Promotion of USP4 to TGF-β1-induced EMT in renal tubular epithelial cells is regulated by Akt through stabilizing TβRI

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Abstract

Objective
We aimed to explore the role of ubiquitin-specific peptidase-4 (USP4) in TGF-β1 induced epithelial-mesenchymal transition (EMT) during renal fibrosis, and investigated that if Akt inactivation exerted a critical effect on EMT via USP4/TβRI pathway.

Methods
USP4, pAkt and TβRI proteins in the obstructed kidneys from unilateral ureteral obstruction (UUO) rats were detected by immunohistochemistry assay or western blot method. E-cadherin, α-SMA, USP4 and pAkt protein expression in NRK-52E cells at different concentration of TGF-β1 were detected at different time. Further, NRK-52E cells were transfected with USP4-specific siRNA (si-USP4), and then stimulated with 10 ng/ml TGF-β1 for 24h to detect E-cadherin, α-SMA, E-cadherin and TβRI by immunofluorescent double staining assay. Pretreated with PI3K inhibitor LY294002, protein expression levels of pAkt, E-cadherin, α-SMA were detected. Meanwhile, the location of USP4 was visualized by immunofluorescent assay in NRK-52E cells.

Results
The expression of USP4 and TβRI was significantly upregulated in the tubular epithelial cells of UUO rats. We also found that TGF-β1 upregulated USP4 and activated Akt in NRK-52E cells during EMT. *In vitro,* downregulation of USP4 inhibited TβRI expression and partially reversed EMT stimulated by TGF-β1. In the meantime, blunted phosphorylation of Akt with LY294002 promoted the E-cadherin expression, and inhibited α-SMA expression in response to TGF-β1. However, inactivation of Akt could reverse EMT process, but failed to induce USP4 to shuttle between the nucleus and the cytoplasm in NRK-52E cells stimulated by TGF-β1.

Conclusions
These data implied that USP4 was a harmful molecule induced by TGF-β1, regulated by Akt activation and promoted TGF-β1-induced EMT via TβRI in tubular epithelial cells during renal fibrosis.

Introduction

Renal interstitial fibrosis (RIF) is one of the common pathological features of chronic kidney diseases (CKD) and indicates a poor prognosis. Studies attempt to shed new light on cellular and molecular mechanisms underlying the development of RIF. Renal fibrosis is characterized by excessive extracellular matrix (ECM) and myofibroblasts accumulation in interstitial region. The origins of myofibroblasts have always been a hot topic in renal fibrosis[1]. Experiments by Iwano et al. demonstrated that 36% of myofibroblasts origin from tubular epithelial cells through partial epithelial mesenchymal transition (pEMT)[2]. The pEMT of tubular epithelial cells refers to a process losing epithelial cell-cell adhesion and acquiring mesenchymal markers. Mounting evidence supports that pEMT is required for the progression of renal interstitial fibrosis[3].

The EMT process is activated by transforming growth factor β1 (TGF-β1) and mediated predominantly via Smad or non-Smad signaling pathways[4]. Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway is very important to regulate cell growth, survival, and metabolism[5]. AKT can be phosphorylated by PI3K to phosphorylated Akt (pAkt) and then exerts a positive role in organ fibrosis, such as pulmonary fibrosis, liver fibrosis and myocardial fibrosis. Recent studies reveal that PI3K/AKT signaling serves to regulate EMT independent of TGF-β1/Smad signaling. In addition, Akt activation plays an essential role in ECM deposition during renal fibrosis [6].

Deubiquitination process refers to proteolysis of ubiquitinated substrates directed by deubiquitinating enzymes (DUBs), which have the capacity to detach ubiquitin or ubiquitin-like proteins (UBLs) from target proteins. DUBs are classified into six subclass types according to their Ub-protease domain structure and mechanism of action[7]. The ubiquitin specific proteases (USP) is the biggest subfamily of DUBs, with approximately 60 human members. Ubiquitin specific protease 4 (USP4), also known as UNP or Unph, is one of the first identified DUBs in mammals. Self-deubiquitination of USP4 makes it very stable to engage different cellular signaling pathways[8]. The transforming growth factor-β (TGF-β) type I receptor (TβRI) is a critical mediator of TGF-β1 signal transduction, stability maintenance of which is regulated by ubiquitylation-mediated degradation by DUBs[9]. A direct interaction of USP4 with TβRI mediates deubiquitylation and stabilization of TβRI at the plasma membrane, and regulates activity status of TGF-β transduction pathways[10-11]. Some previous studies showed that USP4 is aberrantly expressed and might act as an oncogene in multiple types of cancer, such as in human sarcoma, colorectal cancer, hepatocellular carcinoma and breast cancer[12-18]. Peter et al. find out that USP4 can facilitate development of TGF-β-induced EMT in vitro in breast cancer cells[19]. However, the role of USP4 in epithelial mesenchymal transition during renal interstitial fibrosis remains still unknown. Considering current knowledge about USP4, we hypothesized that USP4 might play an important role in renal tubular EMT stimulated by TGF-β1. Therefore, the main aim of this study was to investigate the impacts of USP4 on TGF-β1-induced EMT in NRK-52E cells.

