of miR-152 in the 3' UTR of HPIP as predicted on miRBase. (F) miR-152 suppressed the expression of a luciferase reporter gene harboring the 3' UTR of HPIP. $^{\#}$P<0.05 vs. the control group, $^{\#\#}$P<0.05 vs. the TGF-β1 (24 h) group, *P<0.05 vs. the TGF-β1+NC group. HPIP, hematopoietic pre-B-cell leukemia transcription factor (PBX)-interacting protein; miRNA, microRNA; TGF-β1, transforming growth factor β1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control.

least significant difference test, where appropriate. GraphPad Prism (version 6.0; GraphPad Software Inc., La Jolla, CA, USA) was used to generate all graphs. All experiments were repeated at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

TGF-β1 induces downregulation of miR-152 and initiation of EMT in HK-2 cells. The expression profile of miR-152, as well as EMT-associated genes in TGF-β1-treated HK-2 cells was investigated. The results of RT-qPCR (Fig. 1A) demonstrated that the expression of miR-152 was significantly down-regulated in HK-2 cells following stimulation with TGF-β1 (10 ng/ml) for 24 and 48 h, compared with the control group (P<0.05 and P<0.01, respectively). As expected, the expression of the epithelial marker E-cadherin was decreased, whereas mesenchymal markers, including αSMA and collagen I were upregulated in HK-2 cells treated with TGF-β1 (10 ng/ml; Fig. 1B), especially for 48 h. Therefore, HK-2 cells treated with 10 ng/ml TGF-β1 at 48 hwere used in the following experiments.

miR-152 regulates the EMT in TGF-β1 treated HK-2 cells. In order to elucidate the role of miR-152 in TGF-β1-induced EMT in HK-2 cells, the expression of miR-152 was altered by transfection with miR-152 mimic. As presented in Fig. 2A, miR-152 expression was significantly increased in the TGF-β1+miR-152 mimic group compared with the TGF-β1+NC group (P<0.01). The effects of miR-152 on the expression of EMT markers were also tested. mRNA expression of E-cadherin was increased, while mRNA expression of αSMA and collagen I were suppressed in miR-152-overexpressing HK-2 cells compared with the control HK2 cells stimulated with TGF-β1 (Fig. 2B). Consistent with the results of RT-qPCR, E-cadherin, αSMA and collagen I protein expression demonstrated a similar response (Fig. 2C and D). Collectively, the results of the present study indicate that miR-152 serves a role in the regulation of EMT.

Overexpression of miR-152 suppresses the expression of HPIP in HK-2 cells. To determine the downstream target(s) of miR-152, miRBase, miTarget and TargetScanS were employed, and the putative complementary sequence to miR-152 was

