# Insulin-like growth factor binding protein-3 specific aptamer alleviates unilateral ureteral obstruction-induced renal fibrosis

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# Insulin-like growth factor binding protein-3 specific aptamer alleviates unilateral ureteral obstruction-induced renal fibrosis

Directed by Professor Shin-Wook Kang

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This certifies that the Doctoral Dissertation of Mi Jung Lee is approved.

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When I took the first step as a nephrologist, I hope to be a scholar who would investigate both clinical and basic research. PhD was my first challenge for basic research, but the degree program was much tougher than I expected. Still, I have walked to the place where my heart led and I wish I got a little closer to the way I dreamed of.

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<ABSTRACT>

# Insulin-like growth factor binding protein-3 specific aptamer alleviates unilateral ureteral obstruction-induced renal fibrosis

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Background: Insulin-like growth factor binding protein-3 (IGFBP-3) is a predominant IGFBP among the six IGFBPs. Emerging evidence indicates that IGFBP-3 is a mediator of fibrosis and has cell– and tissue–specific effects in various cell types and organs. However, to date, the effect of IGFBP-3 on renal tubulointerstitial fibrosis has not been evaluated. This study was to investigate whether IGFBP-3 is involved in tubular epithelial cell injury induced by transforming growth factor (TGF)-βl *in vitro* and renal fibrosis induced by unilateral ureteral obstruction (UUO) *in vivo*.

Furthermore, the effect of IGFBP-3 inhibition by specific DNA aptamers on attenuation of renal fibrosis was also examined.

Methods: *In vitro*, proximal tubular cells (NRK-52E) were treated with or without TGF-βl (10 ng/mL). IGFBP-3 inhibition was performed by IGFBP-3 small interfering RNA (siRNA) (50 nM) and IGFBP-3–specific binding DNA aptamers (200 nM) for 48 h, respectively. *In vivo*, twenty-four C57BL/6 mice were categorized into 4 groups of 6 mice each: a sham operation with diluent (control), a sham operation with IGFBP-3 aptamers, a UUO operation with diluent, and a UUO operation with IGFBP-3 aptamers. The animals were treated intraperitoneally with either the diluent or IGFBP-3 aptamers (1 mg/kg) 1 h before and every 48 h after sham or UUO surgery. Mice were sacrificed at 7 days after operation, and their left kidneys were removed. Fibrosis–related proteins and IGFBP-3–dependent signaling cascades, including mitogen–activated protein (MAP) kinases were examined by western blots in cultured NRK-52E cells and the kidneys. Masson's trichrome and immunohistochemical staining were used to evaluate renal tubulointerstitial fibrosis in the kidneys.

Results: In TGF-βl–stimulated NRK-52E cells, mRNA and protein expressions of IGFBP-3 were up–regulated in dose– and time–dependent manners. TGF-βl induced fibrosis–related protein expression, including fibronectin and type I collagen, and these increases were reduced by IGFBP-3 siRNA treatment in renal proximal tubule cells. IGFBP-3–specific aptamers significantly abrogated fibrosis–related protein expression up–regulated by TGF-βl in NRK-52E cells. In the kidneys of UUO mice, IGFBP-3 and fibrosis–related protein expression were increased compared with sham–operated mice. IGFBP-3–specific binding aptamers significantly alleviated these changes. Masson's trichrome and immunohistochemical staining also showed an attenuation of renal tubulointerstitial fibrosis in UUO mice treated with IGFBP-3 aptamers. In both TGF-βl–treated NRK-52E cells and UUO mice, IGFBP-3 aptamer treatment significantly reduced the activation of the p38 MAP kinase pathway.

Conclusion: IGFBP-3 is closely implicated in the pathogenesis of renal tubulointerstitial fibrosis, and its inhibition by a specific binding aptamer may offer a therapeutic potential for various kidney diseases associated with renal fibrosis.

Key words: aptamer, insulin-like growth factor binding protein-3, renal tubulointerstitial fibrosis, p38 mitogen–activated protein kinase, unilateral ureteral obstruction

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## I. INTRODUCTION

Renal fibrosis is the final pathologic manifestation by which a wide variety of chronic kidney diseases progress to end-stage renal disease, regardless of the initial causes.[1] It is therefore important to identify initiating or aggravating factors associated with the pathogenesis of renal tubulointerstitial fibrosis in the management of chronic kidney disease. Insulin-like growth factor binding protein-3 (IGFBP-3) is the most prominent member of the six members of the IGFBP family (IGFBP-1 to -6) that binds to insulin-like growth factor (IGF) with high affinity and specificity.[2,3] IGFBP-3

binds circulating IGF-I, prolonging the half-life of IGFs while modulating their availability.[2,3] In addition to IGF–dependent activity, IGFBP-3 also exerts IGF–independent biological effects that involve neither binding of IGFs nor modulation of IGF receptors.[2,3] In particular, IGFBP-3 has been reported to be a significant mediator of organ fibrosis in various cell types.[4-8] IGFBP-3 expression was found at high levels in fibroblasts of patients with systemic sclerosis and idiopathic pulmonary fibrosis, and silencing of IGFBP-3 was associated with inhibition of the pro-fibrotic signaling pathway.[4-8] In the kidney, IGFBP-3 mediates apoptosis in mesangial cells, podocytes, and proximal tubular epithelial cells stimulated by tumor necrosis factor-α, transforming growth factor (TGF)-β1, or high glucose.[9-11] Based on the fibrosis–mediating effect of IGFBP-3 in various fibrotic diseases and increased expression in pathologic environments of the kidney, I hypothesized that IGFBP-3 may be implicated in renal tubulointerstitial fibrosis and its inhibition may be a potential therapeutic approach for kidney fibrosis.

Aptamer is a single–stranded DNA or RNA that can bind specifically with a cognate molecular target.[12] Due to highly specific binding abilities, aptamer has been investigated as a propitious tool for targeted therapies.[12] Moreover, thermal or chemical stability, low immunogenicity, and cost-effectiveness make aptamers more readily applicable to clinical medicine compared with antibodies.[12] Therefore, the present study investigated whether IGFBP-3 is a significant mediator of renal tubulointerstitial fibrosis. In addition, the effect of IGFBP-3 inhibition using specific aptamers on attenuating renal fibrosis was evaluated in both renal tubular cells and unilateral ureteral obstruction (UUO) mice.