Materials and methods

Unilateral Ureteral Obstruction Model

Twenty male Wistar rats weighing approximately 200 to 220g were obtained from Beijing Vital River Experimental Animals Technology Ltd. (Beijing, China) and housed under SPF-laboratory conditions. A model of unilateral ureteral obstruction (UUO) was established following an approved protocol by the Institutional Animal Care Committee and Use committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, as described previously[20]. In brief, the upper and lower third of the left ureter were ligatured with 4-0 silk sutures, and then the middle third was cut off. As controls, sham-operated rats underwent the same surgery except ureter ligation and division. To investigate the severity and time course of UUO-induced renal fibrosis, five rats in sham and UUO groups were sacrificed separately on the 7th or 14th day after operation. And then, the left kidneys were harvested. Formalin-fixed paraffin-embedded kidney tissue slides were prepared for histological evaluation. Other parts were frozen immediately in liquid nitrogen and stored for protein extraction.

Cell culture and treatment

Renal tubular epithelial cell line (NRK-52E) was obtained from the American Type Culture Collection. NRK-52E cells were maintained in high glucose DMEM culture media with 10% fetal bovine serum in a humidified incubator with 5% CO_2 at 37℃. The cells were divided into normal control group, TGF-β1 group (TGF-β1), LY294002 group (TGF-β1+LY294002) and si-USP4 group (TGF-β1+si-USP4). NRK-52E cells were treated *in vitro* with different concentrations of TGF-β1 (1, 2.5, 5, 10 ng/ml) for 12 hours. In LY294002 group, cells were pretreated with 20 μg/ml LY294002 (Calbiochem) for 3 hours before 10 ng/ml TGF-β1 (PeproTech) treatment. In si-USP4 group, cells were pretreated with si-USP4 transfected for 6 hours before 10ng/ml TGF-β1 treatment. The cells were detached and collected at different time (15min, 30min, 1h, 3h, 6h, 12h and 24h) for total protein extraction.

SiRNA for USP4 and transfection

The 21-nucleotide small interfering RNA (5′-UAGACUGACCCAGCUGGAAdTdT-3′) targeting USP4 (si-USP4) was designed and synthesized (Viewsolid, Beijing, China). NRK-52E cells were transfected with the si-USP4 NRK-52E cells using Lipojet™ reagents according to kit instructions (SignaGen Laboratories). Twenty-four hours post transfection, the cells were stimulated with 10 ng/ml TGF-β1 for

additional 24 hours in order to observe effects of USP4 silencing on cells.

Western blot

Total protein from tissue or cells was extracted by RIPA lysis buffer. After denaturing, the samples were loaded in the Tris-gel for electrophoretic separation. After that, the protein was transferred onto the PVDF membrane. Then the membrane was incubated in primary antibody with PBST buffer at 4℃ overnight. The expression amount of protein was measured by ECL detection system. Antibodies for USP4, Akt and pAkt was purchased from CST company. E-cadherin antibody was purchased from BD company. α-SMA antibody was from Boster company. TβRI primary antibody was from Proteintech company. GAPDH antibody was purchased from CST company and used as an inner reference. Relative amounts of each protein were compared in each group.

Immunohistochemistry and immunofluorescence

To visualize the expression, we performed the immunohistochemistry and immunofluorescence assay. The acquired kidney was prepared as formalin-fixed paraffin-embedded tissue. Then the tissue samples were sliced to 10μm thickness and then dewaxed. The slices were boiled for 20min in citrate buffer for antigen retrieval. The non-specific antigen was blocked by 5% BSA for 60min at room temperature. Following that, the primary antibody in 5% BSA was added to slices at 4℃ overnight. After, the slices were washed in PBS for three times. For immunohistochemistry, the HRP-conjugated secondary antibody was incubated for 60 min at room temperature and then performed with DAB assay according to the instruction. While for immunofluorescence, the fluorescent secondary antibody was incubated away from light. Slides were viewed with Olympus optical fluorescence microscope.

Statistical analysis

All experiments were carried out independently at least three times. All values were presented as mean ± standard deviation. The statistical analyses were performed using one-way analysis of variance (ANOVA) by SPSS 19.0 software, and P values<0.05 was considered significant statistically.

Results

1. Expression of pAkt and USP4 increases in UUO rats

To examine the role of USP4 in renal fibrosis, dynamic expression of USP4 protein in established unilateral ureteral obstruction rats was analyzed. Masson trichrome staining showed a significant increase of collagen components in renal interstitium after obstruction for 7 or 14 days (Fig 1A). Immunohistochemistry results showed that the expression of pAkt in UUO rats was significantly upregulated compared with the sham group as early as on day 7 and continued (Fig 1B). Furthermore, the expression of USP4 protein in renal tubular epithelial cells increased progressively over time following UUO (Fig 1C). However, protein expression of USP4 in sham rats was rare. Western blot assay was performed to quantify USP4 protein expression during development of renal fibrosis. The results showed that USP4 expression on day 14 was more than that on day 7 (Fig 1D). In this experiment, we found that expressions of both pAkt and USP4 increased along with renal fibrosis in UUO rats.

Fig. 1 Expressions of pAkt and USP4 during renal fibrosis in sham and UUO rats. **A** Masson staining of renal fibrosis, magnification ×200. **B** Immunohistochemistry staining of pAkt expression, magnification ×200. **C** Immunohistochemistry staining of USP4 expression, magnification ×200. **D** Western blot analysis of USP4 expression in the kidney of sham-operated and UUO rats, the bars showed the relative expression quantity normalized to GAPDH and compared to sham rats. Representative data from five rats were shown as mean ± standard deviation. **P<0.01 compared to sham rats.