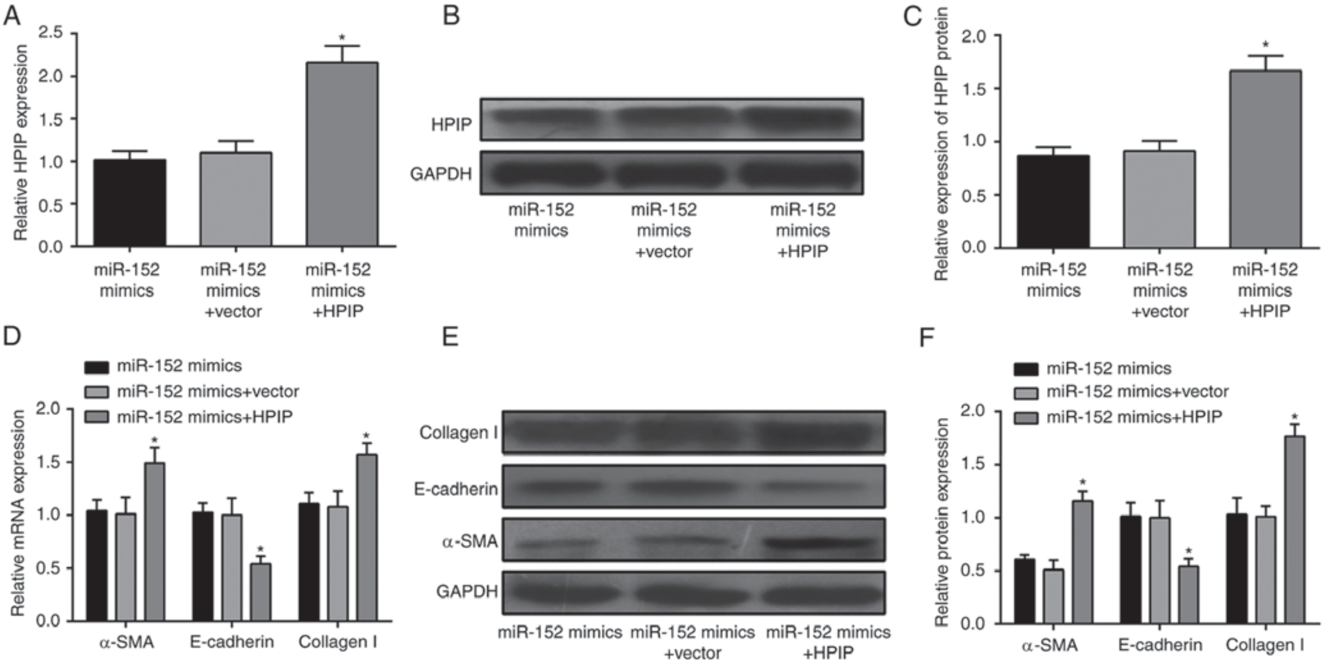

Figure 4. Upregulation of HPIP partially reverses miR-152-mediated EMT inhibition in TGF-β1 treated HK-2 cells. (A) Alterations in HPIP mRNA expression in cells transfected with the HPIP open reading frame expression clone or pcDNA-3.1 empty vector. (B) Western blotting and (C) quantitative HPIP protein expression was increased in HK-2 cells transfected with HPIP compared with those transfected with the control. (D) Relative expression of αSMA, E-cadherin and collagen I mRNA in HK-2 cells co-transfected with miR-152 mimic and HPIP or an empty vector, detected by reverse transcription quantitative polymerase chain reaction from three independent experiments. (E) Western blots and (F) quantitative analysis demonstrate the reversion of the expression pattern of EMT markers in miR-152 mimic-treated HK-2 cells following overexpression of HPIP. *P<0.05 vs. the miR-152 mimic+vector group. HPIP, hematopoietic pre-B-cell leukemia transcription factor (PBX)-interacting protein; miRNA, microRNA; EMT, epithelial-mesenchymal transition; αSMA, α-smooth muscle actin.

identified in the 3'-UTR of HPIP mRNA. The encoded protein has been reported to be involved in the TGF-β1-induced EMT in a variety of cancer cells (20). Therefore, HPIP expression was investigated in HK-2 cells with or without TGF-β1 treatment. HPIP mRNA expression levels were significantly increased in TGF-β1-treated cells compared with the control group (Fig. 3A). Additionally, western blotting analysis demonstrated that HPIP protein expression was upregulated following stimulation with TGF-β1 (Fig. 3B). Subsequently, the effects of miR-152 overexpression on HPIP in HK-2 cells were investigated. HPIP was significantly downregulated at protein (Fig. 3C) and mRNA (Fig. 3D) levels following overexpression of miR-152. A dual luciferase reporter assay was performed to validate the results. Fig. 3E demonstrates the putative position of the miR-152 target site in the 3' UTR of HPIP mRNA. Relative luciferase activity was significantly reduced by co-transfection with miR-152 mimic and luciferase reporters containing 3' UTR mRNA of HPIP, while the inhibition was abolished when the nucleotides were mutated in the 3'-UTR (Fig. 3F). The above results demonstrate that miR-152 regulates HPIP expression by directly targeting its 3' UTR in HK-2 cells.