## II. MATERIALS AND METHODS

### 1. Cell cultures

Renal proximal tubular epithelial cells (NRK-52E) were used *in vitro*. NRK-52E cells were cultured in Dulbeco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 26 mM $NaHCO_3$ at 37 ℃ in humidified 5% $CO_2$ in air. Subconfluent NRK-52E cells were serum–restricted for 24 h, after which the media were replaced by 1.5% FBS-DMEM for the control group and the same media containing TGF-β1 (10 ng/mL) (R&D Systems, Minneapolis, MN, USA) for the intervention group, with or without IGFBP-3 small interfering RNA (siRNA) (50 nM) (Invitrogen, Carlsbad, CA, USA) or IGFBP-3–specific aptamers (200 nM) (Aptamer Science, Pohang, Korea). Cells were harvested 48 h after the media change. IGFBP-3 siRNA was transfected with RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All experimental groups were cultured in triplicate.

### 2. Animal experiments

All animal studies were conducted under a protocol approved by the Committee for the Care and Use of Laboratory Animals at CHA University College of Medicine, Seongnam, Korea. Animal experiments were conducted in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication No. 85–23, revised 1985). Twenty-four C57BL/6 mice were categorized into 4 groups of 6 mice each: a sham operation group with a diluent (control), a sham operation group with IGFBP-3 aptamers, a UUO group with diluent, and a UUO group with IGFBP-3–specific binding aptamers. The animals were treated intraperitoneally with either a diluent or IGFBP-3 aptamer (1 mg/kg) at 1 h before and every 48 h after sham or UUO surgery. Mice were sacrificed 7 days after operation, and the left kidneys were removed for histological evaluation and molecular biological analysis.

**3. Generation of a renal tubulointerstitial fibrosis animal model by UUO**

UUO mice were prepared as previously described.[13] Briefly, each C57BL/6 mouse was anesthetized with zolazepam hydrochloride, a left-flank incision was made to expose to the left ureter, which was ligated with 6-0 silk sutures at two points, and a cut made between the ligatures. The peritoneal membrane and skin were then sutured. Sham operations were similar to UUO procedures, with the exception of the ligation and cutting of the ureter.

**4. IGFP-3–specific binding aptamers**

IGFBP-3–specific binding DNA aptamers were purchased from Aptamer Sciences Inc. (Pohang, Republic of Korea). To evaluate their specific binding ability to IGFBP-3, Cy3–labeled aptamers were used. Cy3–labeled IGFBP-3 aptamers were constructed by labeling them with Cy3 at the 5′ ends. For animal studies, a polyethylene glycol (PEG)–conjugated aptamer was used to reinforce *in vivo* biological stability by conjugating 40 kDa PEG to the 5′ end and inverted dT to the 3′ end.

**5. Quantitative real–time polymerase chain reactions**

RNA extraction from NRK-52E cells and the kidney sampling were carried out as described in a previous study.[14] A cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany) was used to obtain first–strand cDNA. The reactions were run on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), with a total volume of 20 μL containing 10 μL of SYBR Green PCR Master Mix (Applied Biosystems), 5 μL of cDNA, and 5 pmol sense and antisense primers. The primers used for IGFBP-3 and 18s amplification were IGFBP-3 sense 5′-GGCCACCAAGAATCACT-3′ antisense 5′-GGAGGTAAGGGTATAT-3′; and 18s, sense 5′-AACTAAGAACGGCCATGCAC-3′, antisense 5′-CCTGCGGCTTAATTTGACTC-3′. Each reaction tube contained 25 ng of RNA for amplification. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. The polymerase chain reaction (PCR) conditions were 35 cycles of denaturation for 30 min at 94.5 ℃,

annealing for 30 s at 60 ℃, and extension for 1 min at 72 ℃. Initial heating for 9 min at 95 ℃ and final extension for 7 min at 72 ℃ were used for PCR amplification. Each sample was run in triplicate in separate tubes and a control without cDNA was run in parallel with each assay. After real–time PCR amplification, the temperature was increased from 60 ℃ to 95 ℃ at a rate of 2 ℃/min to construct a melting curve. The cDNA content of each specimen was determined using the comparative cycle threshold method with 2-ΔΔCT. The results were given as the relative expression normalized to the expression of 18s rRNA in arbitrary units.

**6. Western blot analysis**

For western blots, cultured cells and the homogenized whole kidneys were lysed in a sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol), treated with a Laemmli sample buffer, heated at 100 ℃ for 5 min, and electrophoresed in an 8%–12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA). The membrane was then incubated in a blocking buffer A (1× PBS, 0.1% Tween-20, and 8% nonfat milk) at room temperature for 1 h, followed by overnight incubation at 4℃ in a 1:1,000 dilution of primary antibodies to IGFBP-3 (Abcam, Cambridge, UK), fibronectin (DAKO, Glostrup, Denmark), type I collagen (Southern Biotech, Birmingham, AL, USA), phospho-extracellular-signal-regulated kinase (ERK)/ERK, phospho-p38/p38, phospho-c-Jun N-terminal kinase (JNK)/JNK (Cell Signaling Technology, Denver, MA, USA), or β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA). The membrane was then washed once for 15 min and twice for 5 min in 1× PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A, which contained a 1:1,000 dilution of horseradish peroxidase–linked goat anti-rabbit or anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL,

USA). Band densities were measured with TINA imaging software (Raytest, Straubenhardt, Germany).

