Tamoxifen ameliorates renal tubulointerstitial fibrosis by modulation of estrogen receptor α-mediated transforming growth factor-β1/Smad signaling pathway

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ABSTRACT

Background. After insult to the kidney, a renal fibrotic process is initiated with sustained inflammation, fibroblast activation and accumulation of extracellular matrix (ECM). Tamoxifen has been used as an anti-estrogen for the prevention and treatment of breast cancer. In this study, we investigated the protective effects of tamoxifen on unilateral ureteral obstruction (UUO)-induced renal tubulointerstitial fibrosis and its molecular mechanism.

Methods. Renal fibrosis was induced by UUO in 7-week-old C57BL/6 mice. Tamoxifen (50 mg/kg) was given by oral gavage for 5 days before induction of renal fibrosis. Tamoxifen treatment was continued for 14 days after UUO operation. Histologic changes were examined by periodic acid–Schiff stain and Masson’s trichrome stain. Expression of α-smooth muscle actin, vimentin, type I collagen, fibronectin and cell adhesion molecules were evaluated by immunohistochemistry and western blot analysis. We also evaluated the effect of tamoxifen on estrogen receptor (ER)-α-mediated transforming growth factor (TGF)-β1/Smad signaling pathway in vitro.

Results. Renal tubular injury and fibrosis were increased after UUO. Tamoxifen treatment significantly decreased UUO-induced renal tubular injury and fibrosis. Renal fibroblast activation, ECM deposition and inflammation were significantly increased after ureteral ligation. However, tamoxifen treatment significantly decreased UUO-induced renal fibroblast activation, ECM deposition and inflammation by suppression of TGF-β1/Smad signaling pathway in vivo. Tamoxifen decreased TGF-β1-induced fibroblast proliferation and cell migration by modulating ERα-mediated TGF-β1/Smad signaling pathway in vitro.

Conclusion. These findings indicate that tamoxifen has a beneficial effect on UUO-induced tubulointerstitial fibrosis by suppression of renal fibroblast activation via modulation of ERα-mediated renal TGF-β1/Smad signaling pathway.

Keywords: estrogen receptor-α, fibroblast, inflammation, kidney fibrosis, transforming growth factor-β

INTRODUCTION

Progressive renal disease is characterized by glomerulosclerosis, tubular atrophy and dilatation, tubulointerstitial fibrosis and rarefaction of peritubular capillaries [1]. These pathologic changes are commonly found in diabetic kidney disease, hypertension, glomerulonephritis and obstructive uropathy. Regardless of etiology, patients with chronic kidney disease who show irreversible renal dysfunction and uremic symptoms and signs should consider to start renal replacement therapy such as dialysis or renal transplantation [2]. After insult to the kidney, a renal fibrotic process is initiated with sustained inflammation, activation of matrix-producing fibroblasts and accumulation of extracellular matrix (ECM) [3, 4]. Therefore, modulation of renal fi brogenesis may be one of the promising therapeutic targets for attenuating the progression of chronic kidney diseases.

Among the mechanisms of renal fi brogenesis, transforming growth factor (TGF)-β signaling pathway is known to play a pivotal role in the renal tubulointerstitial fibrosis [5, 6]. Once activated, TGF-β stimulates proliferation of fibroblasts and matrix accumulation through binding of TGF-β type I and type II receptor complexes, leading to activation of canonical Smad family and non-canonical TGF-β signaling pathways [7]. Canonical TGF-β/Smad signaling is mediated by phosphorylation of Smad proteins, whereas other downstream cellular responses are MAP kinase, Rho-like GTPase and phosphatidylinositol-3-
kinase/Akt pathways, which are known as Smad-independent pathways [8, 9]. Regulation of TGF-β/Smad signaling by estrogen receptor (ER) has shown that estrogen suppresses TGF-β-induced transcription and cell migration through interaction between Smad3 and ER in human kidney carcinoma cells [10]. Estrogen also inhibits TGF-β signaling in breast cancer cells, promoting Smad2/3 degradation [11].

