

Long Noncoding RNA uc.412 Promotes Mesangial Cell Fibrosis via Binding ELAVL1 in Glomerulosclerosis

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Research

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Abstract

Background: Glomerulosclerosis is a characteristic pathologic feature in chronic kidney disease (CKD). Convincing evidence indicates that the mesangial cells (MCs) play critical role in this process. However, the exact mechanism remains unclear. Using RNA-seq analysis, we previously found that lncRNA uc.412 was involved in the MC proliferation. Here, the effect of uc.412 on glomerular fibrosis and the potential mechanism were explored.

Methods: *In vivo*, CKD mice models were established by 5/6 nephrectomy. The expression of lncRNA uc.412 in CKD was detected by Real-Time PCR. *In vitro*, MCs were intervened with TGF- β 1 (10ng/mL). The uc.412 expression in MCs was detected by in site hybridization. MCs were transfected with uc.412 siRNA or a lentivirus targeting uc.412 and then examined using western blot, Real-Time PCR, RNA pull down assay and immunofluorescence staining.

Results: We found that the expression of uc.412 was significantly increased in CKD mice and is induced by TGF- β 1 via Smad3- dependent signal pathway. Overexpressing uc.412 caused MCs fibrosis and knockdown of uc.412 alleviated TGF- β 1-induced MCs fibrosis. Using RNA pull down analysis, we found that the ELAVL1 was the specific binding protein for uc.412. Moreover, ELAVL1 expression was increased in TGF- β 1-treated MCs and silencing ELAVL1 expression attenuated MCs fibrosis.

Conclusions: Thus, here, we demonstrated that uc.412, which is regulated in a Smad3-dependent mechanism, is significantly increased during progression of CKD via regulating ELAVL1 expression. Our findings provided the therapeutic strategy for treatment of CKD.

Background

Chronic kidney disease (CKD) has become a burden on global social health, owing to increasing morbidity of hypertension, diabetes mellitus and advancing age. Chronic inflammation, glomerulosclerosis and tubulointerstitial fibrosis, causing a drop in kidney function and progressing to end-stage renal disease, are characterized features of CKD [1]. Glomerulosclerosis is a characteristic pathologic feature marked by accumulation of extracellular matrix (ECM). It is conceivable that mesangial cells (MCs) play instrumental role in glomerular fibrotic process. In models of glomerulosclerosis, it is conformed that there is an increase of mesangial compartment size owing to mesangial matrix deposition and MCs proliferation [2]. However, the mechanisms and functions of MCs during the process of sclerosis remain unclear. Exploring mechanism provides targeted therapy for CKD.

It is now clear that transforming growth factor- β 1 (TGF- β 1) played critical role in the pathologic processes of glomerulosclerosis, including autophagy, inflammation and cell proliferation pathways [3], as evidenced by human patients or animal models [4]. In addition, TGF- β 1 is associated with the severity of renal fibrosis [5]. As a profibrotic cytokine, TGF- β 1 has a critical role in promoting accumulation of ECM, in part due to the role of TGF- β /Smad signaling pathway [6]. Emerging evidence demonstrated that TGF- β /Smad pathway is activated in damaged MCs, leading to overproduction of the glomerular matrix [7].

Although many targeted therapies for TGF- β 1 have been conducted, effective treatments for renal fibrosis remain insufficient and needed to be developed urgently.

Long non-coding RNAs (lncRNAs) are defined as non-protein encoding RNA, > 200 nucleotides in length, exerting functional roles in regulating gene expression, including epigenetics, cell cycle and cell differentiation [8]. However, exact functions of lncRNAs are unknown. With the application of high-throughput sequencing (RNA-seq), more and more lncRNAs have been discovered, followed by the identification of the first lncRNA H19 in 1990 [9]. Increasing evidence demonstrates that lncRNAs play significant roles in the development of kidney diseases, including kidney transplantation, kidney repair and renal cell carcinoma [10, 11]. Recent studies have suggested that lncRNAs are upregulated in CKD and are associated with progression of renal fibrosis. In models of CKD, convincing evidence showed that upregulated LINC00667 could promote renal fibrosis through the miR-19b-3p/connective tissue growth factor pathway [12]. Meanwhile, Sun et al. showed that lncRNA Erbb4-IR, which was induced by TGF- β 1, promoted renal fibrosis via a Smad3-dependent mechanism [13]. Moreover, by using RNA-seq, we previously identified that lncRNA uc.412 was upregulated during the process of MCs proliferation [14]. Furthermore, to explore the potential roles of lncRNA uc.412, GO and KEGG pathway analysis were performed. These findings indicate lncRNAs may serve a novel diagnostic and therapeutic target for CKD. However, the biological function of uc.412 in regulating glomerulosclerosis needs further investigation.

The aim of the present study was to explore the effect of uc.412 on MCs. Furthermore, we also investigated the functional role and mechanisms of uc.412 in the pathogenesis of glomerulosclerosis. The therapeutic potential of uc.412-targeted therapy on glomerulosclerosis was also explored.

Materials And Methods

Antibodies and reagents

TGF- β 1 was purchased from Novoprotein (Beijing, China). SIS3 was acquired from MedChemExpress (MCE, CA). Antibodies against collagen I, α -SMA, ELAVL1 and GAPDH were obtained from Abcam (CA). Anti-phospho-Smad3 (p-Smad3), anti-Smad3, and β -tubulin were purchased from Affinity (Changzhou, China).