Overexpression of HPIP partly reverses miR-152-mediated EMT induced by TGF-β. A rescue assay was designed to investigate whether HPIP is involved in the miR-152-mediated regulation of EMT in HK-2 cells. Following transfection with the HPIP ORF clone, mRNA and protein expression levels of HPIP in HK-2 cells were increased compared with cells transfected with the pcDNA-3.1 vector (Fig. 4A-C). Overexpression of HPIP reversed the effects of miR-152 on TGF-β1-induced EMT by decreasing E-cadherin and upregulating αSMA and collagen I mRNA expression (Fig. 4D). EMT-associated proteins demonstrated the same pattern of alterations at the protein level in HK-2 cells (Fig. 4E and F). The above results demonstrate that miR-152 reverses TGF-β1-induced EMT by negatively regulating HPIP expression in HK-2 cells.

Discussion

In the present study, miR-152-mediated HPIP upregulation stimulated TGF-β1-mediated induction of EMT in renal fibrosis (Fig. 5). miR-152 inhibited TGF-β1-induced EMT of human renal tubular epithelial cells through the negative regulation of HPIP. A previous study reported that tubular epithelial and epithelial parenchymal cells of the kidney are involved in the progression of renal fibrosis (21). Tubular epithelial cells demonstrate unique plasticity that enables them to transform form epithelial and mesenchymal phenotypes, and vice versa (22). An increasing number of publications suggest that the pathological process of the EMT of tubular epithelial cells could result in renal fibrosis and chronic renal disease (23,24). Therefore, inhibition of specific pathways involved in the EMT offers a novel therapeutic target to inhibit renal fibrogenesis. Nevertheless, the molecular mechanisms underlying the control of the onset of EMT of tubular epithelial cells remains to be elucidated.

TGF-β1, which can be secreted by all types of renal cells and infiltrated inflammatory cells, is a profibrotic agent in

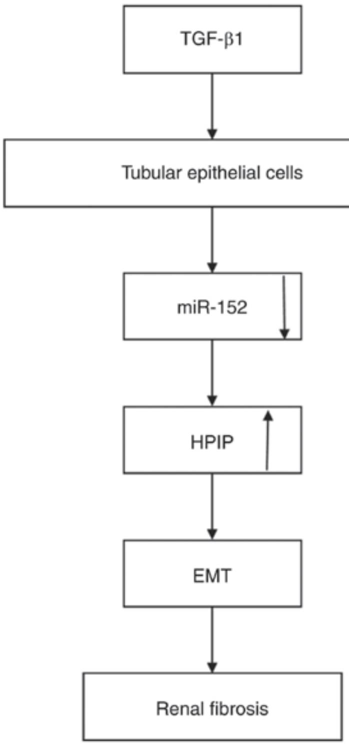

Figure 5. Hypothesized, schematic molecular mechanism underlying the development of renal fibrosis. miR-152-mediated HPIP upregulation resulted in TGF-β1-induced EMT in renal fibrosis. ↑, upregulated expression; ↓, downregulated expression; HPIP, hematopoietic pre-B-cell leukemia transcription factor (PBX)-interacting protein; miRNA, microRNA; EMT, epithelial-mesenchymal transition; TGF-β1, transforming growth factor β1.