**7. Immunohistochemistry and Masson's trichrome staining**

Slices of the left kidney were fixed in 10% neutral-buffered formalin, processed in the standard manner, and sections of paraffin-embedded tissues 5 μm thick were utilized for immunohistochemical (IHC) staining. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in a 10 mM sodium citrate buffer for 20 min using a Black & Decker vegetable steamer. Primary antibodies for IGFBP-3, fibronectin, and type I collagen were diluted to appropriate concentrations with 2% casein in bovine serum albumin and then added to the slides, followed by overnight incubation at 4 ℃. After washing, a secondary antibody was added for 20 min, and the slides were washed and incubated with a tertiary rabbit peroxidase anti-peroxidase complex (DAKO Agilent, Santa Clara, CA, USA) for 20 min. Deaminobenzidine was added for 2 min and the slides were counterstained with hematoxylin. For Masson's trichrome staining, sections of paraffin-embedded tissues 5 μm thick were deparaffinized, hydrated in ethyl alcohol, washed in tap water, and re-fixed in Bouin's solution at 56 ℃ for 1 h. After washing in running tap water for 10 min and staining with Weigert's iron hematoxylin working solution for 10 min, the sections were stained with Biebrich scarlet-acid fuchsin solution for 15 min, followed by a wash for 10 min. Next, the slides were differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min, transferred to an aniline blue solution and stained for 10 min, and allowed to react in a 1% acetic acid solution for 5 min. Semi-quantitative staining scores were based on an examination of at least 10 tubulointerstitial fields using digital image analysis (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA, USA).

**8. Statistical analysis**

All values are expressed as the mean ± standard error of the mean. Statistical analysis was performed using SPSS for Windows Ver. 21.0 (SPSS, Inc., Chicago, IL,

USA). Results were analyzed using a Kruskal–Wallis non-parametric test for multiple comparisons. Significant differences were confirmed by Mann–Whitney U tests. *P* values of less than 0.05 were considered statistically significant.

## III. RESULTS

### 1. IGBP-3 mRNA and protein expression are increased in TGF-β1–stimulated renal tubular cells in dose– and time–dependent manners

To examine changes in IGFBP-3 expression according to TGF-β1 administration, the changes in IGFBP-3 mRNA and protein expression were determined in cultured NRK-52E cells. The mRNA and protein expression of IGFBP-3 were significantly increased at higher doses of TGF-β1 (5 and 10 ng/mL) ($P<0.05$) and after 48 h of TGF-β1 administration ($P <0.05$) (Fig. 1).

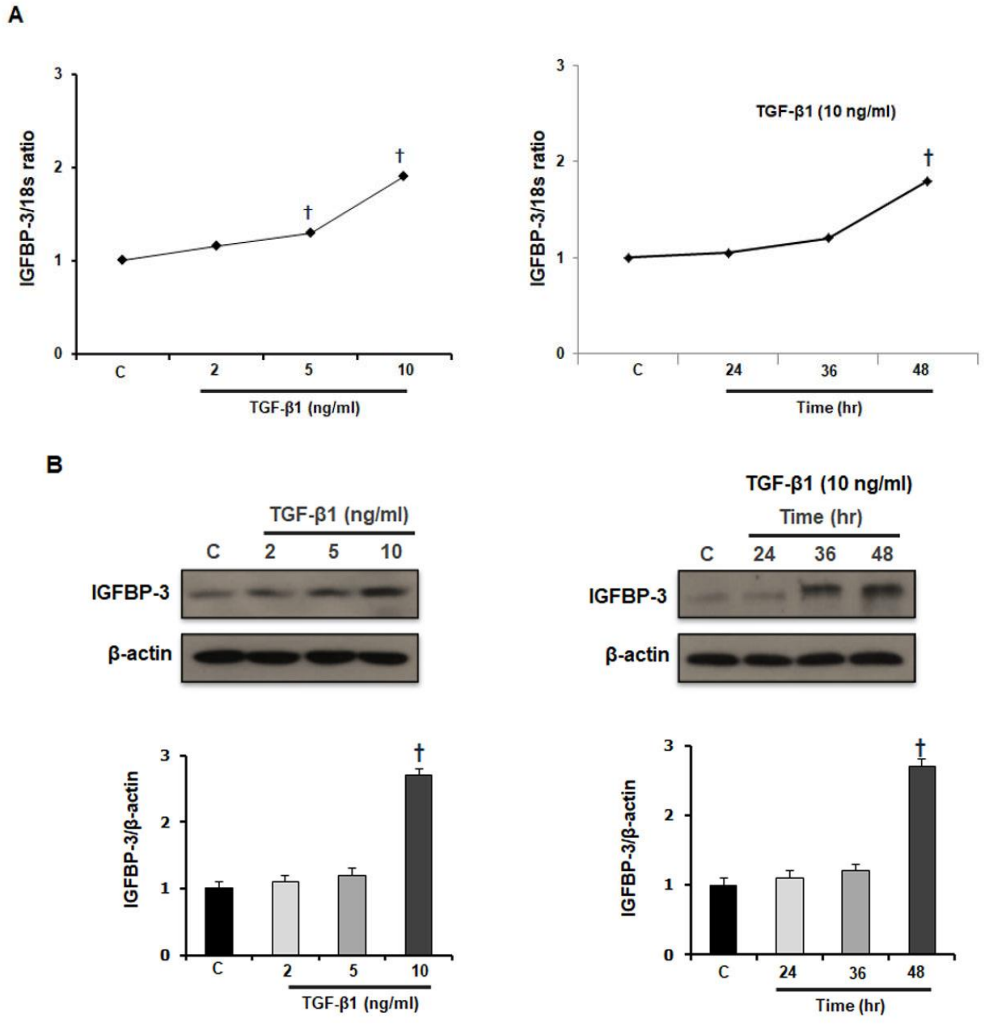


**Figure 1. IGFBP-3 expression in NRK-52E cells according to different doses and time of exposure to TGF-β1.** (A) mRNA levels of IGFBP-3 (n = 6). Compared with control cells, IGFBP-3 mRNA expression was significantly increased at higher doses of TGF-β1 (5 and 10 ng/mL) and after 48 h of administration of TGF-β1 (10 ng/mL). (B) Protein expression of IGFBP-3 was increased significantly in dose– and time–dependent manners in TGF-β1–trated cells. Representative of six blots; C, control; †P <0.05 vs. C.

**2. IGFBP-3 siRNA ameliorates the increase in fibrosis–related protein expression induced by TGF-β1 treatment in renal tubular cells**

The effect of IGFBP-3 inhibition on TGF-β1–induced fibronectin and type I collagen protein expression was determined in cultured NRK-52E cells using IGFBP-3 siRNA. The administration of TGF-β1 (10 ng/mL) significantly increased the protein expression of fibronectin and type I collagen in cultured NRK-52E cells ($P < 0.05$), and these increases were significantly abrogated by IGFBP-3 siRNA treatment ($P < 0.05$) (Fig. 2). These findings suggest that IGFBP-3 is associated with fibrosis of renal proximal tubular cells.