Animals

The study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Experiments were conducted using 6 to 8 weeks old male C57BL/6 mice. The mice were randomly divided into two groups: Group 1, normal mice (control); Group 2, CKD model mice. Furthermore, all mice were allowed free access to same feeding on the same conditions. The CKD mouse model, named as 5/6th nephrectomy, was obtained by two steps. We removed two-thirds of the left kidney during the first week. After one-week recovery, the right kidney was removed. Eight weeks after the operation later, renal parenchyma specimens were collected for subsequent experiments.

Cell culture

Rat mesangial cells (MCs) were cultured in DMEM/12F medium (Gibco, CA) supplemented with 10% fetal bovine serum (Gibco, CA) and 1% penicillin-streptomycin (Gibco, CA). According to different experimental purposes, cells were subjected to different interventions. Cells were stimulated with TGF- β 1 (10 ng/mL) for 48 h. To inhibit Smad3 activity, cells were treated with the Smad3 inhibitor SIS3 (1 μ M).

Transfection of small interference RNA

To examine the role of uc.412, MCs were transfected with 75 nM uc.412 siRNA (sense, 5'-GCCGGCCAUCAGUUGAUUTT-3' and antisense, 5'-AAUCAACUGUAUGGCCGGCTT-3') or negative control siRNA for 24 h using Lipofectamine® 2000 (Invitrogen, CA) according to the manufacturer's protocol. Thereafter, the level of uc.412 was detected by Real-Time PCR. Similarly, to discuss the role of ELAVL1, MCs were transfected with ELAVL1 siRNA (100 nM) (5'-GAGGCAATTACCAGTTTCATT-3'), and the level of targeting protein with knockdown of ELAVL1 was detected by western blot.

Western blotting

Cultured cells and kidney tissues were harvested after treatment, and protein levels were detected by western blot analysis on the basis of established protocols (15). The primary antibodies against Collagen I (1:1000), α -SMA (1:1000), GAPDH (1:2000), phospho-Smad3 (1:1000), Smad3 (1:1000), ELAVL1 (1:1000) and β -tubulin (1:2000) were used. The gel area was analyzed by using Image J.

Real-Time PCR

Total RNA from the treated cells and kidney tissues were extracted by using Trizol (Invitrogen, CA). The level of lncRNA or mRNA was quantified with SYBR Green (Takara) by the StepOne Real Time PCR System (Applied Biosystems). The primers in this study, including rat collagen I, α -SMA, and ELAVL1, were designed by Invitrogen (Carlsbad, CA). LncRNA uc.412 primer sequences are as followed: forward 5'-CTTGAATTCCAAGCAGCACA -3' and reverse 5'- CAGCAATTAATCCCCAAGA -3'.

Immunofluorescence staining

Cells were washed three times with PBS after 24 h treatment, then fixed in 4% paraformaldehyde. Subsequently, cells were permeabilized in the 0.25% Triton X-100 for 5 minutes. The cells were blocked with 5% BSA for 1 hour at room temperature, followed by incubated with primary antibodies against ELAVL1 in PBS containing 1% BSA overnight at 4°C. Next day, the cells were incubated with secondary antibody at room temperature. After washing with PBS, DAPI was used to stain the cell nuclei. The method of paraffin sections (3 μ m) staining was the same as above. Immunostained samples were observed under laser confocal.

Establishment of the uc.412-overexpressing cell line

A lentiviral construct carrying the uc.412 gene was purchased from GeneChem Co. Ltd. (Shanghai, China). To obtain a stable uc.412-overexpressing cell line, Rat MCs were prepared and transfected with uc.412-overexpressing lentiviruses (MOI:20) according to the protocols recommended by the manufacturer. After 48 h, the lentivirus infected cells were selected by incubation with 2 µg/ml of puromycin. Then, the transfected cells were harvested and analyzed for follow-up experiments.

RNA pull down assay

The pull down test kit (Thermo Fisher Scientific, CA) was used according to the manufacturer's instructions [16]. To form secondary structure of RNA, 3 µg of biotin-labeled RNAs were added to amount of structure buffer, heated at 95°C for 2 minutes, put on ice for 3 minutes. Magnetic beads were washed using 500 µl RIP buffer for 3 times, then added to biotin-labeled RNAs overnight. Next day, the mixture was added to cell lysate with RNase inhibitor and further incubated for 1 h. The mixture of beads and RNAs was washed with RIP buffer and boiled in SDS buffer. Then the proteins were detected by western blot.

RNA FISH

To determine the location of uc.412 in MCs, fluorescence in situ hybridization was used for the experiment. Cells were washed with PBS 3 times, and rinsed with SSC 10 minutes. Subsequently, hybridization with DNA probe sets was performed at 50°C overnight according to the manufacturer's instructions. Finally, cells were observed with laser confocal.

Statistical analysis

Data were expressed as mean ± SEM. Statistical analyses were performed using SPSS 22.0 statistical software (IBM Corp.). When two groups were compared, T test analysis was used. When more than two groups were compared, one-way ANOVA analysis followed by Bonferroni's correction was employed to analyze the differences. P-value of < 0.05 was considered to indicate statistically significant differences.

Results

1. The expression of lncRNA uc.412 is increased in CKD mice

Here, we used the 5/6th nephrectomy ablation model to resemble several aspects of human glomerulosclerosis [17]. Histologically, glomerulosclerosis was observed in CKD group (Figure 1a). Furthermore, increased levels of collagen I and α-SMA were observed in the CKD group compared with the control group (Figure 1b). By using RNA-Seq, we previously found that uc.412 played crucial roles in the renal diseases [13]. Hence, to detect the role of uc.412 in the progression of glomerulosclerosis, the level of uc.412 in CKD was analyzed via Real-Time PCR. The result showed that the expression of uc.412 was markedly upregulated in CKD (Figure 1c). Subsequently, to verify its location in the MCs, FISH was performed and we found that uc.412 was uniformly expressed both in cytoplasm and nuclei (Figure 1d). These findings suggested that uc.412 may play a key role in the glomerulosclerosis.