renal cells (25). In the present study, TGF-β1 was used as an inducer of EMT in tubular HK-2 epithelial cells *in vitro*, aiming to investigate its underlying mechanisms of action. Stimulation with 10 ng/ml TGF-β1 resulted in the loss of E-cadherin expression and elevated expression of αSMA and collagen I, signifying the induction of EMT of HK-2 cells. Recently published data focused on the contribution of specific miRNAs to the progression of EMT in renal fibrosis (26,27). In the present study, downregulation of miR-152 in HK-2 cells was observed following stimulation with TGF-β1, which is consistent with the results of a previous study (15). Investigation of miRNA regulation in the kidney will improve the understanding of renal pathology and may eventually lead to the development of novel treatment strategies for reversing renal fibrosis and dysfunction. Results of previous studies revealed a critical role for miR-152 in human diseases, and miR-152 has been classified as an onco-miRNA in a variety of cancers, including breast, gastric and bladder cancers, and glioma (28-30). However, to date, a limited number of studies investigated the role of miR-152 in the urinary system. In a recent study, Lin *et al* (31) demonstrated that miR-152 expression was significantly downregulated in a rat model of peritoneal fibrosis, suggesting its involvement in the pathogenesis of peritoneal fibrosis. Therefore, in the present study, it was hypothesized that miR-152 may serve similar roles in EMT of tubular epithelial cells and the progression of renal fibrosis. In the present study, miR-152 was overexpressed to investigate its role in the modification of the EMT, and it was identified that overexpression of miR-152 prevents TGF-β1-induced EMT in HK-2 cells. These results provide novel insights into the role miR-152 in renal disease.

Subsequently, to determine the potential mechanisms of miR-152 function, downstream targets were investigated and it was demonstrated that the 3' UTR of HPIP contained a sequence complementary to miR-152. HPIP has emerged as an important regulator of organogenesis and tumorigenesis. It has been previously reported that HPIP is highly expressed in a variety of cancers (32-34). Recently, Shi *et al* (35) demonstrated that HPIP silencing suppresses TGF-β1-induced EMT in lung cancer cells by inhibiting activation of mothers against decapentaplegic homolog 2. Similarly, a study conducted by Zhang *et al* (36) demonstrated that HPIP silencing prevents TGF-β1-induced EMT in ovarian cancer cells. This data indicates the regulatory effect of HPIP during TGF-β1-induced EMT. However, the expression profile of HPIP in TGF-β1-treated tubular epithelial cells and the involvement of HPIP in the TGF-β1-induced EMT remain to be elucidated. A recent study by Mai *et al* (37) indicated that overexpression of HPIP promoted EMT, whereas knockdown of HPIP repressed EMT in renal carcinoma cells. Elevated HPIP mRNA and protein levels were observed in TGF-β1-treated HK-2 cells in the present study. The present study also indicated that transfection with the miR-152 mimic resulted in a decrease in HPIP expression at mRNA and protein level, suggesting that miR-152 serves a role in HPIP mRNA degradation and post-transcriptional regulation. One previous study has suggested the regulatory role of miRNA on HPIP (38). Consistent with this study, overexpression of HPIP partially abolished miR-152-mediated suppression of the EMT in HK-2 cells, suggesting that HPIP is a potential therapeutic target for EMT-associated renal fibrosis.

HPIP controls modulation of serine/threonine-protein kinase mTOR phosphorylation and expression in liver cancer (38). Knockdown of HPIP significantly blocked the phosphatidylinositol 4,5-bisphosphate 3-kinase/RAC-alpha serine/threonine-protein kinase signaling pathway in TGF-β1-stimulated ovarian cancer cells (36,39). However, the effects of TGF-β1-induced EMT on the HPIP signaling pathway were not identified in the present study. Further studies are required to elucidate the role and mechanism of HPIP in renal fibrosis. In addition, the present study only investigated the expression profile of miR-152 and HPIP in HK-2 cells. Further studies should validate their expression levels in human renal fibrosis tissues and reveal their role *in vivo*.

In conclusion, the results of the present study provide evidence that miR-152 controls TGF-β1-induced EMT in tubular epithelial cells. Furthermore, it was demonstrated that overexpression of miR-152 downregulates HPIP, contributing to the inhibition of EMT progression. The results of the present study contribute to better understanding of the mechanisms underlying antifibrotic therapies. The results of the present study suggest that upregulation of miR-152 or inhibition of HPIP may be useful strategies for the treatment of renal fibrosis.

Acknowledgements

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