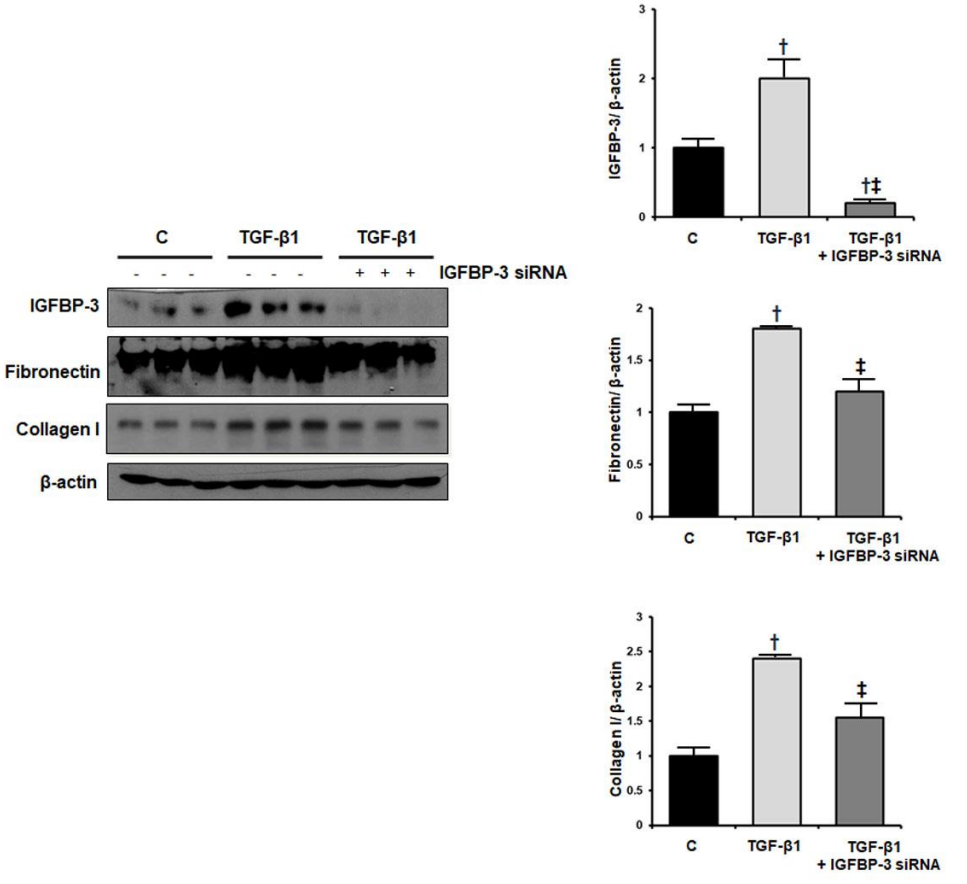


**Figure 2. A representative western blot of fibronectin and type I collagen protein in control, TGF-β1, and TGF-β1 + IGFBP-3 siRNA groups.** Compared with

control cells, there were significantly increases in fibronectin and type I collagen protein expression were evident in TGF-β1 (10 ng/mL)–stimulated NRK-52E cells. IGFBP-3 siRNA (50 nM) significantly reduced the increases in fibrosis–related protein expression. Representative of six blots; C, control; †P <0.05 vs. C; ‡P <0.05 vs. TGF-β1 group.

### 3. IGFBP-3 DNA aptamers specifically binds to IGFBP-3 in renal tubular cells

To evaluate the specific binding of IGFBP-3 DNA aptamers to IGFBP-3, immunofluorescence staining was performed using Cy3–labeled IGFBP-3 aptamers in NRK-52E cells. Cells were treated with or without TGF-β1 or IGFBP-3 siRNA. Cy3–labeled IGFBP-3 aptamers were administered as fluorescence–labeled primary antibodies during immunofluorescence staining. The intensity of Cy3 was significantly greater in TGF-β1–treated cells and reduced by IGFBP-3 siRNA transfection. These findings indicate that IGFBP-3 DNA aptamers binds specifically to IGFBP-3 expressed in NRK-52E cells.

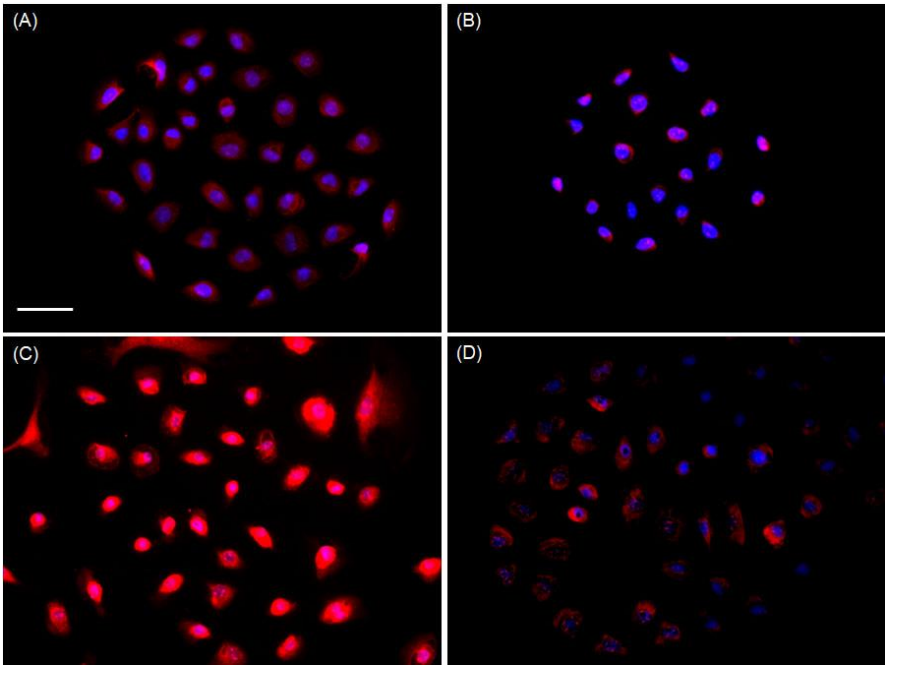


**Figure 3. Specific binding of IGFBP-3 DNA aptamers to IGFBP-3 in NRK-52E cells.** (A) control, (B) control + IGFBP-3 siRNA (50 nM), (C) TGF-β1 (10 ng/mL), and (D) TGF-β1 (10 ng/mL) + IGFBP-3 siRNA (50 nM). Cy3–labeled IGFBP-3 DNA aptamers were administered as primary antibodies to all groups. The intensity of Cy3 was significantly greater by TGF-β1 and diminished by IGFBP-3 siRNA administration. Scale bar, 50 μm; ×400.