2. LncRNA uc.412 is induced in a Smad3-dependent pathway

As demonstrated in Figure 2a, the levels of collagen I, α -SMA, p-Smad3 and Smad3 expression were increased in the TGF- β 1-treated MCs. Meanwhile, we found that uc.412 expression was increased in the TGF- β 1 group (Figure 2b), indicating that uc.412 was induced by TGF- β 1. We next examined the potential mechanism that how TGF- β 1 regulated uc.412 expression. By using SIS3, a specific Smad3 inhibitor, we found that SIS3 could block TGF- β 1-induced Smad3 phosphorylation and therefore obviously inhibited the expression of uc.412 (Figure 2c and 2d). In addition, in the TGF- β 1+SIS3 group, collagen I and α -SMA expression were decreased significantly (Figure 2d and 2e). Thus, it can be seen that TGF- β 1 can contribute to MCs fibrosis via Smad3 pathway and uc.412 can be as its downstream target gene to participate in the development of glomerulosclerosis.

3. LncRNA uc.412 promotes MCs fibrosis

In order to further clarify the pathogenic role of uc.412 in glomerulosclerosis, a lentiviral construct expressing the uc.412 was infected into rat MCs (Figure 3a). Results of western blotting showed that the transfection with uc.412-overexpressing lentivirus significantly induced MCs fibrosis, as shown by increase in the level of collagen I and α -SMA (Figure 3b). Subsequently, uc.412 siRNA was transfected into MCs to inhibit uc.412 expression. Transfection efficiency at different concentrations of uc.412 siRNA was examined, and the appropriate concentration was at 75nM (Figure 3c). Interestingly, we observed that the levels of collagen I and α -SMA expression were effectively decreased in TGF- β 1+uc.412 siRNA group (Figure 3d), which means MCs fibrosis was markedly inhibited, conforming a pro-fibrogenic role of uc.412 in rat MCs.

4. ELAVL1 is the target of LncRNA uc.412

Recent studies have shown that lncRNAs have important functions during various diseases via interacting with specific proteins [18, 19]. Next, RNA pull down assay was performed and identified the protein related to uc.412 by mass spectrometry assays. We found that ELAVL1 was the binding protein for uc.412 (Figure 4a). Previous results using sequencing also showed that ELAVL1 expression was elevated after uc.412 overexpression [14]. Therefore, ELAVL1 was identified as the appropriate binding protein for uc.412. In addition, we detected the level of ELAVL1 expression in uc.412 overexpression group by western blot (Figure. 4b), demonstrating an association between ELAVL1 and uc.412.

5. ELAVL1 has a key effect on the progression of glomerulosclerosis

We next examined ELAVL1 expression in CKD model by immunofluorescent staining with anti-ELAVL1 antibody in paraffin sections of kidney. In CKD group, we found that ELAVL1 expression was significantly increased (Figure 5a). Furthermore, as shown in Figure 5b, western blot analysis revealed that the level of ELAVL1 expression was obviously increased in CKD, indicating that upregulated ELAVL1 is associated with glomerulosclerosis.

6. Knockdown of ELAVL1 inhibits MCs fibrosis

Result of immunofluorescent staining showed that ELAVL1 expression was increased after TGF- β 1 treatment (Figure 6a). Thus, we hypothesized that ELAVL1 was associated with MCs fibrosis. To determine the role of ELAVL1 in MCs fibrosis, siRNA targeting ELAVL1 was specifically transfected into TGF- β 1-stimulated MCs. The results showed that ELAVL1 expression was dramatically decreased in TGF- β 1+ELAVL1 siRNA group, followed by a marked decrease in collagen I and α -SMA mRNA expression (Figure 6b and 6c), accounting for the role of ELAVL1 in glomerulosclerosis.

Discussion

Glomerular fibrosis, the characteristic pathological manifestations of CKD, serves a significant role in the development and progression of CKD [20, 21]. However, because of the limited scope, the mechanisms of glomerular fibrosis remain unclear, resulting in no real change in management or outcomes of patients with glomerular fibrosis. By using RNA-sequencing analysis, we previously revealed that lncRNA uc.412 was involved in mesangial cell proliferation. In this study, we further characterized the potential roles of lncRNA uc.412 in glomerular fibrosis. Interestingly, we found that lncRNA uc.412 was significantly increased and was located in the mesangial cell. Furthermore, we revealed that lncRNA uc.412 played a critical role in this pathophysiological process. Therefore, the exact molecular mechanism was clarified.

It is now clear that TGF- β 1, a profibrotic cytokines, is associated with glomerular fibrosis [22, 23]. However, the mechanism of TGF- β 1 in fibrosis is unclear. Smad3, the downstream target of TGF- β 1, has been confirmed the distinct effects on regulating fibrosis [24]. Here, Smad3 activation and phosphor-Smad3 formation under the condition of TGF- β 1 treatment indicates that TGF- β 1/Smad3 signaling pathway exerts a positive effect on the profibrotic function. To explore the potential relationship between TGF- β 1 and lncRNA uc.412, a Smad3-specific inhibitor (SIS3) was used. Interestingly, we found that SIS3 prevented the TGF- β 1-induced lncRNA uc.412 expression in MCs, suggesting that lncRNA uc.412 maybe the downstream effector of TGF- β 1/Smad3 signaling. Furthermore, MC fibrosis was attenuated markedly after inhibition of lncRNA uc.412 function. These findings indicated that lncRNA uc.412 was induced by TGF- β 1/Smad3, which provides another strategy for the treatment of related kidney diseases.

