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Original Article

Knockdown of eIF3a inhibits collagen synthesis in renal fibroblasts via Inhibition of transforming growth factor-β1/Smad signaling pathway

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Abstract: Renal fibrosis is characterized by an exacerbated accumulation of deposition of the extracellular matrix (ECM). The eukaryotic translation initiation factor (eIF) 3a is the largest subunit of the eIF3 complex and has been involved in pulmonary fibrosis. However, the role of eIF3a in rental fibrosis is still unclear. Therefore, in this study, we investigated the role of eIF3a in rental fibrosis and explored the underlying mechanism. Our study found that eIF3a was up-regulated in renal fibrotic tissues and transforming growth factor (TGF)-β1-treated HK-2 cells. In addition, knockdown of eIF3a significantly inhibited TGF-β1-induced expression levels of α-smooth muscle actin (α-SMA) and collagen I. Furthermore, knockdown of eIF3a attenuated TGF-β1-induced Smad3 activation in HK-2 cells. Taken together, these results suggest that knockdown of eIF3a inhibits collagen synthesis in renal fibroblasts via inhibition of TGF-β1/Smad signaling pathway, and eIF3a may be a potential molecular target for the treatment of renal fibrosis.

Keywords: Eukaryotic translation initiation factor (eIF) 3a, renal fibrosis, TGF-β1

Introduction

Renal fibrosis is generally recognized as a common pathological feature of chronic kidney disease (CKD) [1]. It is characterized by the accumulation of extracellular matrix (ECM), primarily consisting of collagen IV and fibronectin [2]. Despite recent therapeutic advances in the management of renal fibrosis, however, effective therapy for renal fibrosis is still lacking. This is largely attributed to a lack of complete understanding of the exact mechanisms for renal fibrosis. Therefore, further understanding of the molecular mechanisms of renal fibrosis and the development of new therapeutic tools based on these mechanisms are required.

A growing body of evidence demonstrated that the transforming growth factor (TGF)-β pathway is a key mediator of progressive renal fibrosis [3-5]. The expression of TGF-β1 was elevated in animal models of renal fibrosis [6]. TGF-β1 exerts its biological functions through interaction with TGF-β1 receptors, which are composed of type I (TbetaRI) and type II (TbetaRII) receptors [7]. Activated TbetaRI induces phosphorylation of Smad3, which forms a complex with Smad4, and then is translocated to nuclei where it drives expression of TGF-β target genes such as collagens. So, inhibition of TGF-β signaling may be an important therapeutic approach for renal fibrosis.

The eukaryotic translation initiation factor (eIF) 3a is a 170 kDa protein and presents the largest subunit of the eIF3 complex. It has been suggested to play roles in regulating translation of a subset of mRNAs and in regulating cell cycle progression and cell proliferation [8, 9]. eIF3a is upregulated in various cancers, including breast, cervical, colon, esophageal to lung, and gastric cancers [10-12]. Most recently, one study reported that the expression of eIF3a was obviously increased in lungs of pulmonary fibrosis rats accompanied by up-regulation of α-smooth muscle actin (α-SMA) and collagens [13]. However, the role of eIF3a in rental fibrosis is still unclear. In this study, the role of eIF3a in rental fibrosis was investigated. We found that knockdown of eIF3a inhibits collagen synthesis in rental fibroblasts via inhibition of TGF-β1/Smad signaling pathway.