Tamoxifen, selective estrogen receptor modulator (SERM), has anti-estrogenic effect on mammary gland and uterus and can be used to treat ER positive breast cancer patients after surgery or radiation therapy [12, 13]. In addition to the anti-estrogenic effect, the SERMs exhibit estrogenic effects on cardiovascular and skeletal systems, reducing incidence of coronary artery disease and risk of osteoporotic fracture in women [12, 14, 15]. Therefore, SERMs such as tamoxifen and raloxifene display dual biologic responses depending on tissues [12]. In addition, tamoxifen has been successfully used for treating fibroserotic disorders such as idiopathic retroperitoneal fibrosis [16], encapsulating peritoneal fibrosis [17], fibrosing mediastinitis [18] and desmoid tumors [19]. Studies have also indicated that tamoxifen suppresses human dermal fibroblast proliferation [20], excessive ECM production in mesangial cells [21] and renal fibrosis in the hypertensive nephrosclerosis model [22]. However, the protective effect of this agent on renal fibrosis in relation to ER and TGF-β1/Smad signaling pathway is still elusive. Here, we investigated the effect of tamoxifen on unilateral ureteral obstruction (UUO)-induced renal tubulointerstitial fibrosis via modulation of ERα-mediated TGF-β1/Smad signaling pathway.

**MATERIALS AND METHODS**

**Animal experiment**

The animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chonbuk National University, Jeonju, Korea (CBU 2011-0029). Male C57BL/6 mice (7 weeks old, weighing 20–22 g) were purchased from Orient Bio Inc. (Seoul, Korea) and maintained in a room under controlled temperature (23 ± 1°C), humidity, lighting (12 h light/12 h dark cycle) and free access to water. The mice were divided into four experimental groups: sham and UUO groups with vehicle treatment (n = 15 each group), and sham and UUO groups with tamoxifen treatment (n = 15 each group). Tamoxifen (Sigma Chemical Co., St Louis, MO) was suspended in 100 μL ethanol and dissolved in 900 μL corn oil (Sigma Chemical Co.). Tamoxifen was administered by daily oral gavages (50 mg/kg) for 5 days before UUO operation and continued to 14 days of postoperative periods. Corn oil was used as vehicle.

Renal fibrosis was induced by UUO operation as described previously [23]. Briefly, mice were anesthetized using ketamine (100 mg/kg; Huons, Seoul, Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, Korea), and then an incision was made in

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**FIGURE 1**: Effect of tamoxifen (TAM) on UUO-induced renal tubular injury and fibrosis. (A) Representative PAS-stained sections of kidneys from sham and UUO-operated mice treated with vehicle (Veh) or TAM. Semi-quantitative scoring of tubular injury showed more damage in UUO mice treated with Veh compared with UUO mice treated with TAM (n = 15 per group). Data are expressed as mean ± SD. ***P < 0.001 versus Veh or TAM; **P < 0.01 versus Veh. (B) Representative Masson’s trichrome stained sections of kidneys from sham- and UUO-operated mice treated with Veh or TAM. Bar chart shows area fraction (%) of tubulointerstitial fibrosis in the sham- and UUO-operated kidneys after 2 weeks of surgery. Ten randomly chosen, non-overlapping fields at a magnification of ×200 were quantified (n = 15 per group). Data are expressed as mean ± SD. ***P < 0.001 versus Veh or TAM; **P < 0.01 versus UUO. Sham, sham-operated mice; UUO, unilateral ureteral obstruction operated mice.
the midline of the abdomen. The right proximal ureter was exposed and ligated at two separated points by using 3-0 black silk. Sham operation was performed using the same method without ligation of ureter. Two weeks after UUO, the mice were anesthetized and the obstructed kidney was harvested, prepared for histologic examinations and stored at –80°C for western blot analysis and cytokine assay.