**4. IGFBP-3–specific binding aptamers attenuate fibrosis–related protein expression in TGF-β1–stimulated renal tubular cells**

To test the effect of IGFBP-3–specific binding aptamers on renal tubular cell fibrosis, IGFBP-3 aptamers (200 nM) were administered in NRK-52E cells. Fibrosis–related protein expression, including that of fibronectin and type I collagen, was significantly lower in TGF-β1–treated NRK-52E cells (P <0.05) (Fig. 4).

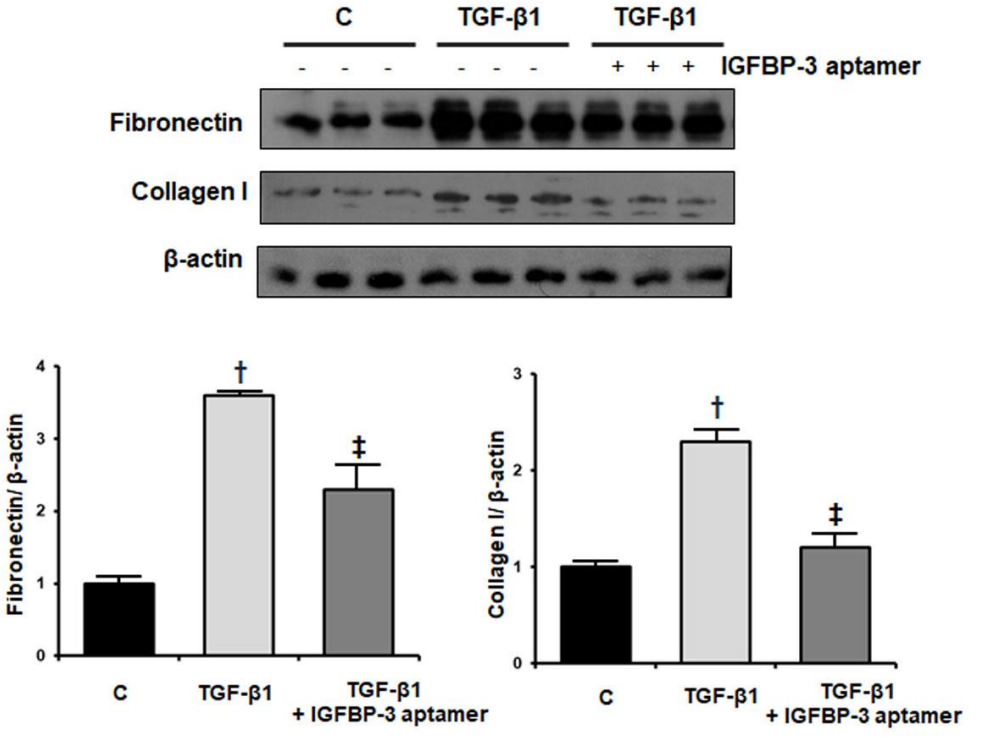


**Figure 4. A representative western blot of fibronectin and type I collagen in control, TGF-β1, and TGF-β1 + IGFBP-3 aptamer groups.** Compared with control cells, there were significant increases in fibronectin and type I collagen protein expression in TGF-β1 (10 ng/mL) –stimulated NRK-52E cells. The increases were significantly abrogated by administration of IGFBP-3–binding aptamers (200 nM). Representative of six blots; C, control; †P <0.05 vs. C; ‡P <0.05 vs. TGF-β1 group.

### 5. IGFBP-3–specific aptamers ameliorate p38 mitogen-activated protein kinase activation in TGF-β1–stimulated renal tubular cells

To examine the mechanism responsible for IGFBP-3–mediated renal tubular fibrosis, mitogen-activated protein (MAP) kinases, including phospho-ERK/ERK, phospho-JNK/JNK, and phospho-p38/p38 proteins, were measured in cultured NRK-52E cells. Compared with control cells, phosphorylation of p38 increased significantly in TGF-β1–stimulated NRK-52E cells ($P < 0.05$), and IGFBP-3 aptamers significantly reduced phosphorylation of p38 ($P < 0.05$). The expressions of phospho-ERK and phospho-JNK did not change according to IGFBP-3 aptamer treatment (Fig. 5).