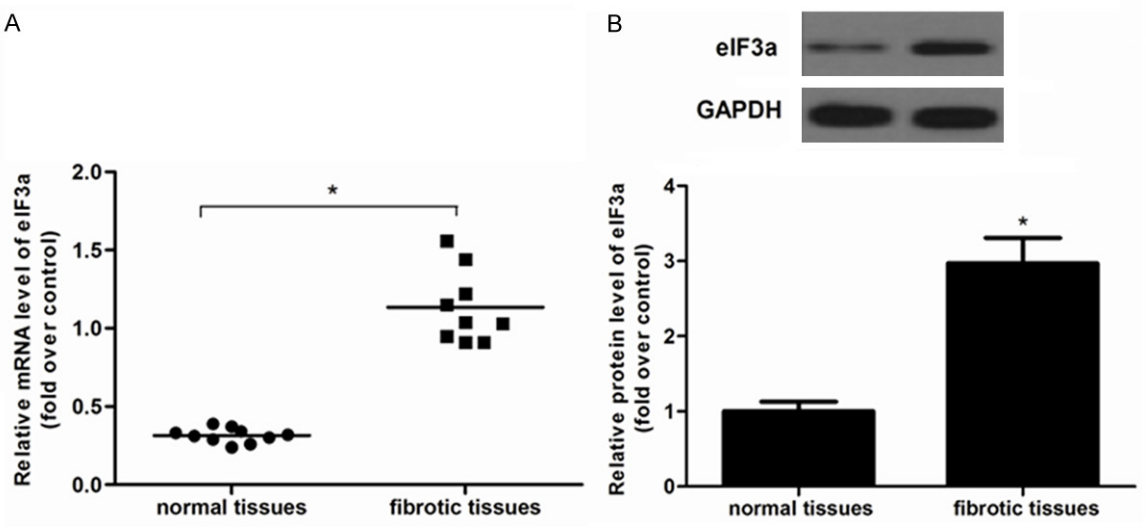

Figure 1. eIF3a expression in renal fibrosis tissues. A. RNA was harvested from healthy and fibrotic human renal tissues, and eIF3a expression was assessed by qRT-PCR. B. Represent western blots of eIF3a in healthy and fibrotic human renal tissues. The levels of proteins were normalized based on the GAPDH levels. *P<0.05 vs. control group.

Materials and methods

Specimen collection

Renal biopsy samples were collected by transparietal puncture from 10 healthy individuals and 10 patients with renal fibrosis, diagnosed on clinical, biological, and histological grounds. All subjects provided written informed consent, and the specimen collection procedure was approved by the Medical Ethics Committee of the Huadu District People's Hospital, Southern Medical University.

Cell culture and treatment

The human proximal tubular epithelial cell line (HK-2) was obtained from American Type Cell Collection (ATCC). Cells were maintained in six-well plates (Sarstedt, Germany) in Keratinocyte-Serum Free (KSF, Invitrogen, USA) Medium cultured at 37°C in a humidified 5% CO_2 atmosphere. For TGF-β1 treatment, HK2 cells were starved for 24 h by incubation with 0.5% fetal bovine serum (FBS) containing DMEM and then were treated with TGF-β1 (R & D Systems, Minneapolis, MN) at a concentration of 20 ng/ml for various periods of time and for 24 h with various concentrations.

siRNA transfection

siRNA-eIF3a and siRNA-scramble were purchased from Cell Signaling (Beverly, MA). For transfection, cells were plated and grown to 70-90% confluency without antibiotics, and then incubated with a mixture of siRNA and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in 100 μl serum-free DMEM, according to the manufacturer's protocol. The transfection efficiency was examined by Real-Time PCR and Western blotting.

Real-time PCR

Total RNA was extracted from kidney tissues and cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then, 2 μg of total RNA was transcribed to first-strand cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The following primers were used: eIF3a, 5'-TCAAGTCGCCGGACGATA-3' (sense), 5'-CCTGTCATCAGCACGTCTCCA-3' (antisense); α-SMA, 5'-CTATTCCTTCGTGACTACT-3' (sense), 5'-ATGCTGTTATAGGTGGTGGTT-3' (antisense); collagen I, 5'-CCAACTGAACGTGACCA-AAAACCA-3' (sense), 5'-GAAGGTGCTGGGTAGG-GAAGTAGGC-3' (antisense); and GAPDH 5'-GG-CAAATTCAACGGCACAGTC-3' (sense), 5'-GCTGA-CAATCTTGAGTGAGTT-3' (antisense). The protocol comprised 42 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 1 min each. Reactions were run on a real-time PCR system (ABI PRISMH 7700, Applied Biosystems). GAPDH was used as an internal control for sample normalization.

Western blot

Total protein was extracted from renal fibrosis tissues and cells, then washed with ice-cold