Renal histologic examination

The kidney was fixed in 4% paraformaldehyde and embedded in paraffin. The block was cut into 5 μm sections and stained with Periodic acid–Schiff (PAS) stain and Masson’s trichrome. Immunohistochemical staining was performed as described previously [24]. The tissue sections were then deparaffinized with xylene and rehydrated with ethanol. After treatment with the blocking buffer, the slides were incubated overnight at 4°C with anti-mouse type I collagen (Southern Biotech, Birmingham, AL), anti-ER-HR3 (BMA, Augst, Switzerland) or anti-mouse intercellular adhesion molecule (ICAM)-1 (BD Biosciences, San Jose, CA). The kidney sections were treated with DAKO Chromogen (DakoCytomation, Glostrup, Denmark) to visualize the immunocomplexes and counterstained with hematoxylin (Sigma Chemical Co.). For immunofluorescence staining, freshly frozen renal tissues were fixed with 4% paraformaldehyde, permeabilized in 1% Triton X-100 and then incubated with a blocking buffer. The tissue samples were incubated with anti-α-smooth muscle actin (α-SMA; BD Biosciences). The slides were exposed to Cy3-labeled secondary antibody (Chemicon, Temecula, CA). The nuclear staining was performed by using 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR). For morphometric analysis, all of the slides were evaluated by two observers, who were unaware of the origins of samples, using a Zeiss Z1 microscope or Zeiss LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany). The tubular injury was scored into six levels on the basis of the percentage of tubular dilatation, epithelial desquamation and loss of brush border in 10 randomly chosen, non-overlapping fields at a magnification of ×200 under a light microscope: 0, none; 0.5, <10%; 1, 10–25%; 2, 25–50%; 3, 50–75% and 4, >75%. The fibrotic areas and positive area for α-SMA, type I collagen and ICAM-1 were measured in 10 randomly chosen, non-overlapping fields at a magnification of ×200 using Image J software (http://rsb.info.nih.gov/ij). The number of ER-HR3 positive macrophages was counted at a magnification of ×400.

Picrosirius red stain

For evaluation of the collagen deposition after ureteral obstruction, paraffin-embedded tissue sections were stained with picrosirius red [25]. After deparaffinization, sections were hydrated and stained with 0.1% picrosirius red solution (Sigma Chemical Co.) for 1 h. After washing in acidic water, tissue

![Figure 2](http://example.com/figure2.png)

**FIGURE 2:** Effect of TAM on UUO-induced renal fibroblast activation. (A) Representative sections of kidneys from sham and UUO-operated mice treated with Veh or TAM. The sections were stained with α-SMA. Bar chart shows the area fraction (%) of α-SMA in the sham operated and UUO kidneys 2 weeks after surgery. Ten randomly chosen, non-overlapping fields at a magnification of ×400 were quantified (n = 15 per group). Data are expressed as mean ± SD. **P < 0.001 versus Veh or TAM; ††† P < 0.001 versus UUO. (B) α-SMA and vimentin expression in kidney tissue from sham and UUO-operated mice treated with Veh or TAM were evaluated by western blotting. Data from densitometric analyses are presented as the relative ratio of each protein to GAPDH. The relative ratio measured in the kidneys from sham-operated mice treated with Veh is arbitrarily presented as 1. Data are expressed as mean ± SD. **P < 0.001 versus Veh or TAM; †† P < 0.01 versus UUO. Sham, sham-operated mice; UUO, unilateral ureteral obstruction operated mice.
sections were dehydrated and mounted. Sections were examined by a polarizing microscope (Olympus Bx50F4, Tokyo, Japan).

**Western blotting**

Western blot analysis was performed as described previously [26]. Primary antibodies to α-SMA (Sigma Chemical Co.), vimentin (Sigma Chemical Co.), fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), ICAM-1 (Santa Cruz Biotechnology), phospho-Smad2 (Cell Signaling Technology, Danvers, MA, USA), phospho-Smad3 (Cell Signaling Technology), Smad2 (Santa Cruz Biotechnology), Smad3 (Millipore, Temecula, CA, USA) and Smad7 (Cell Signaling Technology) were used. Actin (Sigma Chemical Co.) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Bioworld, Atlanta, GA) were used as internal control. All signals were analyzed by a densitometric scanner (LAS-3000; Fuji Film, Tokyo, Japan).

**Measurement of renal tumor necrosis factor-α and TGF-β1 levels**

The tumor necrosis factor (TNF)-α and TGF-β1 levels were determined by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Cell culture and knockdown of ER-α**

