Antifibrotic Effects of Hepatocyte Growth Factor on Endothelial-to-Mesenchymal Transition via Transforming Growth Factor-Beta1 (TGF-β1)/Smad and Akt/mTOR/P70S6K Signaling Pathways

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Background: The related mechanisms involved in allograft interstitial fibrosis and chronic allograft dysfunction (CAD), following renal transplant, remain largely unknown. Here, we explored the role of hepatocyte growth factor (HGF) treatment on the endothelial-to-mesenchymal transition (EndMT) as a new way to target and prevent kidney fibrosis and improve outcomes for renal transplant recipients.

Method/Material: We extracted proteins and mRNAs from human umbilical vein endothelial cells (HUVECs) and human renal glomerular endothelial cells (HRGECs) treated with transforming growth factor-beta1 (TGF-β1) and/or varying doses of HGF, and assessed the effect of HGF on the EndMT using western blotting, qRT-PCR, and ELISA assays. We utilized cell motility and migration assays to evaluate cell movement, and applied western blotting to assess the mechanism by which TGF-β1 induced the EndMT.

Results: HGF significantly attenuated the development of TGF-β1-induced EndMT in a concentration-dependent way, and weakened the abilities of motility and migration of both HUVECs and HRGECs. Moreover, our results reveal that the antifibrotic effect of HGF on the EndMT was associated with the TGF-β/Smad and Akt/mTOR/p70S6K signaling pathways.

Conclusions: Our study suggests that HGF treatment significantly attenuates the development of EndMT induced by TGF-β1 via the TGFβ/Smad and Akt/mTOR/P70S6K signaling, which provides novel insights into the prevention and treatment of interstitial fibrosis and CAD following renal transplant.

MeSH Keywords: Hepatocyte Growth Factor • Kidney Transplantation • Renal Insufficiency, Chronic

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Background

Kidney transplantation remains the optimal therapy for patients with end-stage renal disease [1]. Nevertheless, the relatively high proportion of kidney allograft loss after transplantation means that improving long-term allograft survival remains one of the critical challenges facing kidney transplantation. Various factors contribute to the loss of kidney allograft function, including rejection, delayed graft dysfunction, and calcineurin inhibitors (CNIs)-related nephrotoxicity [2,3]. Among these, chronic allograft dysfunction (CAD), previously named chronic rejection, is known to play a key role in kidney allograft loss [4].

While CAD is morphologically characterized by progressive interstitial fibrosis and tubular atrophy (IF/TA), and glomerular sclerosis [5], its mechanisms remain unknown. Researchers have shown that the endothelial-to-mesenchymal transition (EndMT) is a crucial factor in the pathogenesis of IF/TA and CAD. The EndMT is a unique subset of the epithelial-to-mesenchymal transition (EMT), whereby cells gradually lose endothelial markers, such as CD31 and VE-cadherin, and gain mesenchymal biomarkers, such as α-smooth muscular actin (α-SMA) and vimentin [6]. The essential role and contribution of EndMT in kidney fibrosis pathogenesis remains largely unclear and controversial. Studies investigating mouse models of both unilateral ureteral obstruction (UUO) and streptozocin (STZ)-induced diabetic nephropathy (DN) have shown that inhibiting the EndMT significantly attenuates kidney fibrosis by blocking the TGF β/Smad pathway [7,8]. Moreover, our group has previously found that TGF-β1 promotes pathogenesis of the EndMT and allograft interstitial fibrosis via the Akt signaling pathway both *in vivo* and *in vitro* [9]. Therefore, targeting the EndMT provides a new way to prevent kidney fibrosis and improve outcomes for renal transplant recipients.

Hepatocyte growth factor (HGF), originally defined as a potent mitogen for mature hepatocyte, functions similarly to renal epithelial cells [10,11]. In acute renal failure, HGF significantly accelerates the repair process of renal tubular cells and rapid recovery of tubular morphology and function [12]. Furthermore, HGF is a potent antifibrotic factor that prevents the deterioration of renal interstitial fibrosis through inhibiting growth factor-beta (TGF-β) expression, myofibroblasts activation, and EMT [13–15]. However, the crucial role of HGF in chronic allograft fibrosis has only recently been explored. Azuma and colleagues reported that HGF treatment significantly decreased fibrogenic events and TGF-β expression in chronic allograft nephropathy (CAN) rats, indicating that HGF treatment provides renal protection and recovery from early allograft injury and greatly contributes to the prevention of CAN [16]. HGF expression has also been significantly associated with fibrosis in human renal allografts from renal transplant recipients, after adjusting for human leukocyte antigen mismatches, highlighting the antifibrotic effects in human renal transplants [17]. However, conflicting results have also been observed. For example, a steady increase in HGF levels has been reported to contribute to the progression of chronic renal disease in diabetes, by decreasing the glomerular filtration rate and promoting the accumulation of extracellular matrix [18]. Therefore, in this study, we aimed to investigate and explore the effects and mechanisms of HGF treatment on the TGF-β1-induced EndMT process, *in vitro*.

Material and Methods

Ethics statement

The study protocol was in accordance with the ethical standards of the Declarations of Helsinki and Istanbul, and the protocol of this study was approved by the local Ethics Committee of the First Affiliated Hospital with Nanjing Medical University (2013-MD-062).