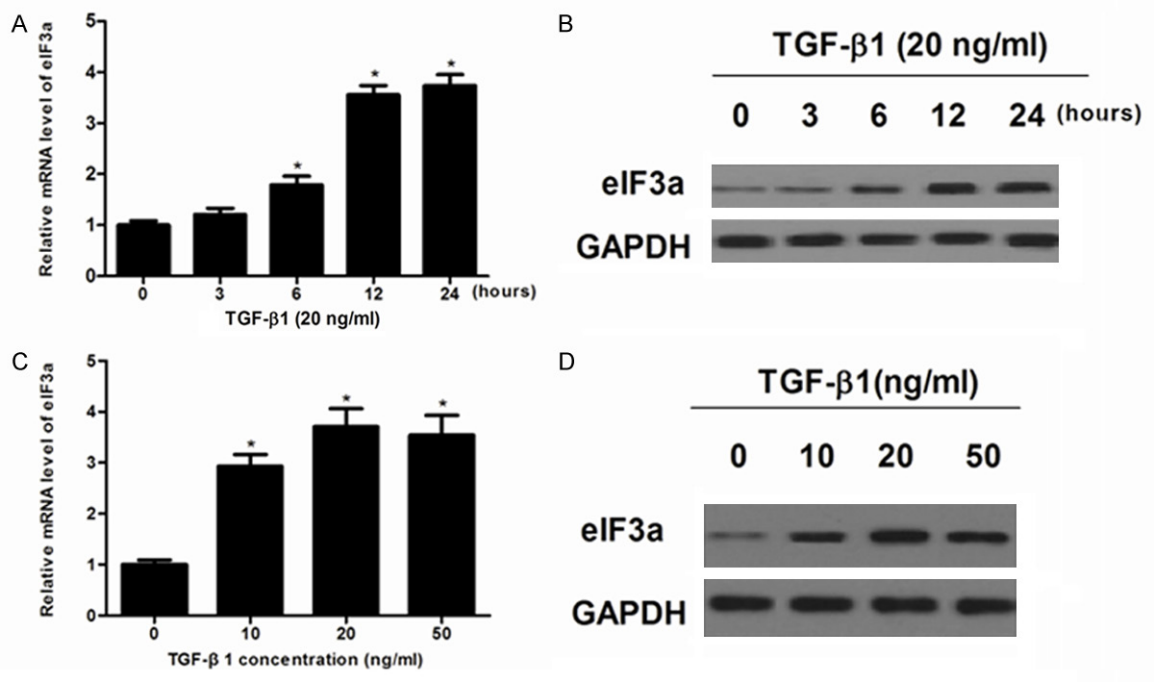

Figure 2. eIF3a is induced rapidly in TGF-β1-treated HK-2 cells. A and B. Time-dependent induction of eIF3a mRNA and protein expression; C and D. concentration-dependent induction of eIF3a by TGF-β1. At least three independent experiments were performed. *P<0.05 vs. no TGF-β1 treatment.

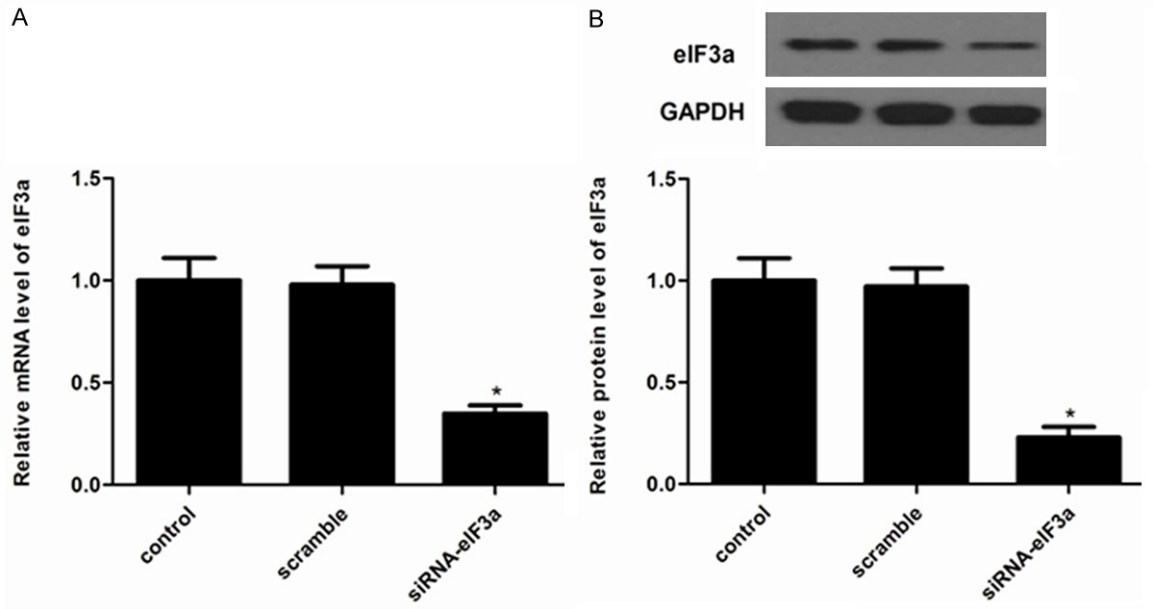

Figure 3. The expression of eIF3a was significantly decreased in siRNA-eIF3a-transfected HK-2 cells. A. eIF3a mRNA expression was detected by qRT-PCR 24 h after transfection with siRNA-eIF3a. B. Western blot analysis of eIF3a expression 24 h after transfection with siRNA-eIF3a. GAPDH was used as a control. At least three independent experiments were performed. *P<0.05 vs. control group.