*In vitro* experiments were performed using rat renal fibroblast cell line (NRK-49F; American Type Culture Collection, Manassas, VA). NRK-49F cells were cultured in Dulbecco’s modified Eagle’s medium with 4 mM l-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 5% (v/v) heat-inactivated fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) at 37°C with 5% CO₂ in 95% air. To investigate the effect of tamoxifen on fibroblast activation, subconfluent NRK-49F cells were incubated with tamoxifen (0.5, 1 and 5 μM) and stimulated TGF-β1 (2 ng/mL; Sigma Chemical Co.) for indicated time periods. To examine the effect of tamoxifen on the ER-mediated TGF-β1/Smad signaling pathway, NRK-49F cells were pretreated with pure ER antagonist, ICI 182,780 (5 μM; Sigma Chemical Co.), or ERα siRNA (On-Target plus; Thermo Scientific, Pittsburgh, PA). For RNA interference study, ERα siRNA (2 nM) or non-target control siRNA (2 nM) were transfected using lipofectamine (Invitrogen, Carlsbad, CA) in subconfluent NRK-49F cells. After knockdown of ERα, cells were

![F I G U R E 3](image-url) Effect of TAM on UUO-induced type I collagen and fibronectin expression. (A) Representative sections of kidneys from sham- and UUO-operated mice treated with Veh or TAM. The sections were stained with type I collagen and picrosirius red with polarizing microscope. Bar chart shows the area fraction (%) of type I collagen and picrosirius red positive areas (%) in the sham operated and UUO kidneys 2 weeks after surgery. Ten randomly chosen, non-overlapping fields at a magnification of ×200 were quantified (n = 15 per group). Data are expressed as mean ± SD. ***P < 0.001 versus Veh or TAM; †††P < 0.001 versus UUO. (B) Fibronectin expression in kidney tissue from sham- and UUO-operated mice treated with Veh or TAM was evaluated by western blotting. Data from densitometric analysis are presented as the relative ratio of each protein to GAPDH. The relative ratio measured in the kidneys from sham-operated mice treated with Veh is arbitrarily presented as 1. Data are expressed as mean ± SD. ***P < 0.001 versus Veh or TAM; †††P < 0.001 versus UUO. Sham, sham-operated mice; UUO, unilateral ureteral obstruction operated mice.
incubated with tamoxifen (5 μM) and then stimulated with TGF-β1 (2 ng/mL) for indicated time periods.

Cell proliferation assay

After 24-h treatment with tamoxifen (0.5, 1 and 5 μM) and TGF-β1 (2 ng/mL), proliferation of NRK-49F cells was determined by a colorimetric assay (Cell Proliferation Kit II; Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. All experimental values were determined from triplicate wells.

Wound healing assay

Subconfluent NRK-49F cells were cultured in 6-well dishes. Before treatment with tamoxifen and TGF-β1, dishes were scratched using a sterile 200 μL pipet tip, causing three separate wounds. The cells were incubated with tamoxifen (0.5, 1 and 5 μM) for 30 min and then stimulated with TGF-β1 (2 ng/mL) for 24 h. Wound lengths were measured using ImageJ program. At 0 h after scratching, this wound length was used as control.

Statistical analysis

Data were expressed as mean ± SD. Multiple comparisons were examined for significant differences using ANOVA, followed by individual comparison with the Tukey’s post hoc test, with P < 0.05 indicating statistical significance.

RESULTS

Tamoxifen decreases UUO-induced renal tubular injury and fibrosis

Because tamoxifen binds to ER for its effects, we determined ERα expression in the sham and UUO kidney. ERα was expressed in sham operated and UUO kidneys, and the expression was not changed at 4, 7 and 14 days after UUO operation (Supplementary data, Figure S1A). Immunohistochemical staining analysis revealed that ERα was expressed in the nucleus of tubular epithelial cells (Supplementary data, Figure S1B). To investigate the effect of tamoxifen on UUO-induced renal fibrosis, we examined kidney section by PAS and Masson trichrome staining and graded the tubular injury by three parameters: tubular dilatation, epithelial desquamation and loss of brush border. Two weeks after surgery, the UUO kidneys from vehicle-treated mice showed destruction of renal tubules with infiltration of mononuclear inflammatory cells and severe tubulointerstitial fibrosis (Figure 1A and B). The obstructed kidneys from tamoxifen-treated mice showed an improvement of tubular damage, a decrease in inflammatory cell infiltration and tubulointerstitial fibrosis. These data suggest that tamoxifen improves UUO-induced tubular injury and fibrosis.