2. TGF-β1 induces EMT accompanied by pAkt and USP4 upregulation in NRK-52E cells

TGF-β1 is considered as one of important inducers of EMT during renal fibrosis. We performed *in vitro* experiment to investigate the expression changes during EMT of NRK-52E cells. After treated with TGF-β1 at concentrates of 0, 1, 2.5, 5, 10 ng/ml for 12 hours, E-cadherin was decreased, while α-SMA and USP4 were increased in NRK-52E cells(Fig 2A). Quantitative analysis showed that expressions of E-cadherin was downregulated gradually with increasing TGF-β1 concentration, while α-SMA and USP4 upregulated significantly at 10 ng/ml TGF-β1 (Fig 2B). Thereafter, we studied the expression of E-cadherin, α-SMA, USP4, pAkt and Akt in NRK-52E cells stimulated with 10 ng/ml TGF-β1 for different time. E-cadherin expression was significantly downregulated while expressions of α-SMA, USP4 and pAkt were upregulated (Fig 2C). Quantitative analysis showed that USP4 increased significantly from 6h and remained at high levels thereafter. In addition, pAkt expression peaked at 6h and weakened but at higher levels (Fig 2D). This experiment indicated that NRK-52E cells upregulated expression of USP4 and pAkt during EMT.

Fig. 2 USP4 and pAkt expression in TGF-β1-induced EMT in a time- and dose-dependent manner. NRK-52E cells were treated with different concentrations of TGF-β1 (0, 1, 2.5, 5 and 10 ng/ml) for 12h or extended periods (0, 15min, 30min, 1h, 3h, 12h and 24h). **A** Protein expressions of E-cadherin, α-SMA and USP4 by western blot assay in NRK-52E cells treated with different concentrations of TGF-β1. **C** Protein expressions of E-cadherin, α-SMA, USP4, pAkt and Akt at each time point. **B, D** Western blot analysis of E-cadherin, α-SMA, USP4, pAkt and Akt in NRK-52E cells with different concentrations or at distinct time points. Bars in **B, D** showed relative expression quantity normalized to GAPDH compared to control cells without TGF-β1 treatment. Exceptionally, bars for pAkt in **D** represented relative expression quantity normalized to total Akt. *P<0.05 compared with normal control cells.

3. USP4 depletion inhibits TGF-β1-induced EMT

To investigate the effect of USP4 on EMT, we transfected si-USP4 to knockdown USP4 in NRK-52E cells and immunofluorescence assay was applied to visualize the expression of E-cadherin and α-SMA in NRK-52E cells. After treatment with 10 ng/ml TGF-β1 for 24h, NRK-52E cells displayed downregulated E-cadherin but upregulated α-SMA expressions. Pretreated with si-USP4, NRK-52E cells stimulated with TGF-β1 could increase E-cadherin while decrease α-SMA expression (Fig 3A). Quantitative analysis using western blot assay showed that si-USP4 increased E-cadherin and decreased α-SMA significantly in TGF-β1 stimulated NRK-52E cells (Fig 3B). These results indicated that si-USP4 inhibited EMT induced by TGF-β1 in NRK-52E cells.

Fig. 3 Effect of USP4 depletion on E-cadherin and α-SMA expression in NRK-52E cells. **A** Immunofluorescence microscopy showed downregulated E-cadherin and upregulated α-SMA expression in NRK-52E cells treated with 10 ng/ml TGF-β1 after 24h, and normalized with transfection with si-USP4. **B** Similar results showed in NRK-52E cells, as detected by Western blot analysis. * P<0.05 comapred with normal control cells. # P<0.05 compared with TGF-β1-induced cells.

4. Akt inhibitor LY294002 inhibits USP4 translocation of USP4 in TGF-β1 induced NRK-52E cells

NRK-52E cells normally expressed little pAkt and USP4 on western blot, but overexpressed pAkt and USP4 after treatment with 10 ng/ml TGF-β1 for 6h. Pretreated with PI3K inhibitor LY294002 (20 μg/ml) before TGF-β1 treatment could downregulate expressions of pAkt, but not USP4. Transfected with USP4-siRNA before TGF-β1 treatment, NRK-52E cells downregulated expression of USP4, but not pAkt (Fig 4 A). Immunofluorescence microscopy showed that TGF-β1 stimulated NRK-52E cells to express USP4 all over the cell. Pretreated with LY294002, NRK-52E cells expressed USP4 primarily in nucleus (Fig 4B). These experiments demonstrated that Akt inhibition had impact on USP4 translocation, but not expression level.

Fig. 4 Effect of Akt inhibition on USP4 expression and cellular location in NRK-52E cells. **A** Western blot analysis showed up regulated USP4 and pAkt expression in NRK-52E cells stimulated with 10 ng/ml TGF-β1 after 6h. Pretreated with 20 ug/ml LY294002 could not downregulate USP4 expression. Meanwhile, USP4 depletion could not downregulate pAkt expression. **B** Immunofluorescence microscopy showed LY294002 inhibited USP4 translocation to the cell membrane in NRK-52E cells. ns, short for no significance.

5. USP4 stabilizing TβRI in response to TGF-β1

We used UUO model to observe the levels of TβRI during renal fibrosis. The results showed that renal tubular epithelial cells expressed a little TβRI in sham rats. TβRI expression increased After obstruction for 7 days, and overexpressed significantly after 14 days (Fig 5A). Using western blot, we quantified TβRI expression. The results showed that TβRI expression increased with prolonged obstruction time (Fig 5B). *In vitro*, NRK-52E cells expressed E-cadherin highly, and TβRI lowly. Treated with TGF-β1 10ng/mL for 24h, NRK-52E cells decreased E-cadherin while overexpressed TβRI. Pretreated with LY294002, NRK-52E cells recovered E-cadherin and downregulated TβRI. However, pretreated with si-USP4, TβRI was downregulated noticeably (Fig 5C). Western blot analysis showed expression of TβRI in NRK-52E cells transfected with si-USP4 was lower than pretreated with LY294002. These results indicated that USP4 could stabilize TβRI in NRK-52E cells in response to TGF-β1 during EMT.