PBS and lysed with RIPA Cell Lysis Buffer (Cell Signaling) containing a phosphatase inhibitor and the protease inhibitor cocktail (Sigma, St. Louis, MO, USA), by incubating on ice for 30 min. Protein concentration was determined using the Bradford method. The samples (30

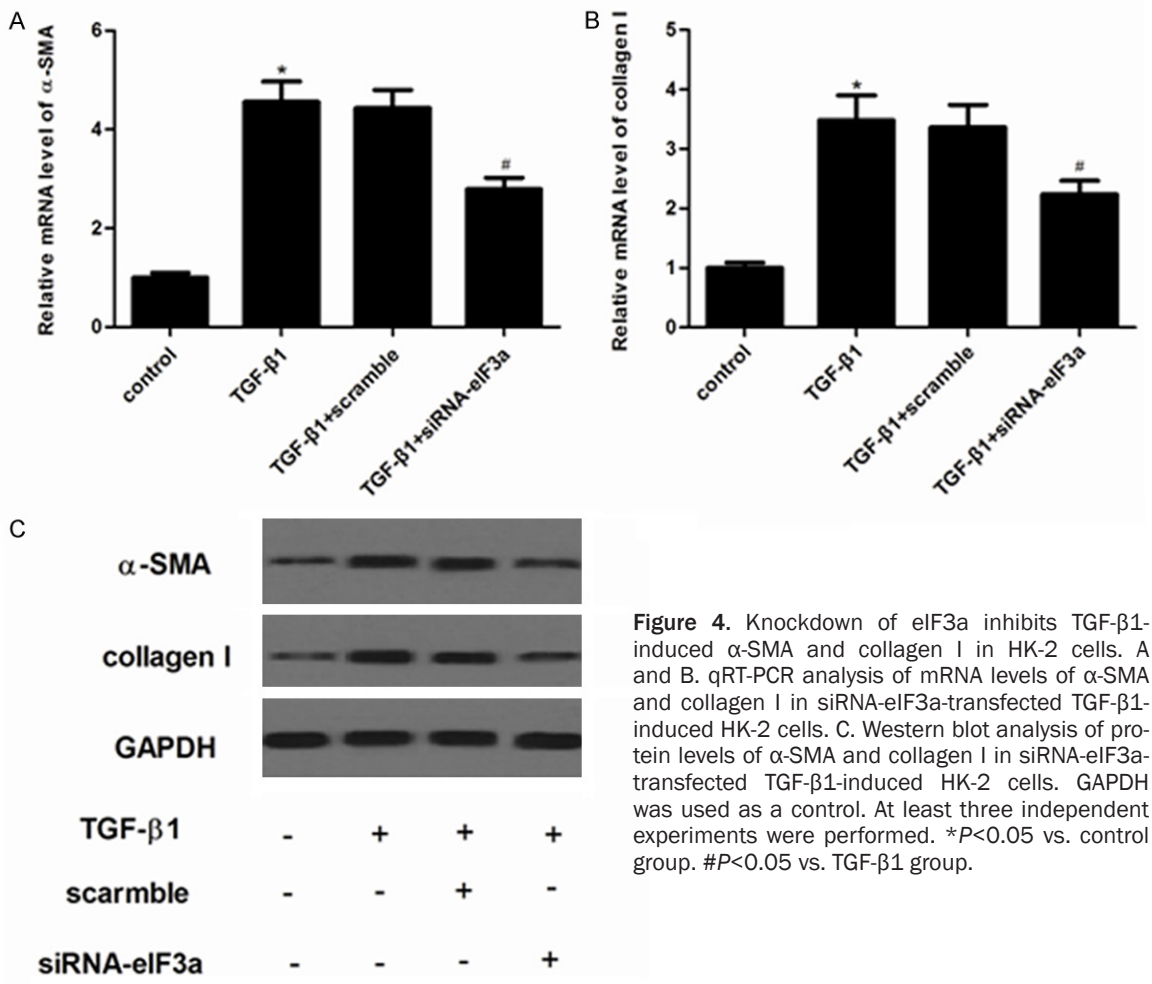

Figure 4. Knockdown of eIF3a inhibits TGF-β1-induced α-SMA and collagen I in HK-2 cells. A and B. qRT-PCR analysis of mRNA levels of α-SMA and collagen I in siRNA-eIF3a-transfected TGF-β1-induced HK-2 cells. C. Western blot analysis of protein levels of α-SMA and collagen I in siRNA-eIF3a-transfected TGF-β1-induced HK-2 cells. GAPDH was used as a control. At least three independent experiments were performed. **P*<0.05 vs. control group. #*P*<0.05 vs. TGF-β1 group.