Tamoxifen modulates UUO-induced renal fibroblast activation and proliferation

After ureteral obstruction, renal interstitial fibroblasts are activated and differentiated into myofibroblasts. Therefore, we assessed the renal interstitial fibroblasts after UUO using anti-S100A4, a fibroblast-specific protein-1 (FSP-1) antibody. The number of FSP-1 positive fibroblasts was increased in the tubulointerstitial areas in the kidney from UUO mice compared with the number in sham-operated kidney. Tamoxifen treatment significantly decreased the number of FSP-1 positive fibroblast infiltration in UUO kidney (Supplementary data, Figure S2). We also evaluated α-SMA positive myofibroblast infiltration. The α-SMA positive myofibroblast infiltration was significantly increased in the vehicle-treated mice. Tamoxifen treatment significantly decreased the UUO-induced increase of α-SMA positive myofibroblast infiltration (Figure 2A).

In western blotting, UUO also increased α-SMA and vimentin expression ~4.7- and 2.8-fold, respectively, compared with those in the kidney of vehicle or tamoxifen mice. Tamoxifen treatment significantly decreased the UUO-induced increase of α-SMA and vimentin expression (Figure 2B). These data indicate that tamoxifen decreases activation of renal fibroblasts and accumulation of myofibroblasts after ureteral obstruction.

Tamoxifen decreases UUO-induced type I collagen and fibronectin expression

Because deposition of ECM is one of the important processes in renal fibrosis, the expression of type I collagen and fibronectin was evaluated. In addition, we assessed collagen fibril deposition by picrosirius red stain with light polarization,
which is a more specific method for detection of collagen fibrils [27]. The expression of type I collagen was significantly increased in the tubulointerstitial areas of obstructed kidney compared with sham-operated kidney. Tamoxifen treatment significantly decreased a UUO-induced increase of type I collagen expression. In UUO kidney, yellow-orange birefringences were increased in tubulointerstitial area, which is suggestive of deposition of type I collagen fibrils. Tamoxifen treatment significantly decreased UUO-induced deposition of type I collagen fibrils (Figure 3A). Western blot analysis showed that fibronectin expression in obstructed kidney was substantially increased ∼4.3-fold, compared with the expression in sham-operated kidney. Tamoxifen treatment significantly ameliorated the UUO-induced increase of fibronectin expression (Figure 3B). These data suggest that tamoxifen modulates UUO-induced ECM expression.

Tamoxifen ameliorates UUO-induced macrophage infiltration and proinflammatory cytokine expression

Interstitial macrophage infiltration is another hallmark of renal fibrosis processes following ureteral obstruction. Therefore, we examined the inflammatory cell infiltration after ureteral obstruction with or without tamoxifen treatment. The number of ER-HR3 positive macrophages was increased in the tubulointerstitial areas of the kidney from UUO mice (Figure 4). Tamoxifen treatment significantly decreased the number of ER-HR3 positive macrophages in the UUO kidney. We also evaluated expression of inflammatory cytokines and cell adhesion molecules. Following ureteral obstruction, ICAM-1 expression was significantly increased in the tubulointerstitial areas (Figure 5A). Tamoxifen reduced significantly the UUO-induced increase of ICAM-1 expression in the UUO kidney. Consistent with the immunostaining data, western blot analysis showed a significant increase of ICAM-1 that was decreased by tamoxifen treatment (Figure 5B). The level of TNF-α significantly increased after UUO (285.4 ± 104.6 pg/100 μg protein) compared with the level in the kidney of vehicle (14.7 ± 0.6 pg/100 μg protein) or tamoxifen (16.9 ± 4.8 pg/100 μg protein) treated mice (Figure 5C). Tamoxifen treatment reduced the UUO-induced increase of TNF-α level (69.4 ± 48.6 pg/100 μg protein). These data suggest that tamoxifen reduces UUO-induced renal inflammation.