Fig. 5 Effect of Akt inhibition on TβRI expression in NRK-52E cells.. **A** Immunohistochemistry staining of TβRI expression in sham and UUO rats, magnification ×200. **B** Western blot analysis of TβRI expression in the kidney of sham-operated and UUO rats, the bars show the relative expression quantity normalized to GAPDH and compared to sham rats. **P<0.01 compared with sham rats. **C** Immunofluorescence images of E-cadherin and TβRI in NRK-52E cells as shown in images above. **D** Western blot analysis of TβRI expression in NRK-52E cells, the bars show the relative expression quantity normalized to GAPDH and compared to normal control group. *P<0.05 compared with TGF-β1 group, #P<0.05 compared with LY294002 group.

Discussion

Renal tubular epithelial cells (TECs) have been widely recognized as vital fibrogenic cells involved in ECM production and the progression of renal interstitial fibrosis[21]. Cumulative studies have reported that the induction of TGF-β1 signaling could activate partial EMT process of renal tubular epithelium by increasing the expression of α-SMA, which leads to renal interstitial fibrosis. In this study, we found that ubiquitin specific protease 4 and Akt signaling activation induced the EMT-related protein expression of renal tubular epithelium after ureteral obstruction (Fig 1BC). Further, the activation of Ubiquitin specific protease 4 could promote the renal fibrosis by regulating the EMT-related protein expression in an Akt-dependent manner. In detail, downregulated E-cadherin and upregulated α-SMA expression caused by TGF-β1 were restored through inhibiting Akt signaling or knockdown of USP4 expression.

Transforming growth factor-β1 (TGF-β1) is recognized as the most potent fibrogenic factor to induce production of excessive extracellular matrix (ECM) components and promote myofibroblast differentiation with involvement in the partial EMT process during renal fibrosis. It should be noted that the status of TGF-β type I receptor (TβRI) is related to EMT program and fibrosis process[22]. The current study found that the expression of TβRI is increased with time in the obstructed kidney (Fig 5AB). Further, TβRI protein expression in NRK-52 cells was upregulated remarkably by TGF-β1(Fig 5C). Meanwhile, the expression pattern of pEMT markers (E-cadherin and α-SMA) was reversed (Fig 2AB). A possible explanation for this might be that the stability of TβRI, as a switch molecule, controls the TGF-β signaling transduction and the biological behaviors of TECs.

TGF-β signaling pathway-related proteins tightly regulate the status of signaling pathway through ubiquitination followed by degradation, especially TβRI. Levels of this receptor on the cell membrane are reduced by Smurf2/Smad7 complex through ubiquitination modification. Another important finding was that USP4 could be detected in high expression in NRK-52E cells stimulated by TGF-β1 (Fig 2). After transfected by USP4-siRNA, TβRI was downregulated and pEMT was alleviated (Fig 3AB). Hence, it could conceivably be hypothesized that USP4 also interacts with TβRI and functions as a deubiquitinating enzyme to reverse its ubiquitination and thus regulate the TβRI levels. A strong relationship between USP4 and TβRI has been reported in previous studies. Zhang L et al.[19] revealed that USP4 interacts directly with TβRI and deubiquitinates it to activate the TGF-β signaling pathway, and subsequently induces the EMT in hepatocellular carcinoma cells. A recent study reveals that short hairpin RNA for USP4 decreases the ubiquitination level of TβRI of human keloid fibroblasts during pathological scars[23].

Zhu J et al.[24] demonstrates that USP4 contributes to EMT of hepatocyte and promotes liver fibrosis through deubiquitination process to activate TGF-β signaling pathway. The level of USP4, targeted to miR-148a, is sustained and subsequently able to stabilize TβRI. In accordance with those studies, our present results confirm the association between USP4 and TβRI. Despite these promising results, these findings may be somewhat limited by research technique. Further research should be undertaken to investigate the protein-protein interaction between USP4 and TβRI in TECs.

To our surprise, USP4 increased significantly not only on plasma membrane, but also in cytoplasm and nuclear region (Fig 4B). The reason for this was not clear but it might have something to do with USP4 protein trafficking and translocation. In HeLa cells, USP4 is initially proved to be phosphorylated by Akt and promoted to distributes more from nucleus to membrane[19]. Further investigation results prove that the interaction between USP4 and TβRI is enhanced by USP4 translocation to the membrane through USP4 phosphorylation induced by Akt. Deep research into the molecular architecture of protein USP4 find that USP4 phosphorylated modification at Ser 445 by pAkt followed by combination to 14-3-3 isoforms induces exportion from nucleus to cytoplasm to execute its deubiquitination capacity. In this study, LY294002, an inhibitor of PI3K/Akt, induced the endogenous USP4 appeared completely in the nucleus, instead of the membrane and cytoplasm (Fig 4B). Accumulative evidences highlight a pivotal role of non-Smad PI3K/Akt pathway in TGF-β1 induced pEMT in renal tubular epithelial cells[25]. TGF-β1-inducible phosphorylation of Akt seems to be depended on to mediate TGF-β1-induced pEMT. However, the mechanisms remain elusive. One study indicates that Akt mediated EMT of human peritoneal mesothelial cells (HPMCs) modulated by USP4 while persuasively enhancing the TβRI stability[26]. Sequence analysis of USP4 identifies an consensus RxRxxS(T) phosphorylation motif for Akt at Ser 445, a conserved domain in USP4 orthologues[19,27]. Therefore, It can be assumed that USP4 was phosphorylated by pAkt and translocated during pEMT of TECs. This finding suggested USP4 play the role of a bridge in the interaction between the Akt signaling pathway and TβRI during pEMT. Our findings provided a novel insight into the molecular mechanism underlying renal fibrosis, and prompted a novel approach to reverse pEMT and alleviation of renal fibrosis through targeting USP4.