Cell lines and treatment

We used human umbilical vein endothelial cells (HUVECs) and human renal glomerular endothelial cells (HRGECs) in our experiments. We extracted primary HUVECs from approximately 4 cm segments from the specific cord without being unclamped. We performed all primary cell extraction procedures according to the local ethical guidelines. Briefly, we rinsed the vessels with 10 mL M199/P/S/G and flushed endothelial cell sheets from the inner surface using M199/P/S/G/0.5% BSA. After centrifugation (two minutes, 300 g), we removed the recovered cells and cultured them on uncoated plastic dishes for two continuous passages. We purchased HRGECs from ATCC (Catalog #4006; Manassas, VA, USA).

We cultured HUVECs and HRGECs in RPMI-1640 medium (Invitrogen, Life Technologies, Grand Island, NY, USA) or DMEM (Invitrogen, Life Technologies, Grand Island, NY, USA), respectively, adding 10% FBS (Gibco, Carlsbad, CA, USA), and 1% penicillin-streptomycin (Invitrogen, Life Technologies, Grand Island, NY, USA) under atmosphere containing 95% air and 5% CO_2 at 37°C. We identified extracted primary HUVECs using the indirect immunofluorescence staining assay of CD31 and visualized the staining using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

To evaluate how HGF influences the development of TGF-β1-induced EndMT in HUVECs and HRGECs, we starved the cells in a serum-free culture before treating with 20 ng/mL HGF, 40 ng/mL HGF and 5 ng/mL TGF-β1 for 48 hours. We extracted total protein and RNA samples for western blot assays and

quantitative real-time PCR (qRT-PCR). Then, we applied antagonist specific for receptor kinase (ALK5) – SB431542 (Cell Signaling Technology, Beverly, MA, USA), and the inhibitor specific for Akt – MK2206 (Selleckchem, Houston, TX, USA). HUVECs and HRGECs were serum-starved overnight, before pretreating with specific inhibitors for one hour, followed by treatment with HGF (40 ng/mL, Santa Cruz, CA, USA) and TGF-β1 (5 ng/mL, Santa Cruz, CA, USA) for 48 hours. The total protein of HUVECs was extracted for western blot analysis. We repeated each experiment for at least three times.

Western blot assay

We performed western blot assays as previously described by Liu XZ [19]. Briefly, we extracted total proteins from cells or tissues and determined protein concentrations using the BCA protein assay (Thermo Scientific, MA, USA). Then, we performed western blotting by incubation with primary antibodies of anti-GAPDH (1: 200, Abcam, Cambridge, MA, USA), anti-α-SMA (1: 2,500, Abcam), anti-CD31 (1: 1,000, Abcam), anti-TGF-β1 (1: 250, Abcam), anti-HGF (1: 500, Abcam), anti-Akt (1: 1,000, Cell Signaling Technology, Beverly, MA, USA), anti-phospho-Akt (Ser473, 1: 1,000, Cell Signaling Technology), anti-mTOR (1: 1,000, Cell Signaling Technology,), anti-phospho-mTOR (Ser2448, 1: 1,000, Cell Signaling Technology), anti-p70S6K (1: 1,000, Cell Signaling Technology), anti-phospho-p70S6K (Thr389, 1: 1,000, Cell Signaling Technology), anti-Erk 1/2 (1: 1,000, Cell Signaling Technology) anti-phospho-Erk 1/2 (Thr202/Tyr204, 1: 1,000, Cell Signaling Technology), anti-c-Jun (1: 1,000, Cell Signaling Technology) and anti-phospho-c-Jun (Ser73, 1: 1,000, Cell Signaling Technology), anti-Smad 2 (1: 1,000, Cell Signaling Technology), anti-phospho-Smad 2 (Ser465/467, 1: 1,000, Cell Signaling Technology), anti-Smad 3 (1: 1,000, Cell Signaling Technology) and anti-phospho-Smad 3 (Ser423/425, 1: 1,000, Cell Signaling Technology), followed by incubation with anti-rabbit or anti-mouse secondary antibody (1: 1,000, Abcam). Relative abundance of proteins was measured, and GAPDH expression was considered as an internal reference and quantified band under the Odyssey infrared imaging system (LI-COR Biotechnology, Nebraska).

Quantitative real-time PCR (qRT-PCR)

We extracted total RNAs using the TRIzol reagent (Invitrogen). We synthesized complementary DNAs using a PrimeScript™ RT Reagent Kit (TaKaRa Biotechnology, Shiga, Japan). qRT-PCR was performed by a SYBR Green PCR Kit (TaKaRa Biotechnology) on a DNA Engine Opticon 2 System (Bio-Rad Laboratories, Hercules, CA, USA). The corresponding primers were as follows: ACTA2: (F) 5'-AAAAGACAGCTACGTGGGTGA-3' and (R) 5'-GCCATGTTCTATCGGGTACTTC-3'; CD31: (F) 5'-AACAGTGTTGAC ATGAAGAGCC-3' and (R) 5'-TGTAAAACAGCACGTCATCCTT-3'. We

normalized mRNA expression against β-actin. We repeated every experiment for at least three times.

Wound healing assay

We performed HUVEC and HRGEC motility assays by six-well culture dishes. We independently scratched HUVECs and HRGECs with pipette tips, and cells were washed with phosphate-buffered saline (PBS) twice. Fresh RPMI-1640 medium or DMEM medium containing HGF (40 ng/mL) and/or TGF-β1 (5 ng/mL) were added to the scratched cells for 0 and 48 hours. We took images by an inverted microscope (Eclipse TS100, Nikon, Japan) at 100x magnification after incubation. We selected and observed at least three fields of the migrated cells randomly. We used the migration index to manual count migrated cells (motility index=migration cell number [0 h]/migration cell number [48 h]). We repeated the assay at least three times independently.