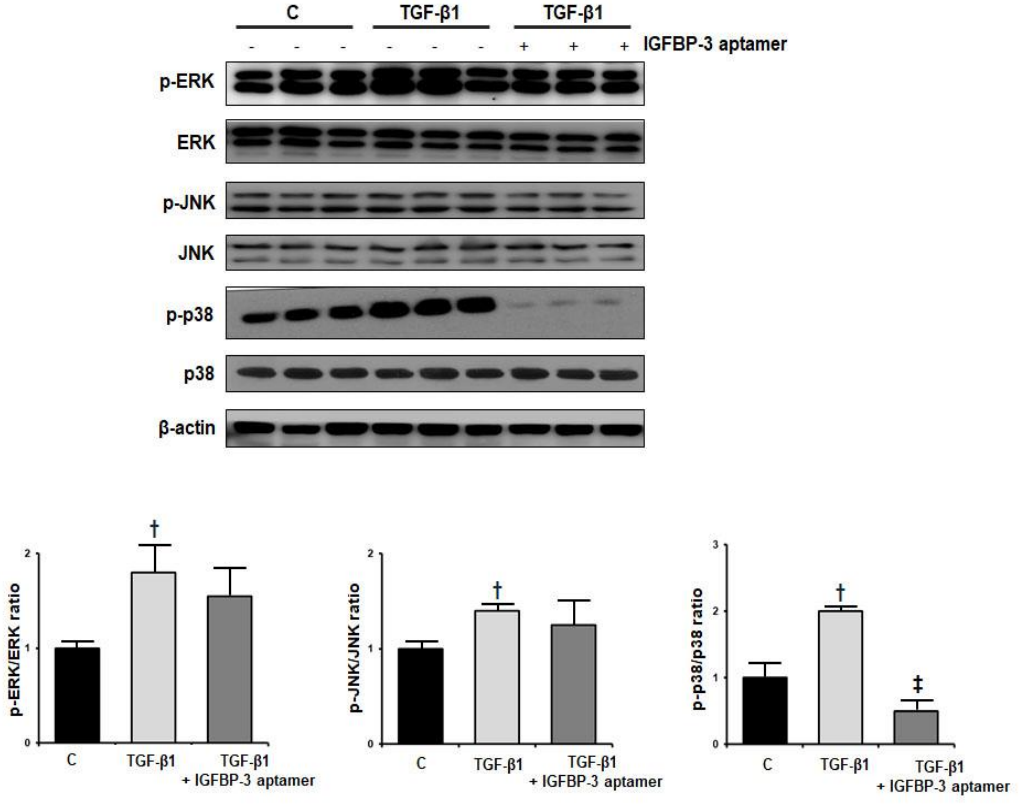


**Figure 5. A representative western blot of phospho-ERK/ERK, phospho-JNK/JNK, and phospho-p38/p38 protein in control, TGF-β1, and TGF-β1 + IGFBP-3–specific aptamer groups.** Compared to control cells, there was a significant increase in phospho-p38/p38 protein expression in TGF-β1 (10 ng/mL)–stimulated NRK-52E cells. IGFBP-3 aptamers (200 nM) significantly ameliorated this increase. Representative of six blots; C, control; †P <0.05 vs. C; ‡P <0.05 vs. TGF-β1 group.

**6. IGFBP-3 mRNA and protein expression are increased in the kidneys of UUO mice**

Compared with expression levels in control mice subjected to sham operations, IGFBP-3 mRNA and protein expression were significantly higher in the left kidney of UUO mice (Fig 6A and 6B). IHC staining for IGFBP-3 supported quantitative PCR and western blot findings. The IGFBP-3 positive area was significantly greater in the kidneys of UUO mice compared with control animals (Figure 6C).

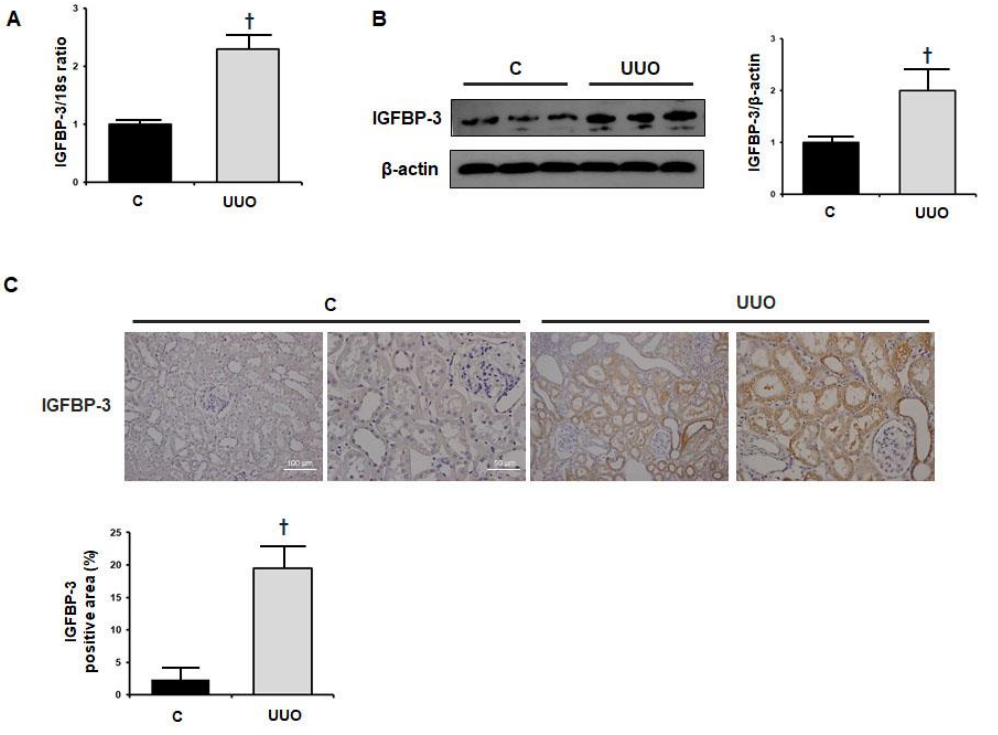


**Figure 6. IGFBP-3 expression in the kidney of sham and UUO mice.** (A) mRNA levels of IGFBP-3 (n = 6) and (B) protein levels of IGFBP-6 (representative of 6 blots). Compared with control mice, IGFBP-3 mRNA and protein expressions increased significantly in the left kidney of UUO mice. (C) IHC staining for IGFBP-3. The IGFBP-3 positive area was significantly greater in the kidneys of UUO mice than in control mice. C, control; UUO, unilateral ureteral obstruction; †P <0.05 vs. C.

**7. IGFBP-3 aptamers ameliorate tubulointerstitial fibrosis in UUO mice**

To examine the effect of IGFBP-3 aptamers on UUO–induced renal tubulointerstitial fibrosis, changes in fibronectin and type I collagen protein expression were determined by western blots and/or IHC. Masson's trichrome staining was also performed. Compared with control mice subjected to sham operations, fibronectin and type I collagen protein expressions were significantly higher in the left kidney of UUO mice, and these increases were significantly ameliorated by IGFBP-3 aptamer treatment (Fig. 7). IHC staining also showed significant increases in fibronectin and type I collagen in the proximal tubules of UUO mice (Fig. 8). These increases were significantly reduced by the administration of IGFBP-3 aptamers, suggesting that the aptamers inhibited the production and accumulation of fibrosis–related proteins in the proximal tubule cells of UUO kidneys. Moreover, Masson's trichrome staining revealed an increase in collagen within the interstitium of UUO mice (Fig. 8). This excessive fibrosis was significantly mitigated in UUO mice treated with IGFBP-3 aptamers.