Tamoxifen decreases UUO-induced TGF-β1 expression via Smad signaling pathway

TGF-β1 is one of the most important cytokines in the renal fibrosis. The TGF-β1 level in the kidney of UUO mice was

FIGURE 5: Effect of TAM on UUO-induced ICAM-1 and TNF-α expression. (A) Representative sections of kidneys from sham- and UUO-operated mice treated with Veh or TAM. The sections were stained with ICAM-1. Bar graph shows the area fraction (%) of ICAM-1 in the sham operated and UUO kidneys 2 weeks after surgery. Ten randomly chosen, non-overlapping fields at a magnification of ×200 were quantified (n = 15 per group). Data are expressed as mean ± SD. ***P < 0.001 versus Veh or TAM; †††P < 0.001 versus UUO. (B) ICAM-1 expression in kidney tissue from sham- and UUO-operated mice treated with Veh or TAM was evaluated by western blotting. Data from densitometric analysis are presented as the relative ratio of each protein to GAPDH. The relative ratio measured in the kidneys from sham-operated mice treated with Veh is arbitrarily presented as 1. Data are expressed as mean ± S.D. **P < 0.01 versus Veh or TAM; ††P < 0.01 versus UUO. (C) Renal TNF-α levels from sham- and UUO-operated mice treated with Veh or TAM were measured by ELISA. The levels were normalized to 100 μg of kidney protein. Data are expressed as mean ± SD (n = 15 per group). **P < 0.01 versus Veh or TAM; ††P < 0.01 versus UUO. Sham, sham-operated mice; UUO, unilateral ureteral obstruction operated mice.
significantly increased (394.9 ± 127.9 pg/100 μg protein) compared with the level in vehicle (54.6 ± 4.2 pg/100 μg protein) or tamoxifen (49.4 ± 1.3 pg/100 μg protein) treated sham mice (Figure 6A). Tamoxifen decreased UUO-induced increase of TGF-β1 (274.2 ± 43.9 pg/100 μg protein). Next, we evaluated downstream signaling molecules of TGF-β1, Smad3 and Smad7. The level of phospho-Smad3 in the kidney of UUO mice was increased ∼2.43-fold compared with the level in sham-operated mice, whereas Smad7 expression was significantly decreased (Figure 6B). Tamoxifen treatment reduced the UUO-induced increase of phospho-Smad3 and the Smad7 level was significantly recovered (Figure 6B). These data suggest that tamoxifen inhibits the UUO-induced activation of TGF-β1/Smad signaling pathway.

Tamoxifen decreases renal interstitial fibroblast proliferation and migration in NRK-49F cells

To address the protective mechanism of tamoxifen in UUO-induced tubulointerstitial fibrosis, we evaluated TGF-β1-induced renal interstitial fibroblast proliferation and migration in vitro using NRK-49F cells. Treatment of TGF-β1 significantly increased proliferation of NRK-49F cells compared with that of vehicle-treated cells (Figure 7A). Tamoxifen treatment decreased TGF-β1-induced proliferation of NRK-49F cells in a dose-dependent manner. Treatment of NRK-49F cells with TGF-β1 also significantly increased cell migration compared with baseline (0 h) (Figure 7B). Tamoxifen treatment significantly inhibited TGF-β1-induced cell migration. These results indicate that tamoxifen inhibits TGF-β1-induced renal interstitial fibroblast proliferation and migration.

Tamoxifen decreases TGF-β1-induced renal interstitial fibroblast activation via ERα-mediated TGF-β1/Smad signaling pathway in NRK-49F cells

To examine TGF-β1-induced renal interstitial fibroblast activation, TGF-β1-induced α-SMA expression in NRK-49F cells was evaluated. Treatment of cells with TGF-β1 for 24 and 48 h increased α-SMA expression in a dose-dependent manner (Supplementary data, Figure S3). Tamoxifen decreased TGF-β1-induced α-SMA expression in a dose-dependent manner (Figure 8A). To access the role of ER in TGF-β1-induced α-SMA expression, we treated cells with pure ERα antagonist, ICI 182,780, or ERα siRNA. Treatment with ICI 182,780 reversed the suppressive effect of tamoxifen on TGF-β1-induced α-SMA expression (Figure 8B). The expression of ERα in NRK-49F cells was significantly decreased by transfection of ERα siRNA compared with non-target siRNA (Supplementary data, Figure S4). Knockdown of ERα in NRK-49F cells by transfection of ERα siRNA abolished the effect of tamoxifen on TGF-β1-induced α-SMA expression (Figure 8C).
We further evaluated the effect of tamoxifen on downstream molecules of TGF-β1 signaling pathway. Treatment of NRK-49F cells with TGF-β1 increased levels of phospho-Smad2 and 3 in a time-dependent manner (Supplementary data, Figure S5). Because phosphorylation of Smad2 and 3 peaked at 30–60 min after TGF-β1 treatment in NRK-49F cells, we chose 30 min for the evaluation of TGF-β1-induced phosphorylation of Smad2 and 3. Tamoxifen significantly decreased the TGF-β1-induced increase of phospho-Smad2 and 3 expressions in a dose-dependent manner (Figure 8D). To verify the effect of tamoxifen on ERα-mediated TGF-β1/Smad signaling pathway, we treated ERα siRNA in NRK-49F cell. After knockdown of ERα, effect of tamoxifen was abolished (Figure 8E). These data indicate that tamoxifen modulates activation of renal interstitial fibroblasts through ERα-mediated TGF-β1/Smad signaling pathway.