μg protein/lane) were separated on 10% SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). After blocking with 10% fat-free milk in TBS (20 mmol/l Tris, 0.15 mol/L NaCl (pH 7.0), 0.1% Tween 20), the membranes were incubated at 4°C overnight with a primary antibody: anti-eIF3a (Abcam, Cambridge, UK) diluted 1:1000; anti-α-SMA (Abcam, Cambridge, UK) diluted 1:500; anti-collagen I (Abcam, Cambridge, UK) diluted 1:1000; anti-Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:800; anti-p-Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:800. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Invitrogen, Carlsbad, CA, USA) and images were captured using LAS 4000 (Fujifilm, Tokyo, Japan).

Statistical analysis

Data depicted in graphs represent the means ± SEM. Differences between groups were determined using ANOVA tests with post hoc Bonferroni tests. $P<0.05$ is considered significant.

Results

eIF3a expression in renal fibrosis tissues

To verify the expression of eIF3a in renal fibrosis, we determined the protein and mRNA levels of eIF3a in renal fibrosis. As shown in **Figure 1A**, the mRNA expression levels of eIF3a in rental fibrosis tissues was increased significantly compared to that of controls ($P<0.05$). Consistent with the results of qRT-PCR, west-

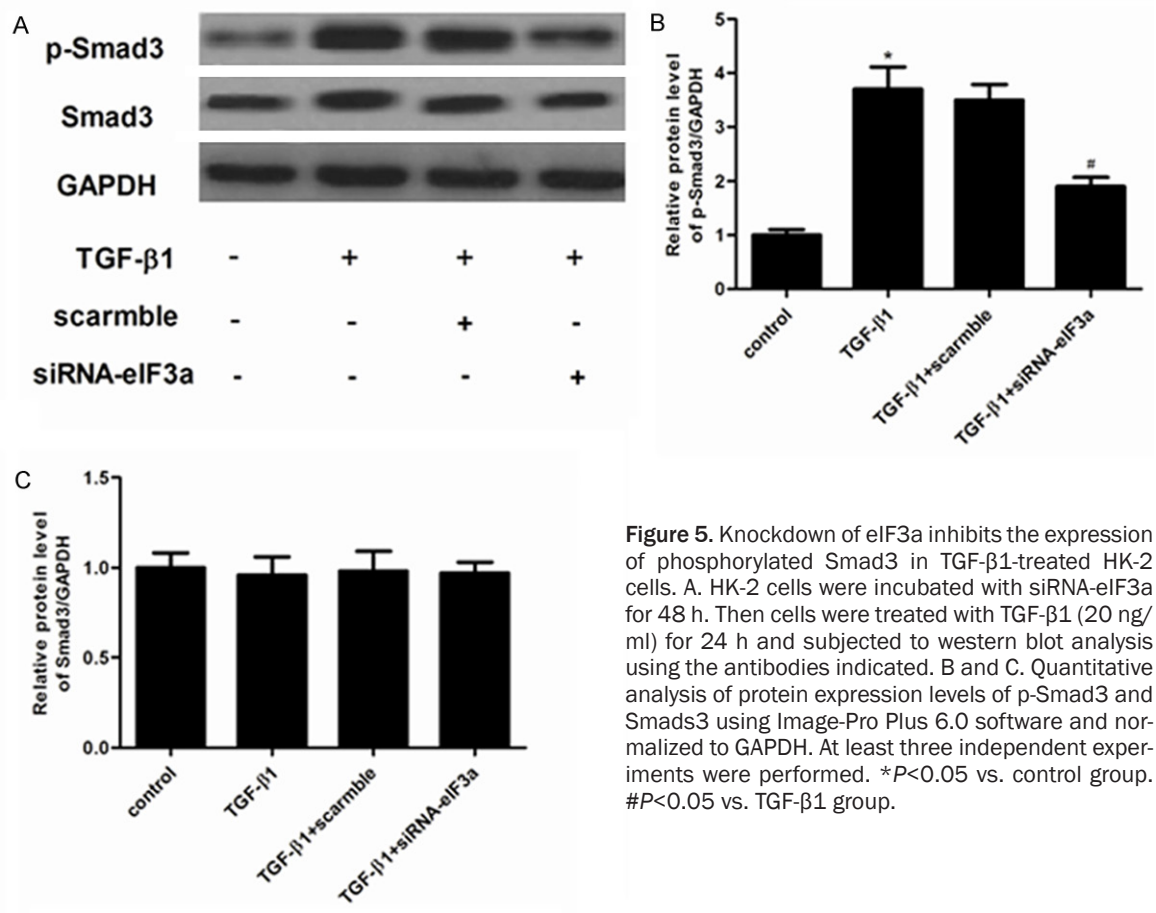

Figure 5. Knockdown of eIF3a inhibits the expression of phosphorylated Smad3 in TGF-β1-treated HK-2 cells. A. HK-2 cells were incubated with siRNA-eIF3a for 48 h. Then cells were treated with TGF-β1 (20 ng/ml) for 24 h and subjected to western blot analysis using the antibodies indicated. B and C. Quantitative analysis of protein expression levels of p-Smad3 and Smads3 using Image-Pro Plus 6.0 software and normalized to GAPDH. At least three independent experiments were performed. *P<0.05 vs. control group. #P<0.05 vs. TGF-β1 group.

ern blot analysis showed that eIF3a protein expression was also upregulated in rental fibrosis tissues, as compared with the control group (**Figure 1B**).

eIF3a is induced rapidly in TGF-β1-treated HK-2 cells