Conclusions

In summary, This study uncovered USP4 as an important determinant for crosstalk between TGF-β and AKT signalling pathways. It indicated that Akt might regulate pEMT in renal fibrosis via various signaling pathways which involved in the Akt/USP4/TβRI axis. This preliminary study shed light on the role of USP4 and the molecular mechanisms involved in renal fibrosis and support the need for further studies. Therefore, blocking USP4 might be a future approach for the prevention and treatment of renal fibrosis.

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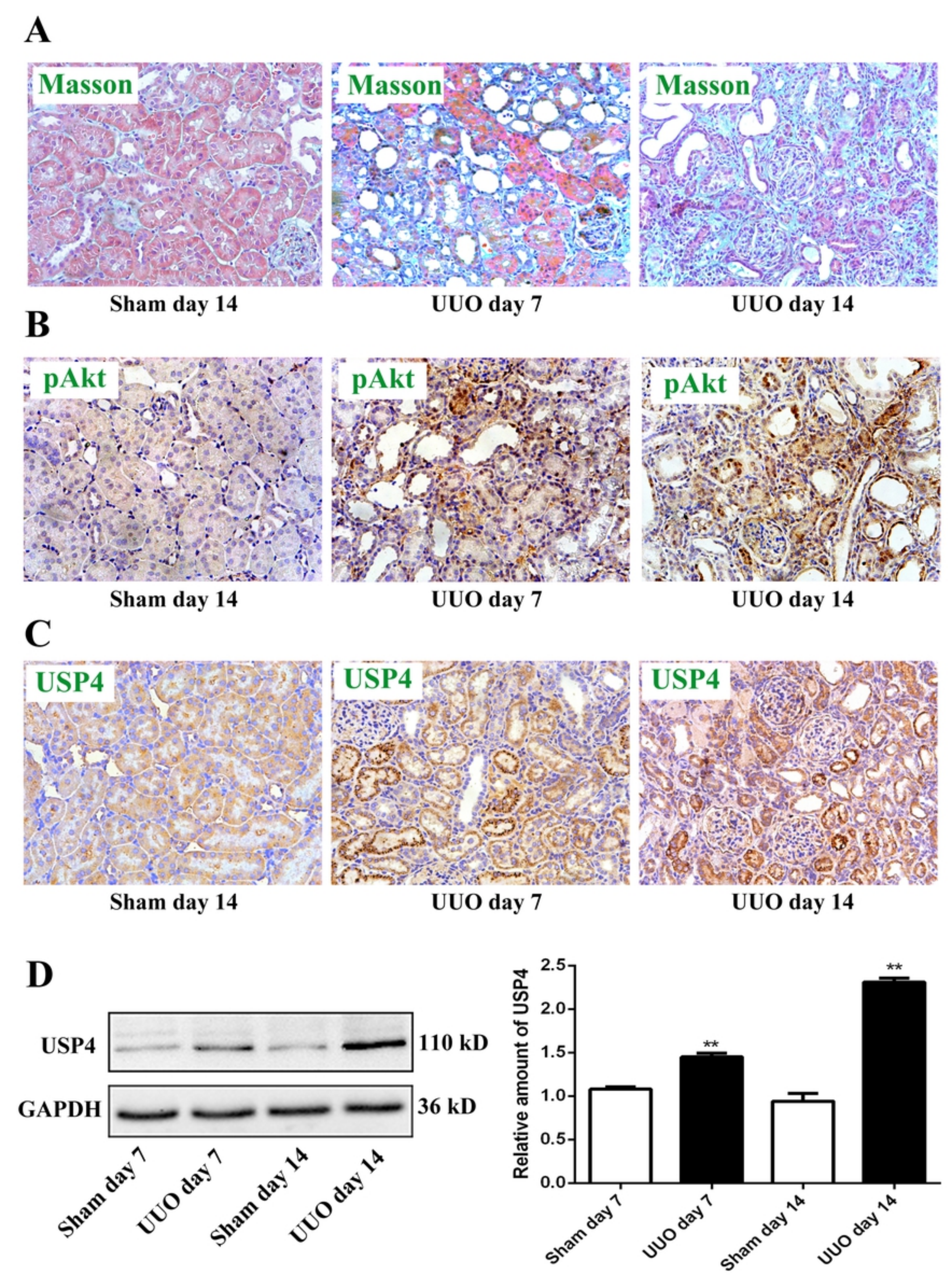
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Figure 1