**DISCUSSION**

Tubulointerstitial fibrosis is a final common feature after chronic renal insults. Therefore, regulation of the renal fibrotic process provides a new insight into the treatment and prevention of chronic kidney diseases. In this study, we evaluated the protective effect of tamoxifen on UUO-induced renal fibrosis, including the molecular mechanisms involved. Our results revealed that tamoxifen decreases UUO-induced renal tubular injury, interstitial matrix deposition and inflammatory processes. Tamoxifen also inhibited TGF-β1-induced renal fibroblast activation through suppression of ERα-mediated TGF-β1/Smad signaling pathway. These findings suggest that tamoxifen is an effective agent for preventing renal fibrosis.

Fibrosis is considered as a result of imbalance between self-limited wound healing process and excessive accumulation
of ECM after initial insult [28]. Renal fibrosis begins from localized activation of inflammatory processes, which include infiltration of inflammatory cells, activation of NF-κB and production of proinflammatory cytokines and chemokines [28–31]. The injured tubules and infiltrated inflammatory cells trigger activation of the profibrotic signaling pathway, which contributes to activation of matrix-producing cells [31, 32]. Among matrix-producing cells, renal interstitial fibroblasts have an important role in renal fibrosis. During the renal fibrosis process, renal interstitial fibroblasts are activated, display proliferative properties and express α-SMA [33]. Acquisition of the myofibroblast phenotype may be one of the stress responses to renal injury such as mechanical strain or fluid shear stress in UUO [32, 34]. Therefore, modulation of renal interstitial fibroblast activation is an important therapeutic target for prevention of renal fibrosis. Our in vitro data suggest that

![Figure 8: Effect of TAM on TGF-β1-mediated renal fibroblast activation by ERα-mediated TGF-β1/Smad signaling pathway.](image)

- **A** A representative immunoblot photograph for α-SMA expression in NRK-49F cells after treatment of Veh, TGF-β1 with or without TAM. Data from densitometric analysis of α-SMA are presented as the relative ratio of each protein to actin. The relative ratio measured from Veh-treated NRK-49F cells is arbitrarily presented as 1. Data are expressed as mean ± SD from three independent experiments. ***P < 0.001 versus Veh or TAM; †P < 0.05 versus TGF-β1; ††P < 0.01 versus TGF-β1.

- **B** Effect of pure ER antagonist, ICI 182,780, on TGF-β1-induced α-SMA expression with or without treatment of TAM in NRK-49F cells. Data from densitometric analysis of α-SMA are presented as the relative ratio of each protein to actin. The relative ratio measured from Veh-treated NRK-49F cells is arbitrarily presented as 1. Data are expressed as mean ± SD from three independent experiments. ***P < 0.001 versus Veh or TAM; †††P < 0.001 versus TGF-β1; ##P < 0.01 versus TGF-β1 + TAM.

- **C** Effect of ERα knockdown on TGF-β1-induced α-SMA expression with or without treatment with TAM in NRK-49F cells. Data from densitometric analysis of α-SMA are presented as the relative ratio of each protein to actin. The relative ratio measured from Veh-treated NRK-49F cells is arbitrarily presented as 1. Data are expressed as mean ± SD from three independent experiments. ***P < 0.001 versus Veh; **P < 0.01 versus Veh; †††P < 0.001 versus TGF-β1; †P < 0.05 versus TGF-β1; ††P < 0.01 versus TGF-β1 + TAM in non-target siRNA-treated NRK-49F cells. (D) A representative immunoblot photograph of phospho-Smad2 or phospho-Smad3 expression in NRK-49F cells after treatment of Veh, TGF-β1 with or without TAM. Data from densitometric analyses of phospho-Smad2 or phospho-Smad3 are presented as the relative ratio of each protein to Smad2 or Smad3. The relative ratio measured from Veh-treated NRK-49F cells is arbitrarily presented as 1. Data are expressed as mean ± SD from three independent experiments. **P < 0.01 versus Veh or TAM; ††P < 0.01 versus Veh; †P < 0.05 versus TGF-β1.