Next, the precise regulatory pathway for this effect was investigated. Recent studies have shown that lncRNAs played a critical effect on the pathogenesis of diseases via interacting with specific proteins [25]. Thus, we explore the exact regulatory pathway using RNA pull down assay. Interestingly, we found that ELAVL1 was the most appropriate binding protein. It has been demonstrated that ELAVL1 can bind to 3'-UTR of mRNA and regulate its transportation and stability [26]. Moreover, ELAVL1 played a crucial role in various kidney diseases, such as glomerular nephropathy, renal fibrosis and renal tumors [27]. However, the detailed mechanism of ELAVL1 is still not well understood. Convincing evidence showed that ELAVL1-dependant REN mRNA expression was increased in the UUO models, which indicated the important role of ELAVL1 in renal fibrosis [28, 29]. In addition, ELAVL1 was found the link to the inflammation-related factors expression, contributing to matrix secretion increase and accelerates fibrosis in mesangial cells [30]. In diabetic kidney diseases, ELAVL1 could induce MC injury to promote hyperglycemia and worse renal function by binding to Nox4 mRNA [31]. Similarly, our study revealed that ELAVL1 could bind to

uc.412 by RNA pull-down analysis, and we found that ELAVL1 expression was increased in uc.412-overexpressing-induced mesangial cells, indicating that ELAVL1 is a downstream target of uc.412. At the same time, in CKD model, ELAVL1 expression was upregulated, conforming that the binding of uc.412 to ELAVL1 may play a crucial role in CKD. In TGF- β 1-stimulated mesangial cells, the expression of ELAVL1 was also increased, while after silencing ELAVL1 expression showed a protective role in the TGF- β 1-induced mesangial cell fibrosis. These findings all provided the evidence that ELAVL1 played an emerging role in the mesangial kidney diseases.

Conclusion

In conclusion, our present findings uncovered a previously unknown mechanism in glomerular fibrosis under CKD conditions. It revealed that upregulation of uc.412 expression was induced by TGF- β 1/Smad3 signaling pathway, and played an important role in glomerular fibrosis. Moreover, we found ELAVL1 was involved in glomerular fibrosis as the downstream effector of uc.412. Inhibiting uc.412 and ELAVL1 contributed to the relieve of glomerular fibrosis. Therefore, our findings represent a promising therapeutic target for kidney disease with glomerular fibrosis.

Abbreviations

CKD	Chronic kidney disease
MCs	Mesangial cells
TGF- β 1	Transforming growth factor- β 1
ECM	Extracellular matrix
LncRNA	Long non-coding RNA
MOI	Multiplicity of infection
α -SMA	Alpha-smooth muscle actin
UUO	Unilateral ureteral obstruction
PBS	Phosphate buffered saline

Declarations

Acknowledgements

Not applicable.

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Availability of data and materials

The datasets analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

W.G., and A.Z. designed the study. J.J. J.R. and M.S. performed the experiments and discussed the results. J.J. wrote the manuscript. C.H., J.W., M.A. and H.S. performed the experiments and analyzed the data.

Ethics approval and consent to participate

The experimental procedures in the present study were approved by Nanjing Medical University (Nanjing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