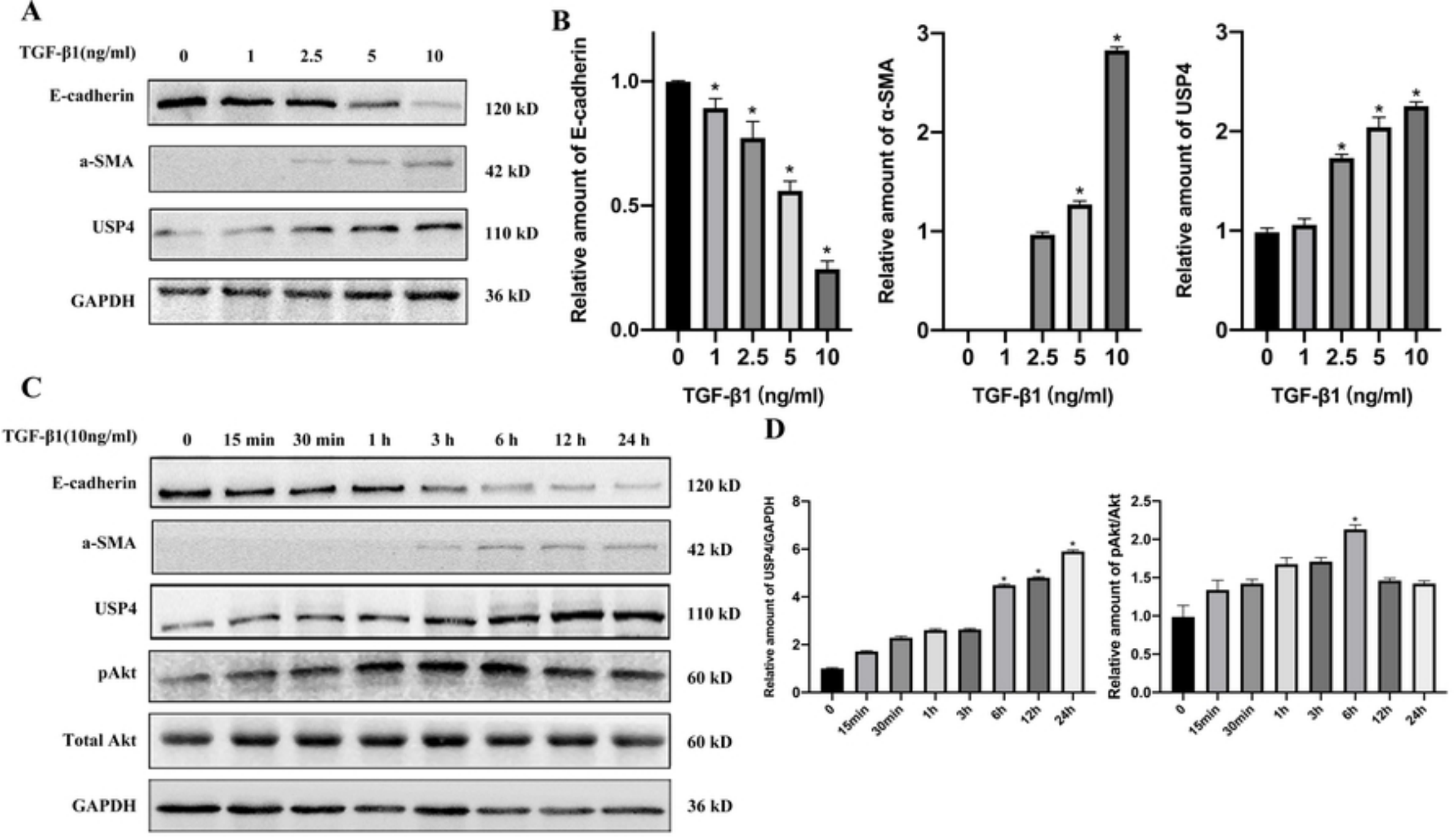
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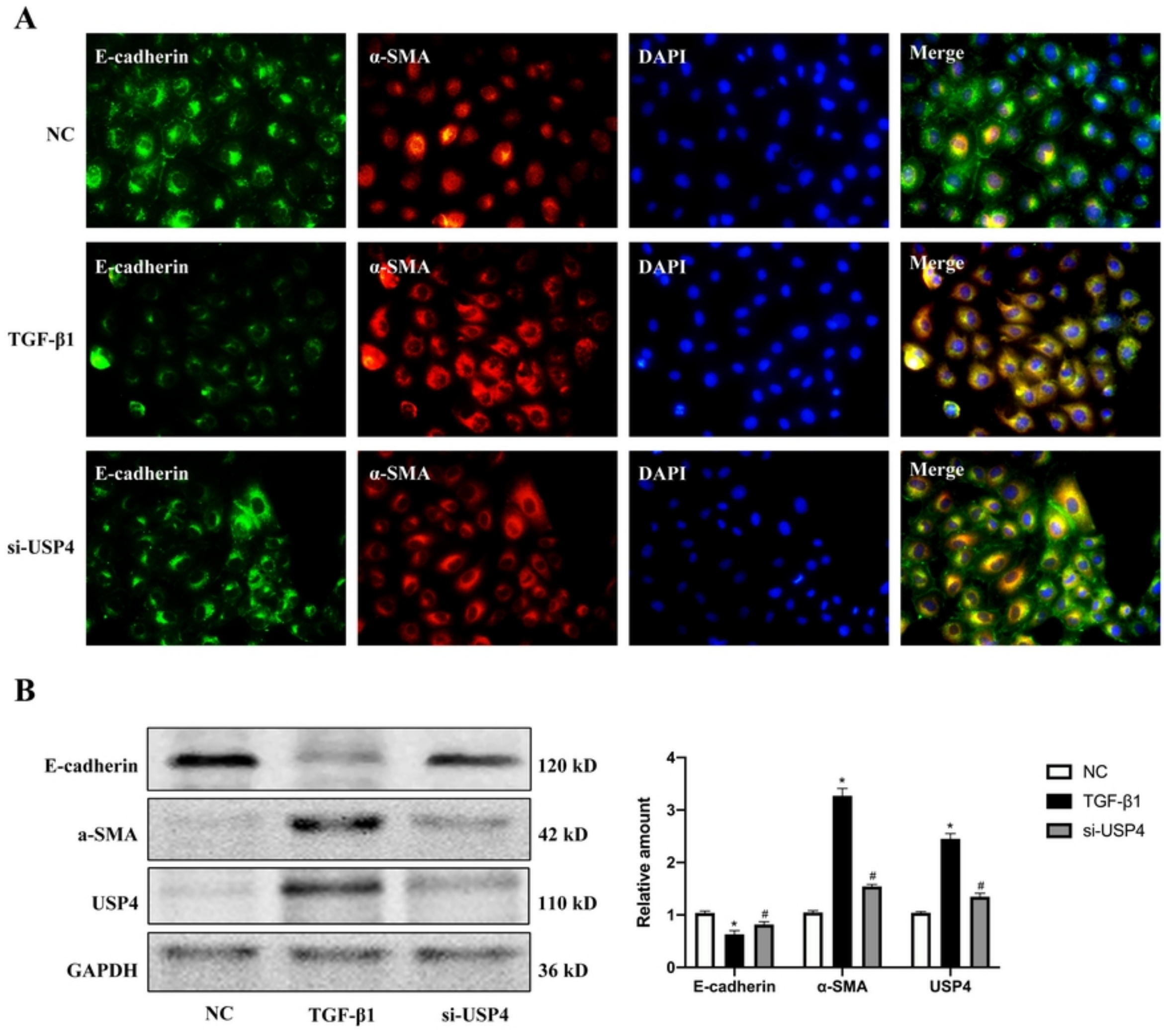
Figure 2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si-USP4