HUVEC and HRGECs migration assay

Next, we analyzed the influence of HGF on the migration ability of HUVECs and HRGECs. Cells were located on the upper surface of polycarbonate filters with 8 µm pores at a density of 5×10^4/upper chamber in medium with HGF (40 ng/mL) and/or TGF-β1 (5 ng/mL). After incubation at 37°C for 48 hours, non-invasive cells were removed using cotton swabs. We quantified the migration of cells by manually counting cells on the lower surface under a phase contrast microscope (Eclipse TS100, Nikon, Japan) at 100× magnification. We used the migration index to manually count the migrated cells (migration index=migration cell number [48 h]/migration cell number [0 h]). We repeated the assay at least three times.

Enzyme-linked immunosorbent assay (ELISA)

Expression levels of collagen-I and fibronectin (FN) in the supernatant of HUVECs and HRGECs treated with HGF (40 ng/mL) and/or TGF-β1 (5 ng/mL) for 48 hours were measured using PeliKine Compact human ELISA kits (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. We repeated the assay at least three times.

Statistical analysis

We expressed all data as the mean ± standard deviations (SD) of three independent experiments. We performed the Student's *t* test to determine the statistical differences between two groups. A two-way analysis of variance (ANOVA), as well as the Dunnett's post hoc test, was used to analyze between multiple treatments and the control group. We considered a *p* value less than 0.05 as statistically significant. All
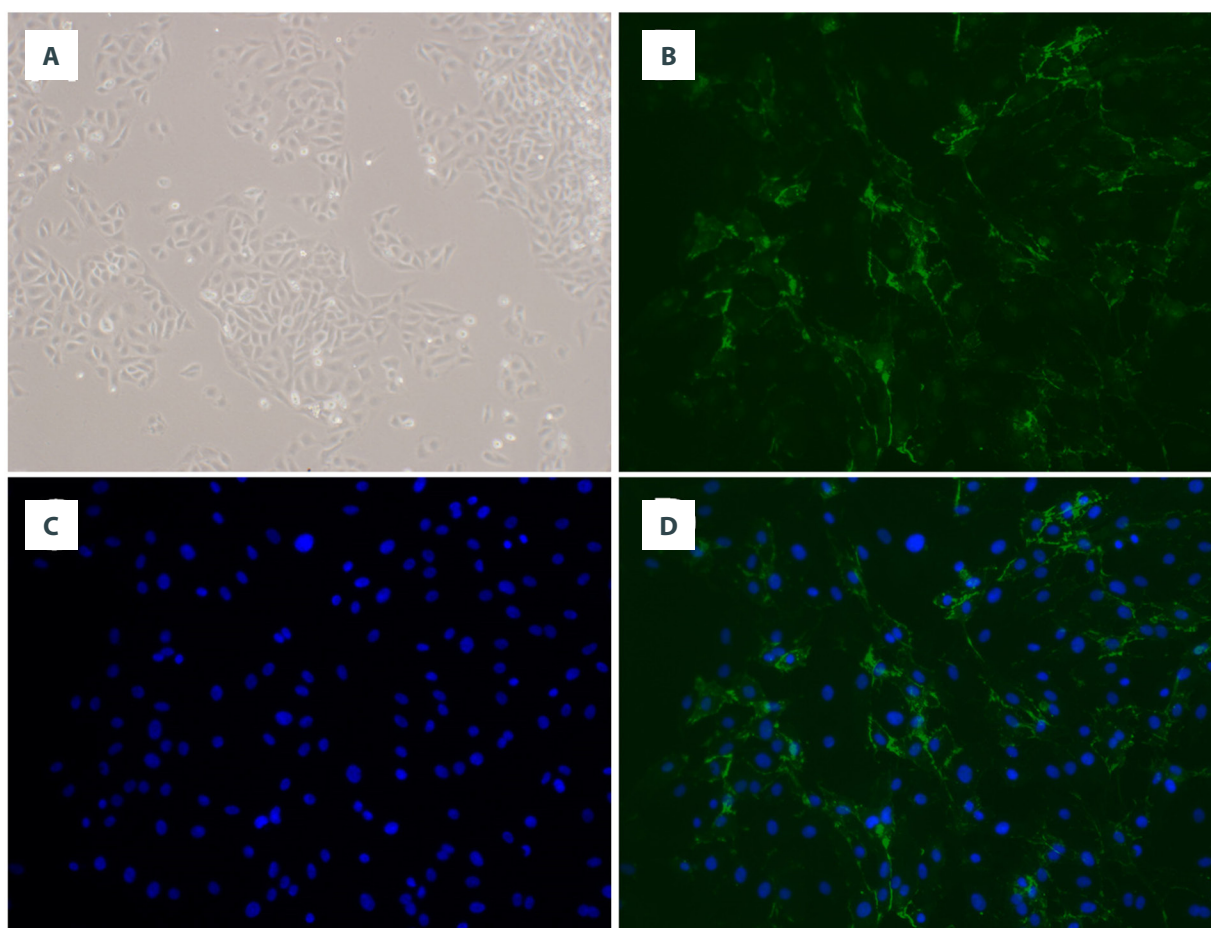
Figure 1. Cells extracted from human umbilical cord segments identified by indirect immunofluorescence staining. (**A**) Extracted cells are morphologically like paving stones under light microscopy (100×). (**B**) CD31 in green light is mainly distributed in the cytoplasm of HUVECs (200×). (**C**) Nuclei of HUVECs are stained with DAPI (blue; 200×). (**D**) Positive staining of merged CD31/DAPI in cells extracted from human umbilical cord segments show that the cells are endothelial cells (200×).