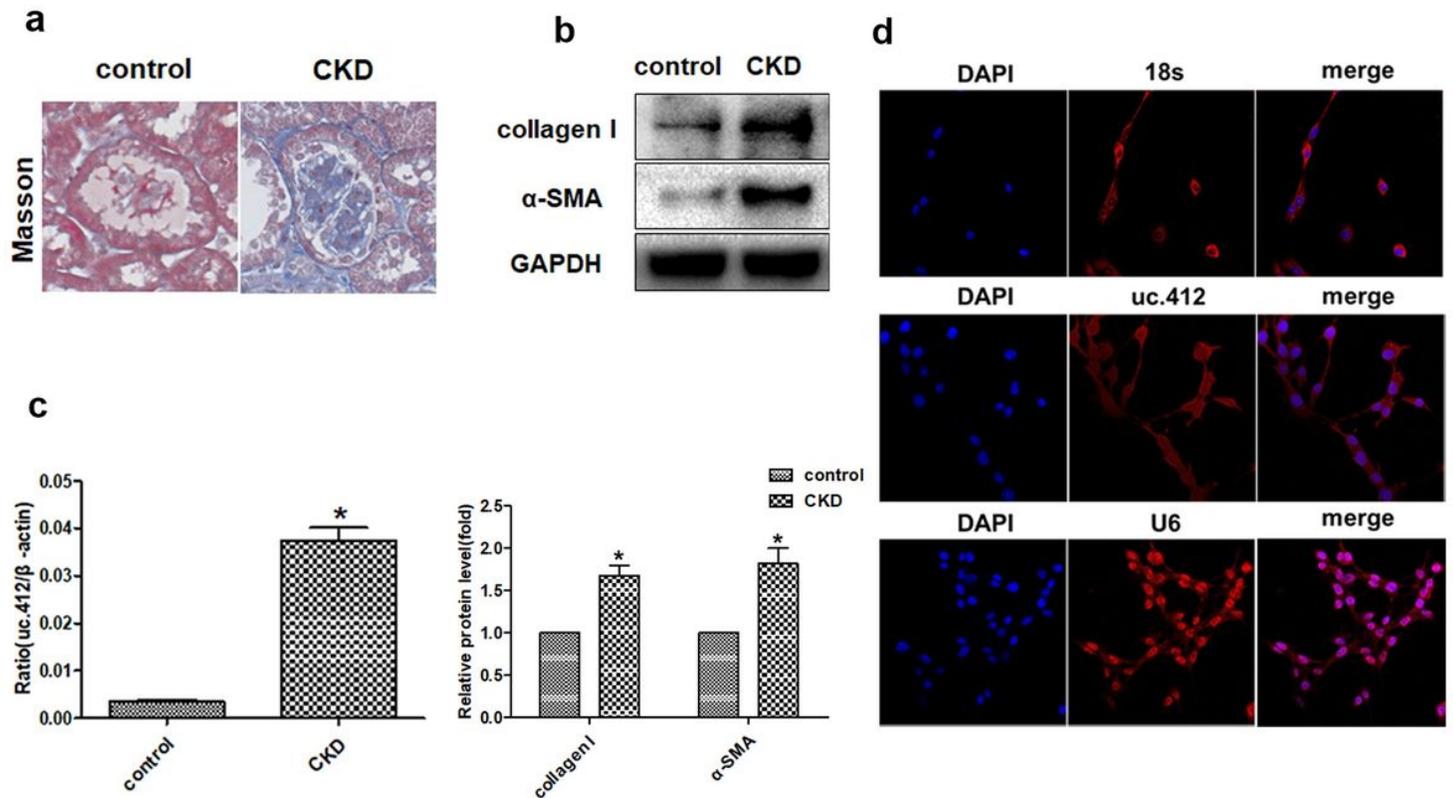


Figure 1

LncRNA uc.412 expression was significantly increased in CKD mice. (a) Representative images of Masson's trichrome staining of the kidney. Magnification, x400. (b) Representative western blots of collagen I and α -SMA. Quantification of western blotting (lower). GAPDH levels were used as standard loading controls. (c) Relative uc.412 expression level in CKD was detected by Real-Time PCR. (d) uc.412

expression in mesangial cells was detected by in situ hybridization. The data are presented as mean \pm SEM. * P <0.05 vs. control group.