Then, we determined the expression of eIF3a in TGF-β1-treated HK-2 cells. As shown in **Figure 1A**, that the level of eIF3a mRNA was increased at 2 h and that the increase was sustained at least until 24 h. Western blotting revealed that eIF3a protein was abundant 6 h after TGF-β1 treatment, and the increase was sustained for at least 24 h (**Figure 2B**). Then, to determine the optimal concentration of TGF-β1, we used various concentrations. When 1 ng/ml was applied to HK-2 cells, the level of eIF3a mRNA started to increase, and the maximal induction was observed at 20 ng/ml TGF-β1 (**Figure 2C**). Moreover, consistent with the mRNA level, the level of eIF3a protein was also increased by TGF-β1 (**Figure 2D**). These results suggested that the elevated level of eIF3a by TGF-β1 was time-and concentration-dependent.

Knockdown of eIF3a inhibits TGF-β1-induced α-SMA and collagen I in HK-2 cells

In order to further investigate role of eIF3a in TGF-β1 stimulation, we constructed eIF3a siRNA to knockdown the expression of eIF3a. The efficiency of siRNA transfection was measured by qRT-PCR and Western blot. As shown in **Figure 3**, siRNA-eIF3a significantly decreased the expression levels of eIF3a. Then, we examined the effect of siRNA-eIF3a on α-SMA and collagen I expression levels in TGF-β1-treated HK-2 cell. As shown in **Figure 4**, TGF-β1 significantly increased the mRNA expression of α-SMA and collagen I, however, siRNA-eIF3a inhibits TGF-β1-induced mRNA expression of α-SMA and collagen I in HK-2 cells (**Figure 4A** and **4B**). Similarly, siRNA-eIF3a also inhibits TGF-β1-induced protein expression of α-SMA and collagen I in HK-2 cells (**Figure 4C**).

Knockdown of eIF3a inhibits the expression of phosphorylated Smad3 in TGF-β1-treated HK-2 cells

It is well know that TGF-β promotes fibrosis activating specific downstream Smad proteins. To elucidate the potential mechanism of eIF3a in rental fibrosis, we investigated the effect of eIF3a on the protein levels of p-Smad3 and Smad3 in TGF-β1-treated HK-2 cells. As shown in **Figure 5**, TGF-β1 significantly increased the expression of phosphorylated Smad3, however, the ratio of p-Smad3 to Smad3 was decreased in the siRNA-eIF3a treatment groups, as compared with the TGF-β1-stimulated HK-2 cells groups.