statistical analysis was performed in the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

Identification of HUVECs

Using light microscopy, our morphological analysis of the HUVEC monolayer revealed a small triangular single cluster, which resembled paving stones (Figure 1A). Our indirect immunofluorescence staining analysis of CD31 revealed that the distribution of CD31 antibody is primarily located in the cytomembrane of the HUVECs (Figures 1B–1D). These results identified the extracted cells to be HUVECs.

HGF attenuates the development of TGF-β1-induced EndMT

We observed a significant increase in α-SMA expression, which is a specific marker in mesenchymal cells, in our HUVECs and HRGECs samples, when treated with TGF-β1, and a remarkable decrease when treated with dose-dependent HGF (Figure 2A, 2E). Conversely, we found CD31 expression in HUVECs and HRGECs significantly decreased with TGF-β1 treatment and increased under dose-dependent HGF intervention (Figure 2A, 2E). Quantitative analysis of α-SMA and CD31, as determined by western blotting, is shown in Figure 2B and 2F, respectively. Furthermore, in our qRT-PCR analysis, we observed similar alternations of α-SMA and CD31 mRNA concentrations in HUVECs and HRGECs (Figure 2C, 2D, 2G, 2H). Taken together, these results suggest that HGF treatment significantly attenuates the development of TGF-β1-induced EndMT.