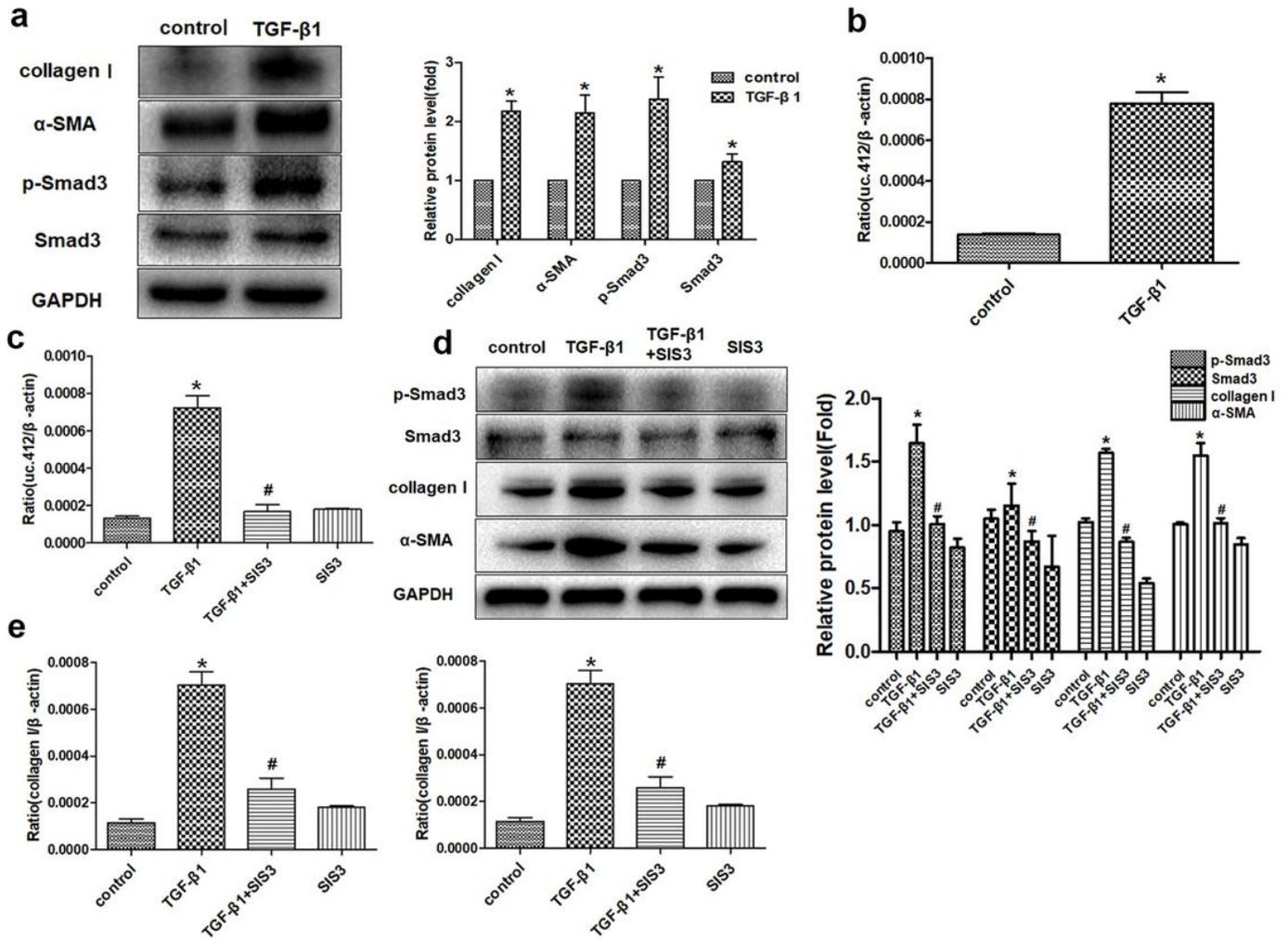


Figure 2

LncRNA uc.412 upregulation was induced through Smad3 signaling pathway. MCs were treated with the Smad3 inhibitor SIS3 (1 μ M) to inhibit Smad3 activity. (a) Representative western blots of collagen I, α -SMA, p-Smad3 and Smad3. Quantification of western blotting (right). (b) and (c) Relative uc.412 expression level was detected by Real-Time PCR. (d) Representative western blots of p-Smad3, Smad3, collagen I and α -SMA. (e) Real-time PCR analysis. GAPDH or β -actin levels were used as standard loading controls. The data are presented as mean \pm SEM. * P <0.05 vs. control group. # P <0.05 vs. TGF- β 1 group;

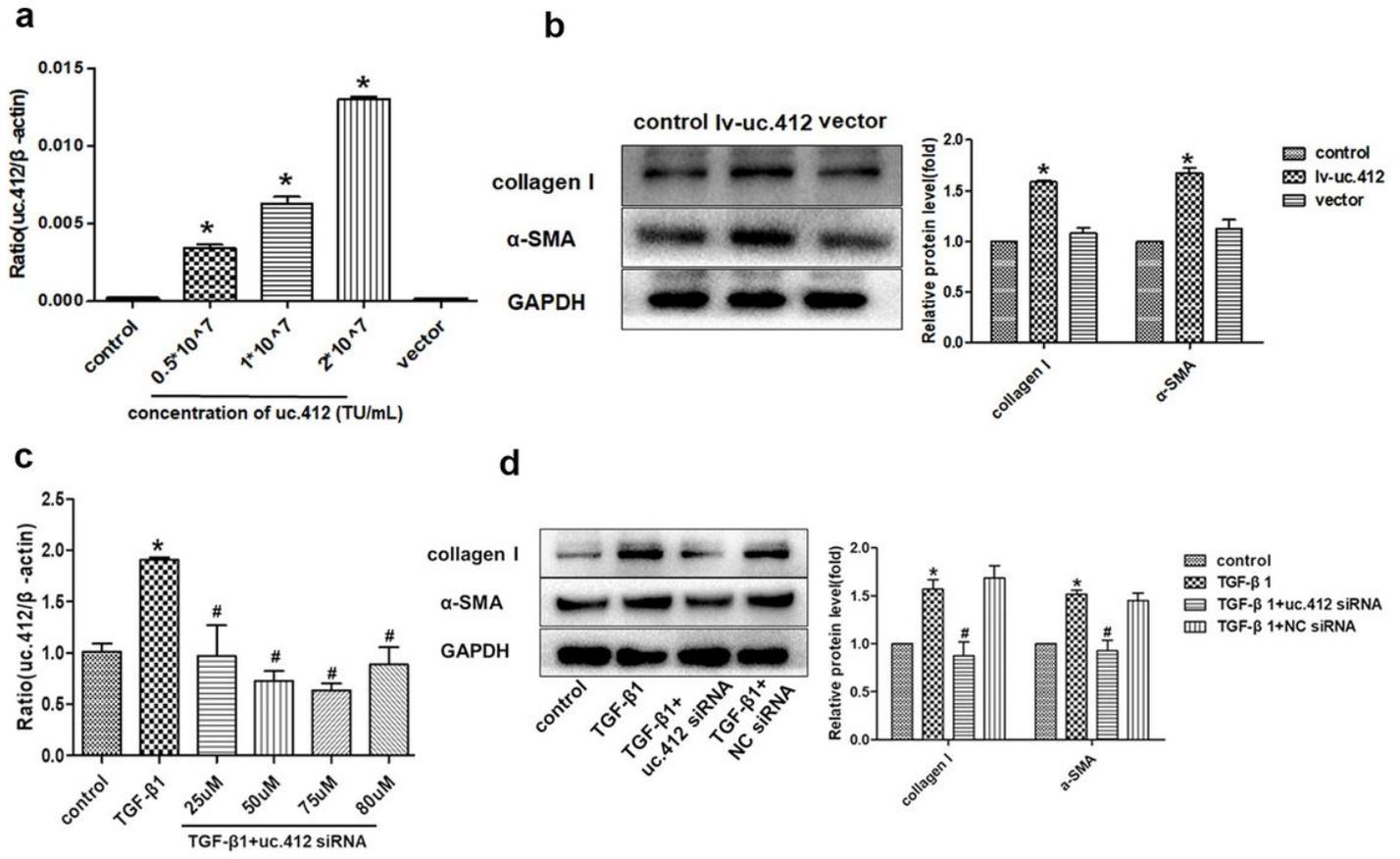


Figure 3

LncRNA uc.412 promotes MCs fibrosis. (a) Relative uc.412 expression level by transfecting uc.412-overexpressing lentiviruses. (b) Representative western blots of collagen I and α -SMA. Quantification of western blotting (right). (c) Transfection efficiency of uc.412 siRNA via Real-time PCR analysis. (d) Representative western blots of collagen I and α -SMA. Quantification of western blotting (right). GAPDH levels were used as standard loading controls. The data are presented as mean \pm SEM. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. TGF- β 1 group.