Figure 3

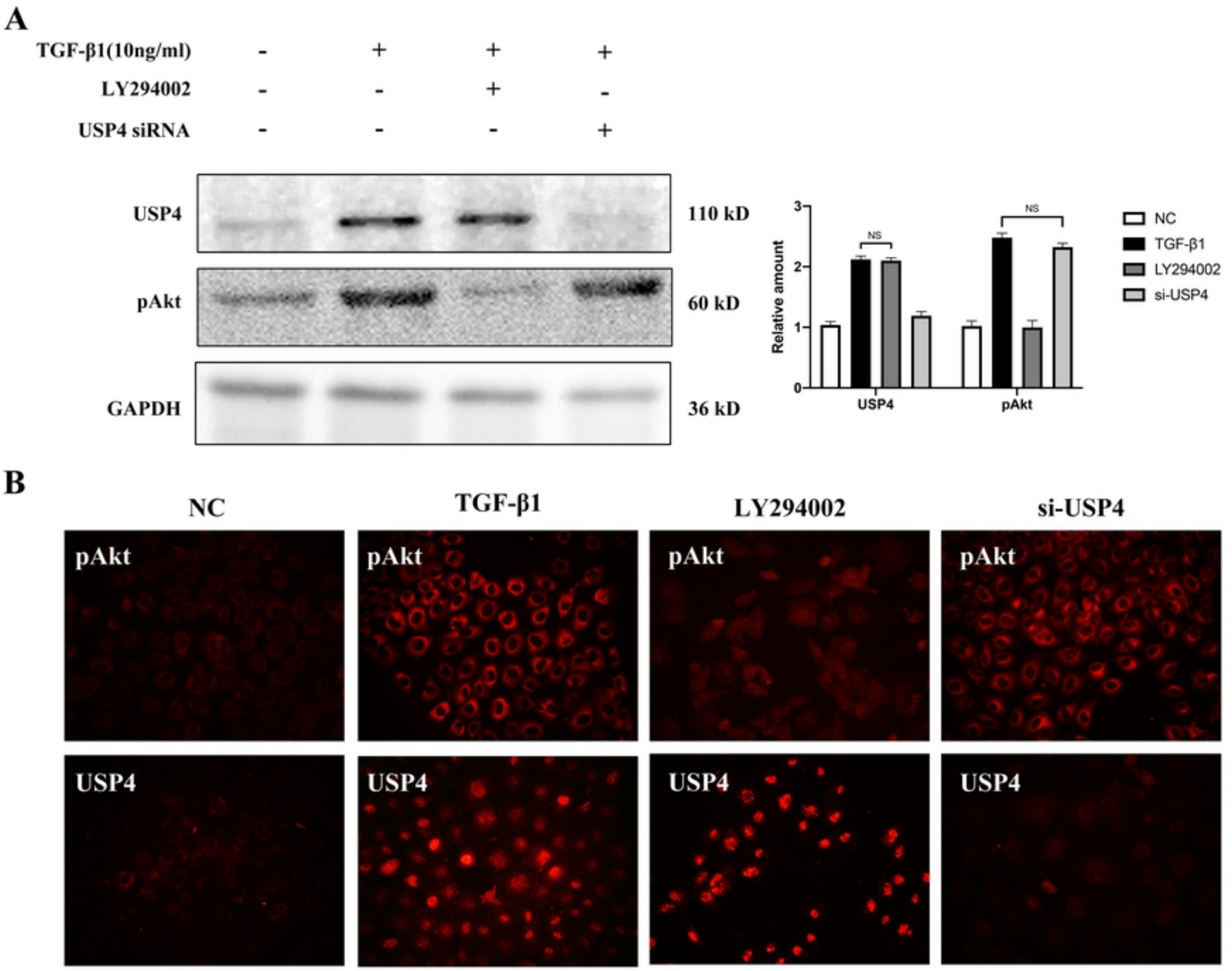
A
TGF-β1(10ng/ml) - + + +
LY294002 - - + -
USP4 siRNA - - - +
USP4
pAkt
GAPDH
110 kD
60 kD
36 kD
Relative amount
NS
NS
USP4
pAkt
NC
TGF-β1
LY294002
si-USP4
B
NC
TGF-β1
LY294002
si-USP4
pAkt
USP4