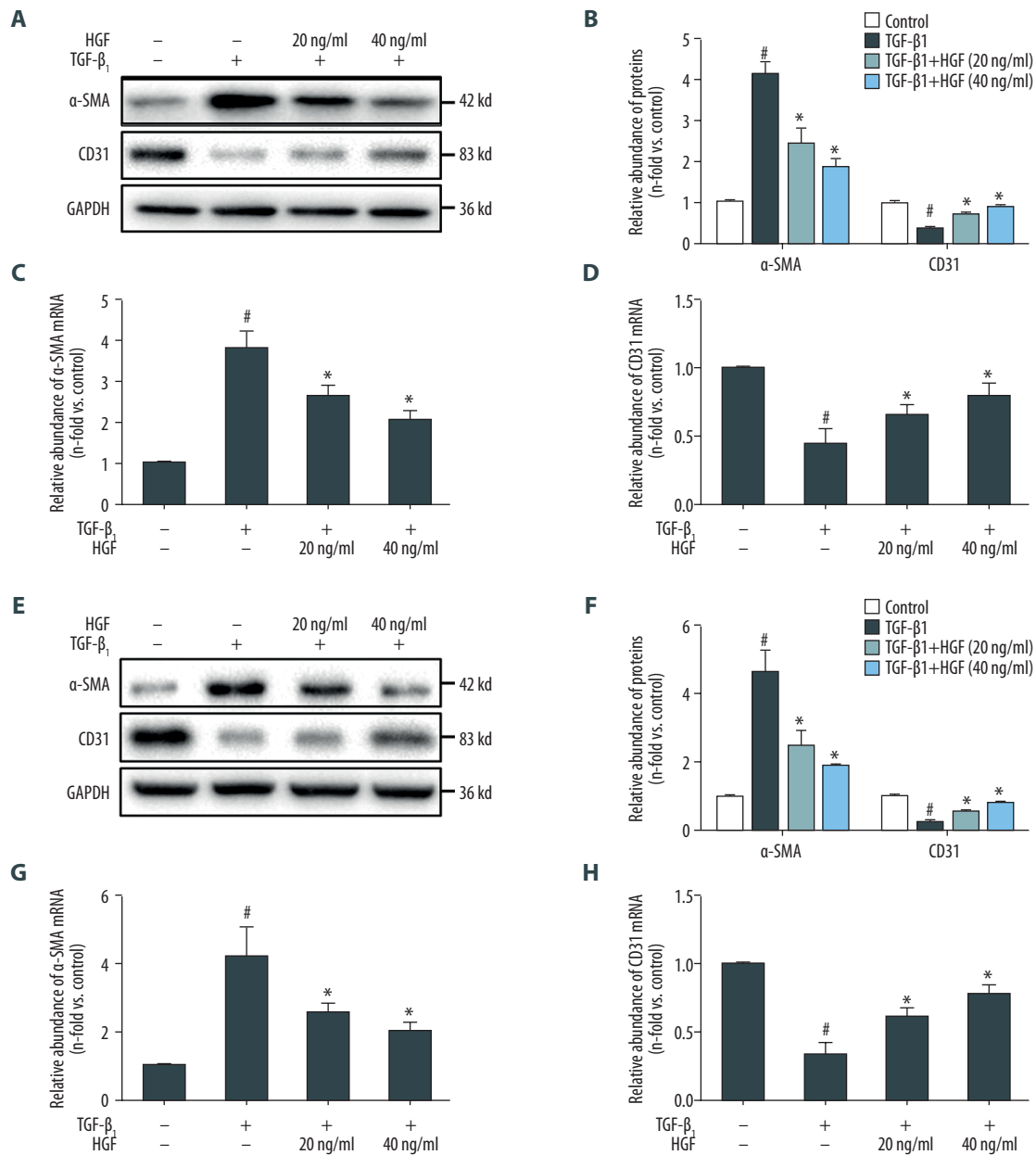
Figure 2. HGF attenuates α-SMA expressions and increases CD31 expression in HUVECs and HRGECs. HUVECs (**A–D**) and HRGECs (**E–H**) were incubated with 5 ng/mL TGF-β1 and/or 20 ng/mL or 40 ng/mL HGF for 48 hours. We analyzed equal amounts of protein from whole cell lysates by western blotting with antibodies against α-SMA, CD31 and GAPDH. We isolated and reversed-transcribed total RNA, and subjected the resultant RNA to quantitative real-time PCR to detect gene expression of α-SMA and CD31. We normalized the quantitative real-time PCR results to β2-macroglobulin. These are expressed as the fold-change relative to unstimulated control cells in HUVECs (**C, D**) and HRGECs (**G, H**). The relative abundance of mRNAs are presented as mean ±SD of three independent experiments. * $p<0.05$, # $p<0.01$, compared with control by Student's t test (**B–D, F–H**).
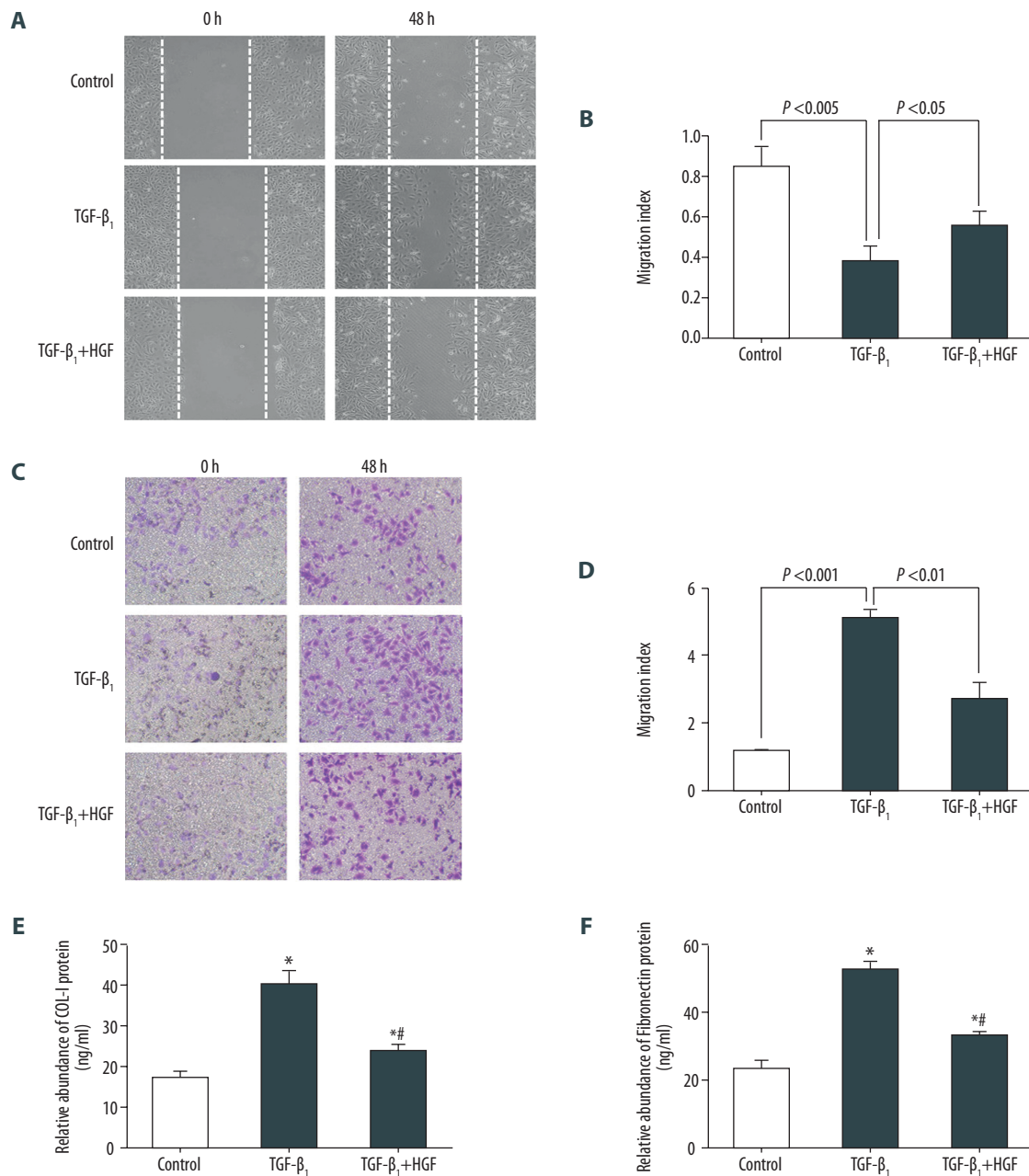
Figure 3. HGF weakens the motility and migration ability of HUVECs and secretion of extracellular matrix. (**A–F**) HUVECs incubated with 40 ng/mL HGF and/or 5 ng/mL TGF-β1 for 48 hours (**A, C, E, F**). (**A, B**) We wounded HUVECs and manually counted migrated cells. The motility index is expressed as the fold-change relative to unstimulated control cells (**B**). (**C, D**) We