Discussion

To the best of our knowledge, this study is the first to demonstrate the critical role of eIF3a in renal fibrosis. We found that the mRNA and protein of eIF3a were significantly up-regulated in renal fibrotic tissues and TGF-β1-treated HK-2 cells. eIF3a was silenced by a specific siRNA; knockdown of eIF3a significantly inhibited TGF-β1-induced expression levels of α-SMA and collagen I. We also observed that knockdown of eIF3a attenuated TGF-β1-induced Smad3 activation in HK-2 cells.

eIF2, composed of α, β, and γ subunits, is a key factor in cap-dependent translation initiation [14]. Wylie et al showed that IF2α is involved in the process of mouse embryonic fibroblast proliferation and differentiation [15]. Among the eIFs family, eIF3a is the largest subunit of eIF3 complex, has been demonstrated that the expression of eIF3a was obviously increased in lungs of pulmonary fibrosis rats [13]. Consistent with these findings, in this study, we found that eIF3a were significantly up-regulated in renal fibrotic tissues and TGF-β1-treated HK-2 cells. Our data implied that eIF3a may play an important role in the development of renal fibrosis.

The pathogenesis of renal fibrosis is a process of excessive accumulation and deposition of extracellular matrix components [16]. Previous studies demonstrated that TGF-β1 could induce α-SMA expression in human renal tubular epithelia cells [17]. Similar results were also found by Lu and colleagues which suggest TGF-β1 time-and dose-dependently induced renal fibroblast collagen type I production [18]. In line with these reports, in this study, we found that TGF-β1 induced expression levels of α-SMA and collagen I, while, siRNA-eIF3a significantly inhibited TGF-β1-induced expression levels of α-SMA and collagen I in HK-2 cells.

TGF-β1 has been identified as a key pro-fibrotic mediator in fibrotic diseases [19]. It initiates canonical and non-canonical pathways to exert multiple biological effects. Among them, Smad signaling is recognized as a major pathway of TGF-β1 signaling in progressive renal fibrosis. During fibrogenesis, Smad3 is highly activated, which is associated with the down-regulation of an inhibitory Smad7 via anubiquitin E3-ligases-dependent degradation mechanism [20]. Activated Smad2/3 proteins form oligomeric complexes with Smad4 proteins and translocate into the nucleus, where they induce the expression of target genes, including ECM proteins, contributing to the development of tubulointerstitial and glomerular fibrosis [21, 22]. It has been reported that blocking TGF-β1 with neutralizing antibody, antisense oligonucleotides, inhibitors, or genetic deletion of receptors can attenuate kidney fibrosis *in vivo* and *in vitro* [23-25]. It has been observed that UUO induces an increase in Smad2 and Smad3 phosphorylation [26]. In this study, we found that knockdown of eIF3a inhibits the expression of phosphorylated Smad3 in TGF-β1-treated HK-2 cells. The results implied that siRNA-eIF3a inhibits collagen synthesis through suppressing inhibiting the TGF-β1/Smad signaling pathway.

In conclusion, we demonstrated that eIF3a was involved in the process of renal fibrosis. Knockdown of eIF3a inhibits collagen synthesis in renal fibroblasts via inhibition of TGF-β1/Smad signaling pathway, and eIF3a may be a potential molecular target for the treatment of renal fibrosis.

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Disclosure of conflict of interest

None.

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References

[1] Kuncio GS, Neilson EG, Haverty T. Mechanisms of tubulointerstitial fibrosis. Kidney Int 1991; 39: 550-556.

[2] Ruiz-Torres MP, Lopez-Ongil S, Griera M, Diez-Marques ML, Rodriguez-Puyol M, Rodriguez-Puyol D. The accumulation of extracellular matrix in the kidney: consequences on cellular function. J Nephrol 2005; 18: 334-340.

[3] Liu N, He S, Ma L, Ponnusamy M, Tang J, Tolbert E, Bayliss G, Zhao TC, Yan H, Zhuang S. Blocking the class I histone deacetylase ameliorates renal fibrosis and inhibits renal fibroblast activation via modulating TGF-beta and EGFR signaling. PLoS One 2013; 8: e54001.

[4] Samarakoon R, Overstreet JM, Higgins SP, Higgins PJ. TGF-β1→ SMAD/p53/USF2→ PAI-1 transcriptional axis in ureteral obstruction-induced renal fibrosis. Cell Tissue Res 2012; 347: 117-128.

[5] Wu CF, Chiang WC, Lai CF, Chang FC, Chen YT, Chou YH, Wu TH, Linn GR, Ling H, Wu KD. Transforming growth factor β-1 stimulates profibrotic epithelial signaling to activate pericyte-myofibroblast transition in obstructive kidney fibrosis. Am J Pathol 2013; 182: 118-131.

[6] López-Hernández FJ, López-Novoa JM. Role of TGF-β in chronic kidney disease: an integration of tubular, glomerular and vascular effects. Cell Tissue Res 2012; 347: 141-154.