Figure 4

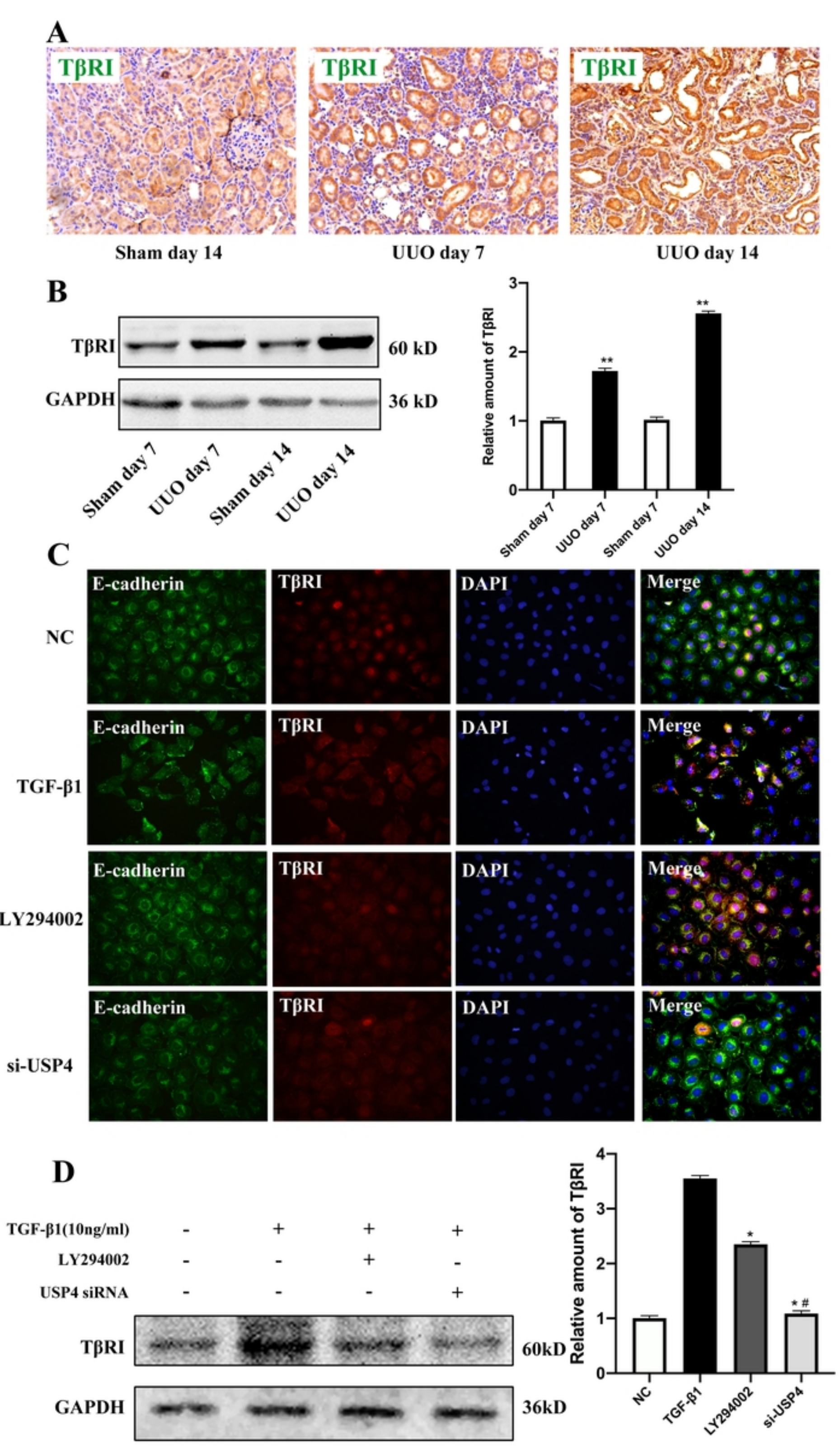
A
TβRI
TβRI
TβRI
Sham day 14
UUO day 7
UUO day 14
B
TβRI
60 kD
GAPDH
36 kD
Sham day 7
UUO day 7
Sham day 14
UUO day 14
Relative amount of TβRI
3
2
1
0
**
**
Sham day 7
UUO day 7
Sham day 7
UUO day 14
C
E-cadherin
TβRI
DAPI
Merge
NC
TGF-β1
LY294002
si-USP4
D
TGF-β1(10ng/ml) - + + +
LY294002 - - + -
USP4 siRNA - - - +
TβRI
60kD
GAPDH
36kD
Relative amount of TβRI
4
3
2
1
0
*
* #
NC
TGF-β1
LY294002
si-USP4

Figure 5