seeded a total of $5×10^4$ HUVECs in the top chamber, and stained and quantified cells that migrated through the membrane. The migration index is expressed as the fold-change relative to unstimulated control cells (**D**). We collected the supernatant of the cultured HUVECs for ELISA to determine total concentrations of collagen-I (**E**) and fibronectin (**F**). Data are presented as mean ±SD of three independent experiments; * $p<0.05$, # $p<0.01$, compared with TGF-β1 group by Student's t test (**B, D, E, F**).
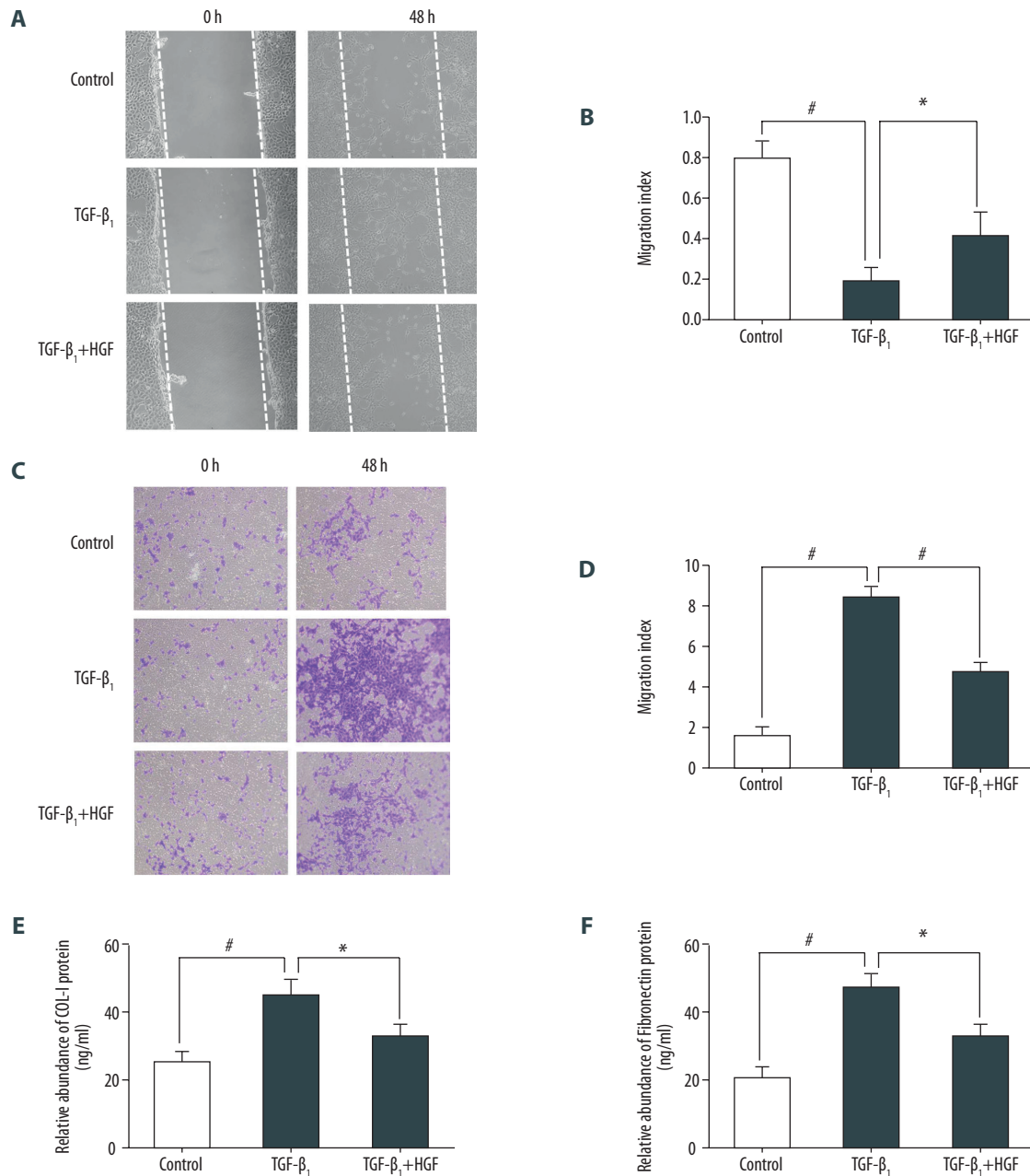
Figure 4. HGF weakens the motility and migration ability of HRGECs and secretion of extracellular matrix. (**A–F**) HRGECs were incubated with 40 ng/mL HGF and/or 5 ng/mL TGF-β1 for 48 hours (**A, C, E, F**). (**A, B**) We wounded HRGECs and manually counted migrated cells. The motility index is expressed as the fold-change relative to unstimulated control cells (**B**). (**C, D**) We seeded a total of 5× 10⁴ HRGECs in the top chamber and stained and quantified cells that migrated through the membrane. The migration index is expressed as the fold-change relative to unstimulated control cells (**D**). We collected the supernatant of the cultured HRGECs for ELISA to determine total concentrations of collagen-I (**E**) and fibronectin (**F**). Data are presented as mean ±SD of three independent experiments; * $p<0.05$, # $p<0.01$, compared with TGF-β1 group by Student's t test (**B, D, E, F**).