[7] Böttinger EP. TGF-β in renal injury and disease. Semin Nephrol 2007; 27: 309-320.

[8] Dong Z, Liu Z, Cui P, Pincheira R, Yang Y, Liu J, Zhang JT. Role of eIF3a in regulating cell cycle progression. Exp Cell Res 2009; 315: 1889-1894.

[9] Dong Z, Zhang JT. Initiation factor eIF3 and regulation of mRNA translation, cell growth, and cancer. Crit Rev Oncol Hematol 2006; 59: 169-180.

[10] Haybaeck J, O'CONNOR T, Spilka R, Spizzo G, Ensinger C, Mikuz G, Brunhuber T, Vogetseder A, Theurl I, Salvenmoser W. Overexpression of p150, a part of the large subunit of the eukaryotic translation initiation factor 3, in colon cancer. Anticancer Res 2010; 30: 1047-1055.

[11] Chen G, Burger MM. p150 expression and its prognostic value in squamous-cell carcinoma of the esophagus. Int J Cancer 1999; 84: 95-100.

[12] Chen G, Burger MM. p150 overexpression in gastric carcinoma: the association with p53, apoptosis and cell proliferation. Int J Cancer 2004; 112: 393-398.

[13] Li XW, Wu YH, Li XH, Li D, Du J, Hu CP, Li YJ. Role of eukaryotic translation initiation factor 3a In bleomycin-induced pulmonary fibrosis. Eur J Pharmacol 2015; 749: 89-97.

[14] TV P, VG K, IB L, EV P, IN S, VI A, CU H. Molecular Mechanisms of Translation Initiation in Eukaryotes. Proc Natl Acad Sci U S A 2001; 98: 7029-7036.

[15] Wylie KM, Schrimpf JE, Morrison LA. Increased eIF2α phosphorylation attenuates replication of herpes simplex virus 2 vhs mutants in mouse embryonic fibroblasts and correlates with reduced accumulation of the PKR antagonist ICP34. 5. J Virol 2009; 83: 9151-9162.

[16] Liu Y. Renal fibrosis: new insights into the pathogenesis and therapeutics. Kidney Int 2006; 69: 213-217.

[17] Chen YX, Li Y, Wang WM, Zhang W, Chen XN, Xie YY, Lu J, Huang QH, Chen N. Phosphoproteomic study of human tubular epithelial cell in response to transforming growth factor-beta-1-induced epithelial-to-mesenchymal transition. Am J Nephrol 2010; 31: 24-35.

[18] Lu J, Shi J, Li M, Gui B, Fu R, Yao G, Duan Z, Lv Z, Yang Y, Chen Z. Activation of AMPK by metformin inhibits TGF-β-induced collagen production in mouse renal fibroblasts. Life Sci 2015; 127: 59-65.

[19] Branton MH, Kopp JB. TGF-β and fibrosis. Microbes Infect 1999; 1: 1349-1365.

[20] Cutroneo KR. TGF-β-induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring. Wound Repair Regen 2007; 15: S54-S60.

[21] Park BB, sun Yoon J, shil Kim E, Choi J, woong Won Y, hye Choi J, Lee YY. Inhibitory effects of eupatilin on tumor invasion of human gastric cancer MKN-1 cells. Tumor Biol 2013; 34: 875-885.

[22] Cho JH, Lee JG, Yang YI, Kim JH, Ahn JH, Baek NI, Lee KT, Choi JH. Eupatilin, a dietary flavonoid, induces G2/M cell cycle arrest in human endometrial cancer cells. Food Chem Toxicol 2011; 49: 1737-1744.

[23] Border WA, Noble NA. Evidence that TGF-&bgr; should be a therapeutic target in diabetic nephropathy. Kidney Int 1998; 54: 1390-1391.

[24] Moon J, Kim H, Cho I, Sheen Y, Kim D. IN-1130, a novel transforming growth factor-β type I receptor kinase (ALK5) inhibitor, suppresses renal fibrosis in obstructive nephropathy. Kidney Int 2006; 70: 1234-1243.

[25] Petersen M, Thorikay M, Deckers M, Van Dinther M, Grygielko E, Gellibert F, De Gouville A, Huet S, Ten Dijke P, Laping N. Oral administration of GW788388, an inhibitor of TGF-β type I and II receptor kinases, decreases renal fibrosis. Kidney Int 2008; 73: 705-715.

[26] Lan HY. Diverse roles of TGF-β/Smads in renal fibrosis and inflammation. Int J Biol Sci 2011; 7: 1056-1067.