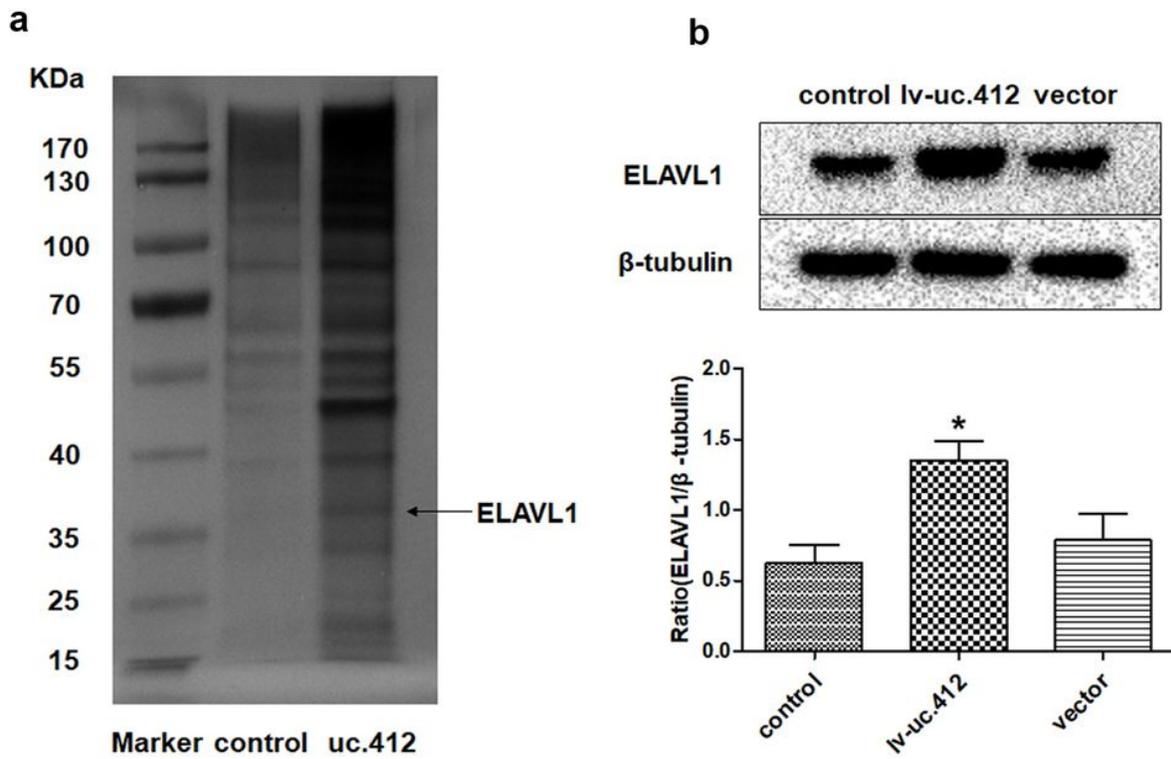


Figure 4

ELAVL1 expression is increased followed by uc.412 overexpression. (a) RNA pull-down assay revealed that the specific association of ELAVL1 with uc.412. (b) Relative ELAVL1 expression was detected by western blot analysis following uc.412 overexpression. Quantification of western blotting (lower). The data are presented after normalization to β -tubulin expression and quantified as mean \pm SEM. * $P < 0.05$ vs. control group.