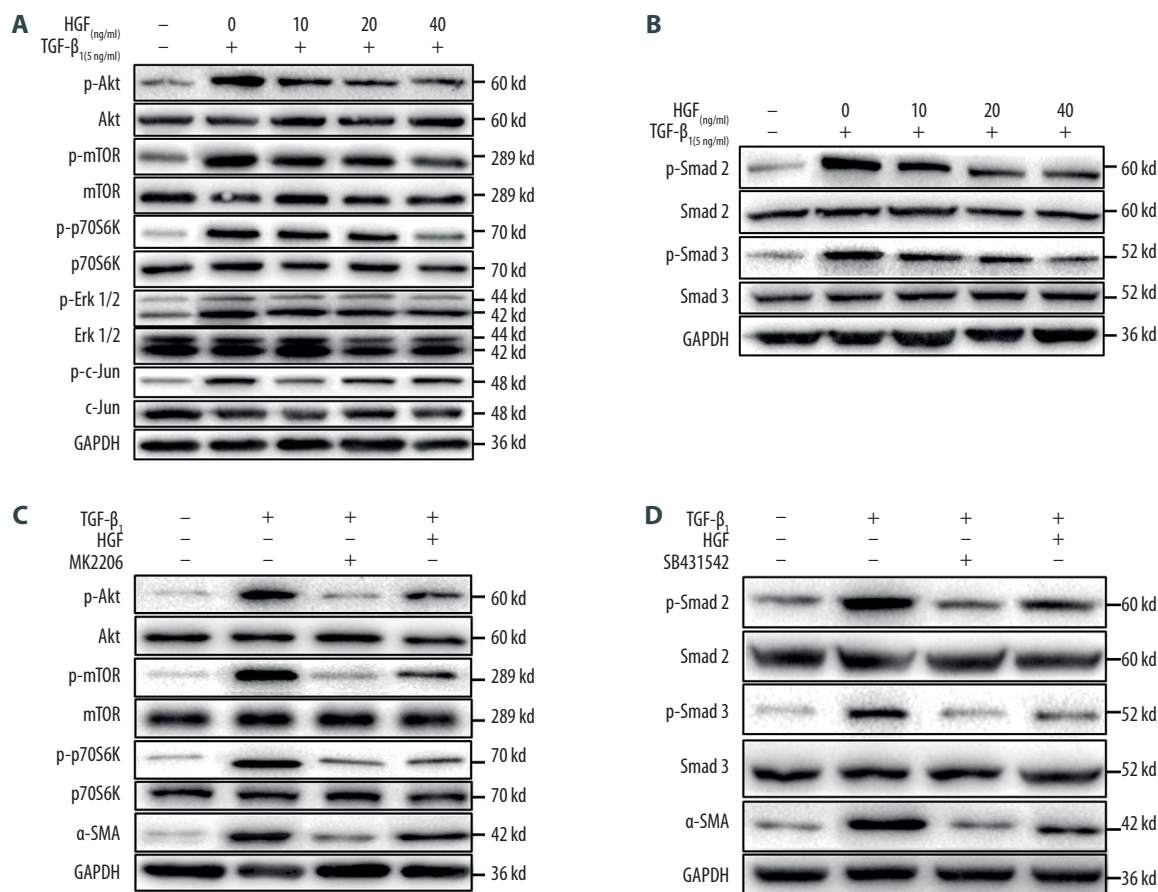
Figure 5. HGF decreases α-SMA expression in HUVECs through TGF-β/Smad and Akt/mTOR/p70S6K signaling pathways. (**A, B**) HUVECs treated with 40 ng/mL HGF and/or 5 ng/mL TGF-β1 for 48 hours. We collected equal amounts of protein from whole cell lysates, which we analyzed by western blotting with antibodies against phosphorylated Smad 2, Smad 3, Akt, mTOR, p70 S6K, Erk1/2, c-Jun. (**C, D**) HUVECs were pretreated for one hour with MK2206 (10 μmol/L) (**C**), and SB431542 (10 μmol/L) (**D**), which are specific chemical inhibitors for Akt and Smad, respectively. Subsequently, we treated cells with 40 ng/mL HGF and/or 5 ng/mL TGF-β1 for 48 hours. We collected cells one hour after HGF and/or TGF-β1 stimulation. We analyzed equal amounts of protein from whole cell lysates by western blotting with antibodies against phosphorylated and total Akt (**C**), phosphorylated and total mTOR (**C**), phosphorylated and total p70S6K (**C**), phosphorylated and total Smad 2 (**D**), phosphorylated and total Smad 3 (**D**). (**C, D**) We collected HUVECs 48 hours after HGF and/or TGF-β1 stimulation and analyzed equal amounts of protein from whole cell lysates by western blotting with antibodies against α-SMA and GAPDH.