- **E** Effect of ERα knockdown on TGF-β1-induced phospho-Smad2 or phospho-Smad3 level with or without treatment with TAM in NRK-49F cells. Data from densitometric analysis of phospho-Smad2 or phospho-Smad3 are presented as the relative ratio of each protein to Smad2 or Smad3. The relative ratio measured from Veh-treated NRK-49F cells is arbitrarily presented as 1. Data are expressed as mean ± SD from three independent experiments. **P < 0.01 versus Veh; †P < 0.05 versus TGF-β1.
tamoxifen modulates not only TGF-β1-induced activation of NRK-49F cells, a renal interstitial fibroblast cell line, but also migration of the cells. The in vivo data also showed that tamoxifen significantly decreased UUO-induced increase of α-SMA, vimentin, type I collagen and fibronectin expression.

Inflammation has an important role in the initiation of renal fibrogenesis [4]. In chronic UUO models, macrophages are infiltrated into the tubulointerstitial areas under the influence of adhesion molecules, chemokines and cytokines [35]. Our UUO model showed that ICAM-1 expression was increased in tubulointerstitial areas, and tamoxifen significantly decreased the UUO-induced increase of ICAM-1 expression. Along with this adhesion molecule expression, the number of ER-HR3 positive macrophages, which is a marker for more mature cells of mononuclear phagocyte system [36], is increased in the tubulointerstitial areas. These findings suggest that tamoxifen decreases UUO-induced inflammatory response via regulation of macrophage recruitment into the renal parenchyma.

TGF-β is a multifunctional cytokine involved in regulation of cell growth, apoptosis, differentiation, migration, ECM production, immune response and angiogenesis [8, 37]. There are three isoforms of TGF-β in mammals, TGF-β1, 2 and 3 [29]. Among them, TGF-β1 isoform is a key player in renal fibrosis. Our in vivo study showed that renal TGF-β1 level was significantly increased by UUO. Tamoxifen treatment effectively decreased the UUO-induced increase of renal TGF-β1 level. In addition, UUO resulted in an increase of phospho-Smad3 indicating that Smad3 is activated. Tamoxifen reduced the UUO-induced increase of phospho-Smad3 level. On the other hand, expression of Smad7 was significantly suppressed after UUO, and tamoxifen significantly recovered its expression.

TGF-β1/Smad signaling pathway may be influenced by functional cooperative interactions between Smad and other kinds of transcriptional factors, kinase receptors and nuclear receptors [38]. Estrogen inhibits TGF-β1 signaling pathway through direct physical and functional interaction between Smad3 and ERα in cancer cells [10]. Other steroid receptor families such as androgen, vitamin D and glucocorticoid receptors also influence TGF-β1 signaling through positive or negative impact on physiological or pathological processes [39–41]. Our in vitro study demonstrated that tamoxifen treatment decreased the TGF-β1-induced increase of α-SMA expression and phosphorylation of Smad2 and 3 in renal interstitial fibroblast cells. However, knocking down of ERα abrogated tamoxifen effect. These observations suggest that tamoxifen regulates TGF-β1-induced activation of renal interstitial fibroblasts via the ERα-dependent TGF-β1/Smad signaling pathway and may act as an ER agonist in renal interstitial fibroblasts.

In conclusion, our studies have demonstrated that tamoxifen attenuates UUO-induced renal tubulointerstitial fibrosis in mice by regulating ECM production, renal inflammation and TGF-β1/Smad protein expression. Our results also suggest that the protective mechanism underlying tamoxifen treatment involves the regulation of renal fibroblast activation via ERα-dependent TGF-β1/Smad signaling pathway. The clinical implication of SERMs in fibrosis may be a novel therapeutic option for preventing and treating chronic kidney diseases.

SUPPLEMENTARY DATA
Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT
None declared.

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