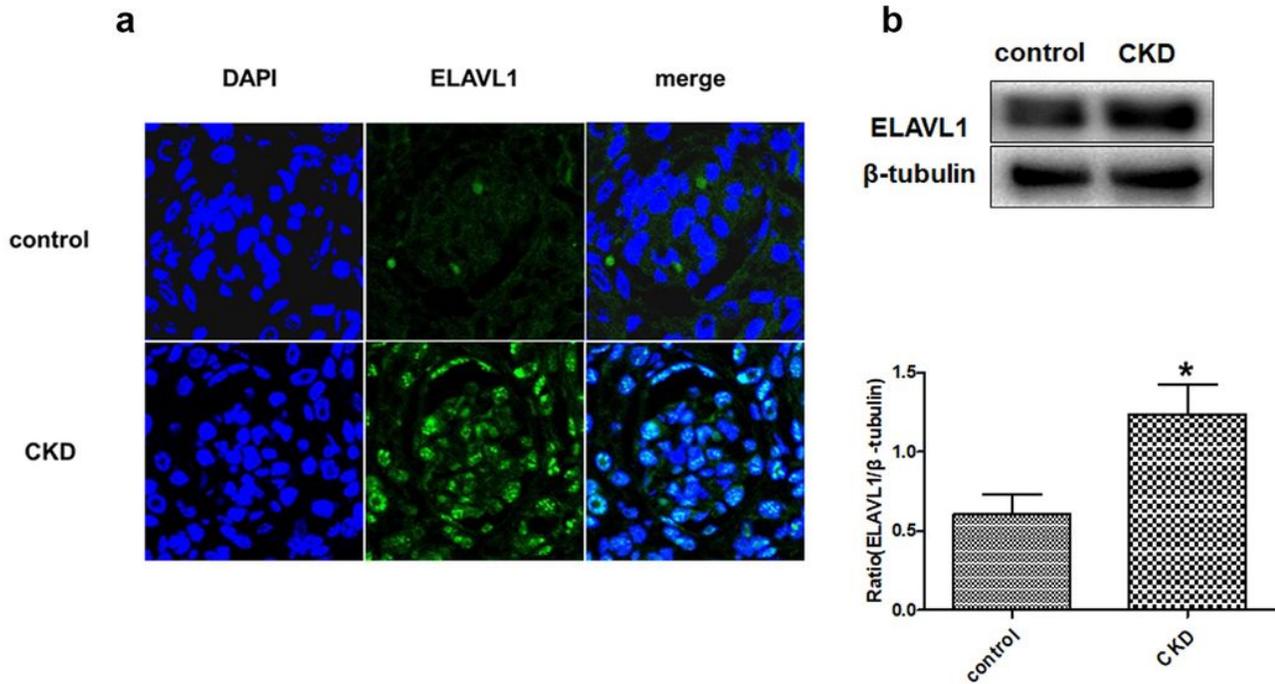


Figure 5

ELAVL1 expression is upregulated in CKD. (a) Immunofluorescence results (magnification, x400) indicating the expression of ELAVL1 in each group. Blue, nuclear staining (DAPI); green, ELAVL1 staining. (b) Relative protein level of ELAVL1 in the control and CKD groups. The data are presented after normalization to β -tubulin expression. The data was quantified as mean \pm SEM. * $P < 0.05$ vs. control group.

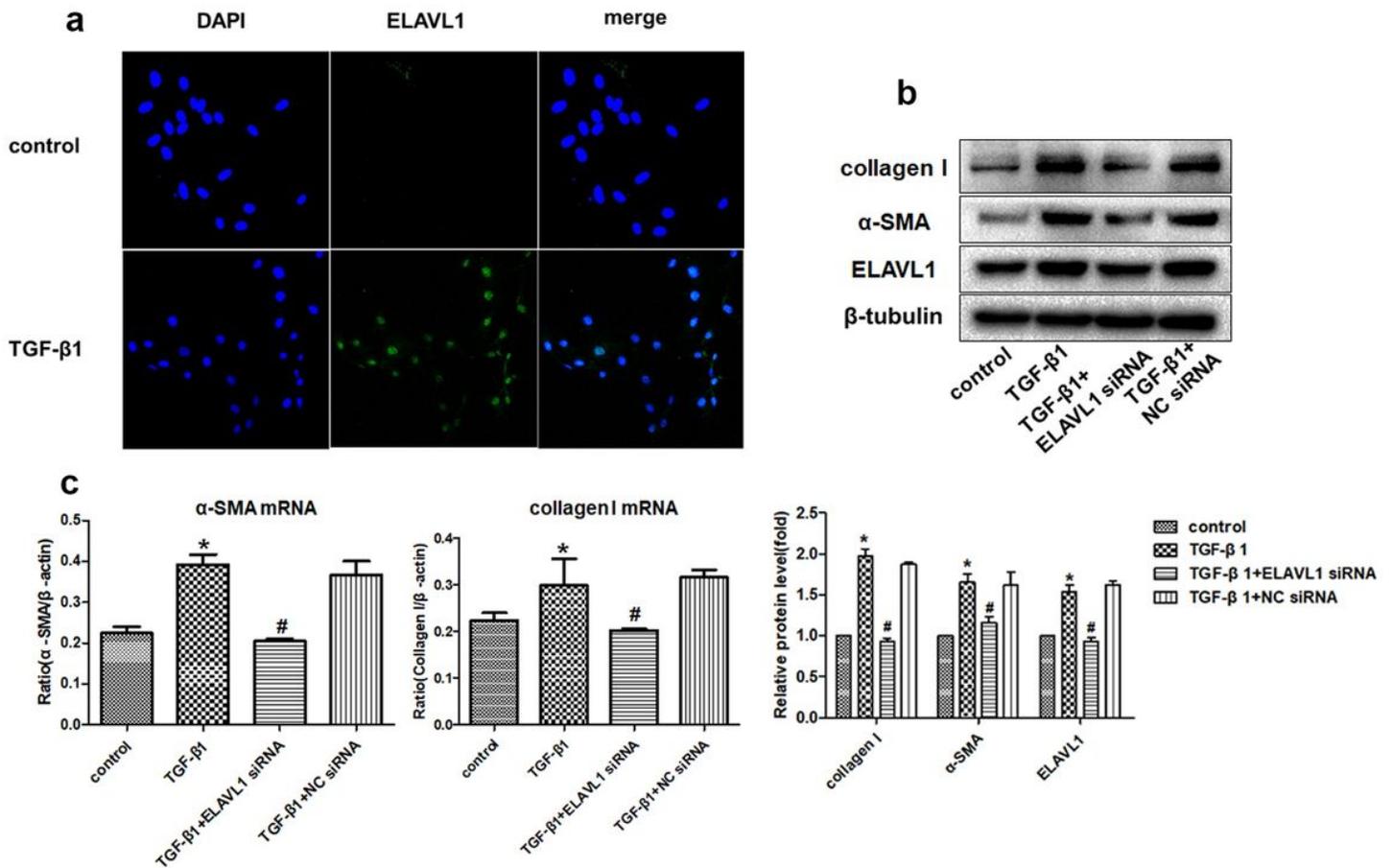


Figure 6

Knockdown of ELAVL1 ameliorates TGF-β1-induced mesangial cell fibrosis. (a) Immunofluorescence results (magnification, x400) indicating the expression of ELAVL1 in the control and CKD group. Blue, nuclear staining (DAPI); green, ELAVL1 staining. (b) The levels of ELAVL1, α-SMA and collagen I expression were detected by western blot. Quantification of western blotting (lower). (c) Relative mRNA levels of collagen I and α-SMA in each group. The data are presented after normalization to β-tubulin or β-actin expression and quantified as mean ± SEM. *P<0.05 vs. control group. #P<0.05 vs. TGF-β1 group;