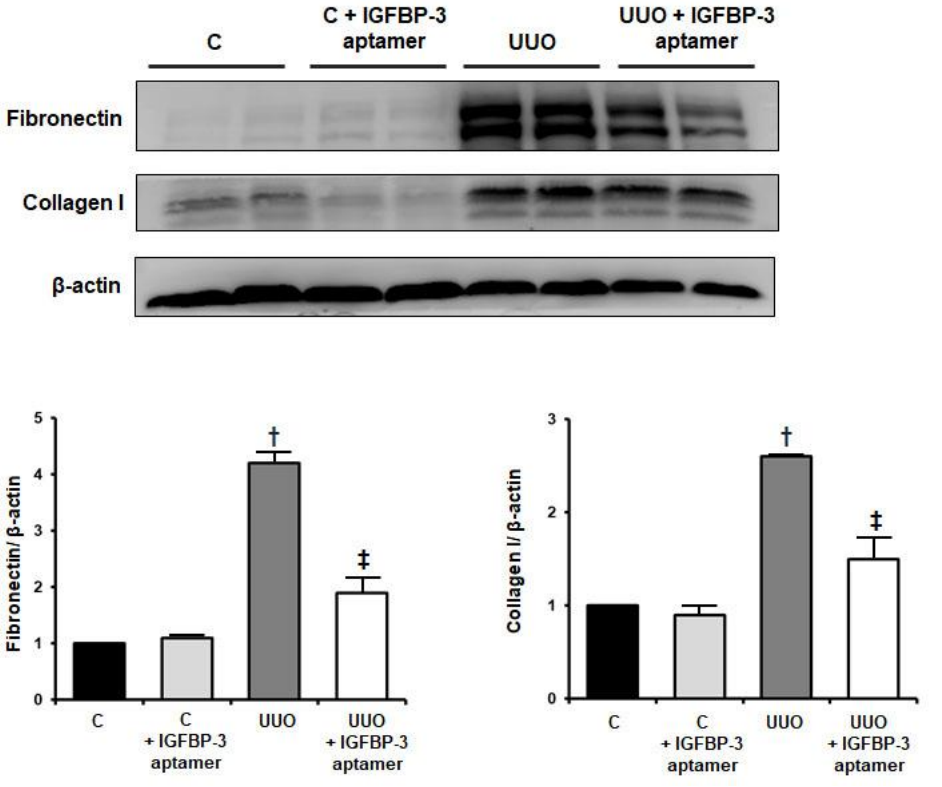


**Figure 7. A representative western blot of fibronectin and type I collagen in the left kidney of control mice, control + IGFBP-3 aptamers, UUO, and UUO + IGFBP-3 aptamer mice.** Compared with control mice, there were significant increases in fibronectin and type I collagen protein expression in UUO mice, and these increases were significantly alleviated by IGFBP-3 aptamer treatment (1 mg/kg) Representative of six blots; C, control; UUO, unilateral ureteral obstruction; †P <0.05 vs. C and C + IGFBP-3 aptamer group; ‡P <0.05 vs. UUO group.