HGF weakens the motility ability of endothelial cells

Both HUVECs and HRGECs showed significant inhibition of chemokinesis motility when treated with a combination of TGF-β1 and HGF for 48 hours, compared with TGF-β1 treatment alone (Figures 3A, 3B, 4A, 4B). Likewise, we observed remarkably weakened chemotactic response and migration ability in HUVECs and HRGECs when treated with a combination of TGF-β1 and HGF for 48 hours (Figures 3C, 3D, 4C, 4D).

HGF reduces the secretion of extracellular matrix (ECM) in endothelial cells

To determine the effects of HGF intervention on the secretion of ECM, which have an impact on the development of interstitial fibrosis, we performed the ELISA assay to examine the expression of two basic elements in ECM: collagen-I and FN in the supernatant of both HUVECs and HRGECs culture medium. We found that HGF treatment for 48 hours significantly inhibited ECM secretion in both HUVECs and HRGECs, compared with TGF-β1 treatment alone (Figures 3E, 3F, 4E, 4F).
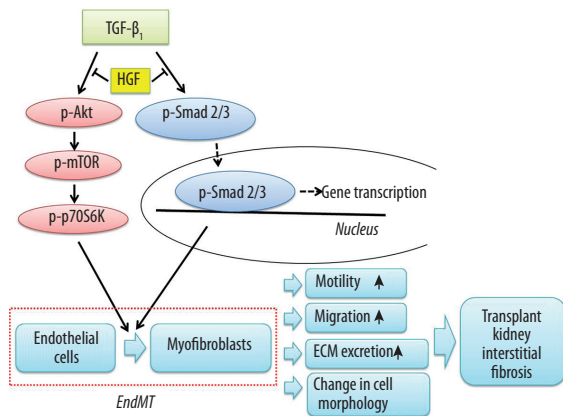
Figure 6. A proposed model illustrating the antifibrotic effects of HGF on TGF-β1-induced EndMT.

HGF attenuates the development of EndMT via TGFβ/Smad and Akt/mTOR/P70S6K signaling pathways

To investigate the roles of various canonical pathways in kidney fibrosis during HGF intervention, we assessed the phosphorylation of key regulators among these pathways. We found that phosphorylated Akt, mTOR, P70S6K, Smad 2 and Smad 3 significantly increased in a dose-dependent manner (Figure 5A, 5B).

Furthermore, we analyzed the selective inhibition of the Akt and TGFβ/Smad pathways before treatment with HGF and/or TGF-β1. Our results showed that the phosphorylation of Akt and regulators in the downstream, mTOR and P70S6K, were all activated with TGF-β1 treatment, and significantly inhibited with the addition of HGF (Figure 5C, 5D). This indicates that HGF attenuates the pathogenesis of EndMT via the TGFβ/Smad and Akt/mTOR/P70S6K signaling pathways.

Discussion

Renal interstitial fibrosis is a key process in the progression of kidney fibrosis and chronic allograft fibrosis. There are four principal cellular protagonists involved in the renal fibrosis progression: epithelial cells, endothelial cells, myofibroblasts, and immune cells [20]. Each plays a crucial role in the response to renal injury, initially aimed to repair and protect the integrity of kidney tissue. The proportion of myofibroblasts is strongly correlated with the severity of renal fibrosis, which provides valuable information when investigating effective therapies that attenuate renal interstitial fibrosis and chronic allograft fibrosis [21]. Moreover, an immense effort has been made to extensively elucidate the molecular and cellular mechanisms of EMT, which has been recognized as primary fibroblast-generating process during the kidney fibrosis progression [22]. EndMT,

similarly to EMT, leads to the loss of endothelial cells, which could be considered as the principal cause of the decreased number of peritubular vessels during the progress of tubular atrophy [23]. Inhibition of EndMT by SIS3, a Smad3 inhibitor, has been reported to attenuate the development of fibrosis in diabetic nephropathy [8]. Consistent with these results, our study suggested mesenchymal cells differentiated from endothelial cells to be an important source of myofibroblasts. Afterwards, further studies addressing the consequences of EndMT on endothelial functions are needed to fully reveal the mechanisms involved in EndMT during renal allograft fibrosis.

There is a large amount of literature demonstrating the role of HGF as an antifibrotic factor preventing tissue fibrosis in various animal models of chronic kidney diseases [24–26]. In this study, we prove that HGF is strongly associated with antifibrotic effects on kidney fibrosis development, and the change in motility and migration ability of endothelial cells, in addition to the signaling pathways of TGFβ/Smad and Akt/mTOR/P70S6K, may be involved in the antifibrotic process (Figure 6). Activation of the TGFβ/Smad pathway, a canonical pathway in tissue fibrosis, is known to be a crucial factor and significantly correlated with the induction of excessive accumulation of ECM [27–29]. Previously, we have shown that the Akt pathway is activated during TGF-β1-induced EndMT in renal allograft fibrosis [9]. Here, we investigated the effect of HGF on the activation of Akt and its down-stream modulators and reported the notable relationship between Akt/mTOR/P70S6K pathways and kidney fibrosis, which is consistent with our previous findings *in vivo* and in *vitro* [30,31]. These findings strongly implicate the antifibrotic effects of HGF in chronic allograft fibrosis. Furthermore, numerous studies have reported that HGF significantly affects EMT development in tissue fibrosis [32–34]. Here, we reveal a significant correlation between HGF and EndMT, which remains an important process in tissue fibrosis. Our findings also provide evidence for the essential role and contributions of endothelial cells to the myofibroblats pool in kidney fibrosis. We are currently processing these findings in the rat model of chronic allograft nephropathy, to confirm these findings *in vivo*.

Conclusions

In conclusion, our study suggests that HGF treatment significantly attenuates the development of TGF-β1-induced EndMT through the TGFβ/Smad and Akt/mTOR/P70S6K signaling, affecting the motility and migration of endothelial cells. These findings provided novel insights to the prevention and treatment of allograft interstitial fibrosis and CAD following renal transplant.

Conflict of interests

None.

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