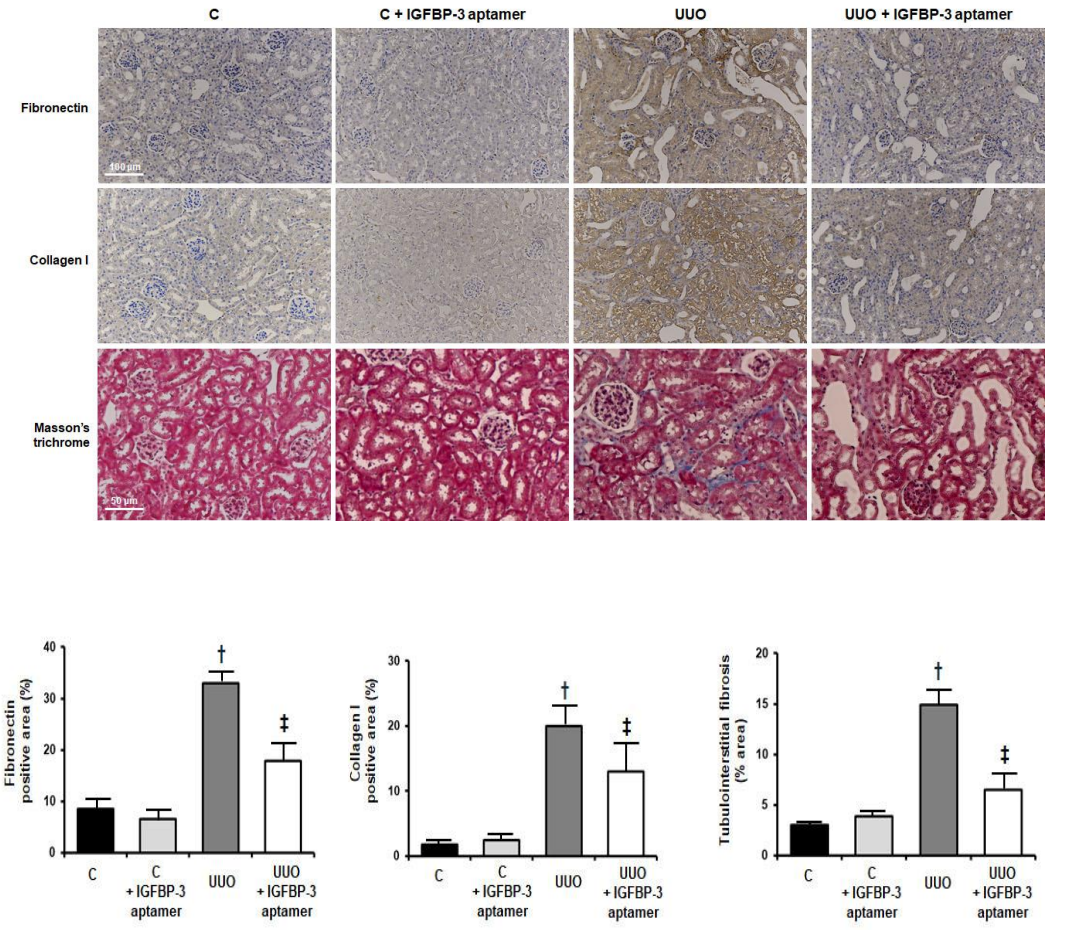


**Figure 8. IHC staining for IGFBP-3, fibronectin, and type I collagen and Masson's trichrome staining with the left kidney tissues of sham–operated control mice, sham–operated control + IGFBP-3 aptamers, UUO, and UUO + IGFBP-3 aptamer mice.** Compared with control and sham–operation mice with IGFBP-3 aptamer treatment, the positive areas of fibronectin and type I collagen in proximal tubules were significantly larger in UUO mice. IGFBP-3 aptamer treatment significantly reduced these changes. Masson's trichrome staining also revealed a decrease in renal interstitial fibrosis in UUO mice treated with IGFBP-3 aptamers (1 mg/kg). C, control; UUO, unilateral ureteral obstruction; †$P < 0.05$ vs. C and C + IGFBP-3 aptamer group; ‡$P < 0.05$ vs. UUO group.

**8. IGFBP-3 aptamers attenuate the activation of p38 MAP kinase in UUO mice**

The impact of IGFBP-3 aptamers on MAP kinase pathways was explored in UUO mice. As seen in Figure 9, phospho-p38 protein expression was significantly increased in the left kidneys of UUO mice compared with mice subjected to sham operation ($P < 0.05$), and the phosphorylation of p38 MAP kinase was significantly reduced by IGFBP-3 aptamer treatment ($P < 0.05$) (Fig. 9).

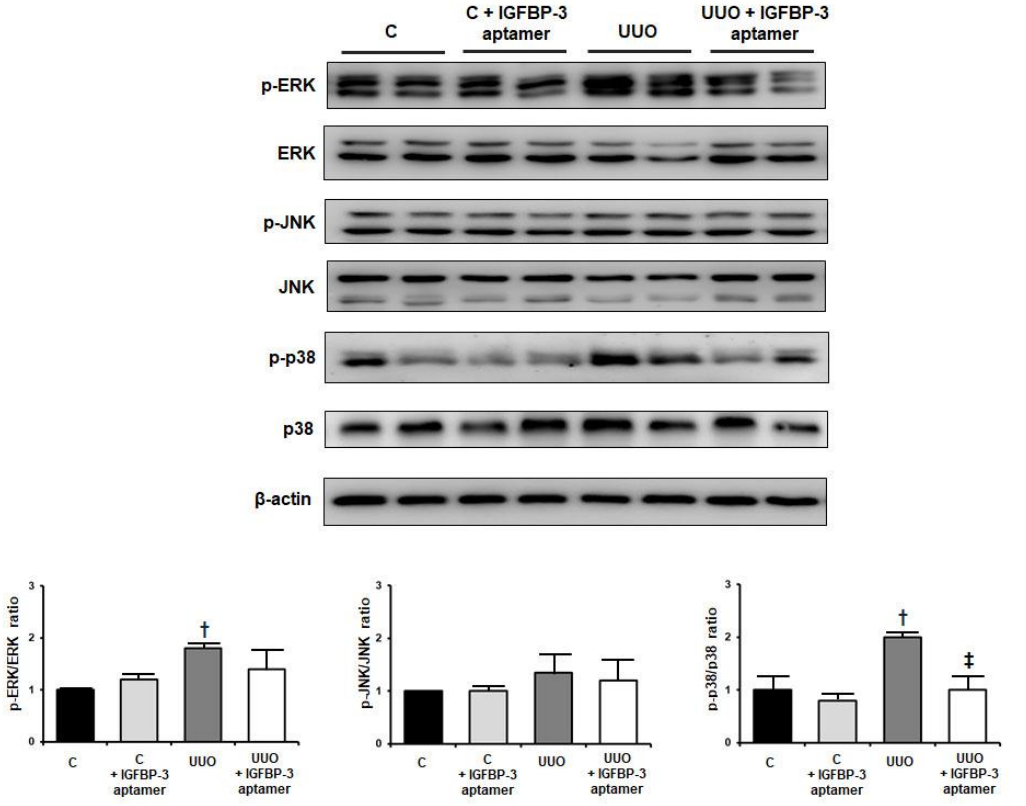


**Figure 9. A representative western blot of phospho-ERK/ERK, phospho-JNK/JNK, and phospho-p38/p38 protein in the left kidney of sham-operated control mice, sham-operated mice + IGFBP-3 aptamers, UUO mice, and UUO + IGFBP-3 aptamer mice.** Compared with sham-operated control mice, there was a significant increase in phospho-p38/p38 protein expression in UUO mice, and this increase was significantly reduced by treatment with IGFBP-3 aptamers.

Representative of six blots; C, control; UUO, unilateral ureteral obstruction; †P <0.05 vs. C and C + IGFBP-3 aptamer group; ‡P <0.05 vs. UUO group.

## IV. DISCUSSION

Expression of IGFBP-3 increased in TGF-β1–stimulated renal proximal tubular cells and the kidneys of UUO mice. The increased expression of fibrosis–related proteins in TGF-β–treated tubular cells and the kidneys of UUO mice were significantly abrogated by IGFBP-3 inhibition using IGFBP-3–specific binding aptamers. Phosphorylation of p38 induced by TGF-β1 and UUO was significantly reduced by administration of IGFBP-3 aptamers, suggesting that the anti-fibrotic effect of IGFBP-3 aptamers can be attributed to inhibition of the p38 MAP kinase pathway. These results speculated that IGFBP-3 is significantly associated with the pathogenesis of renal tubulointerstitial fibrosis and that IGFBP-3 inhibition by specific binding aptamers is a promising therapeutic strategy for various kidney diseases associated with renal fibrosis.

Renal tubulointerstitial fibrosis is characterized by inexorable production and progressive accumulation of the extracellular matrix (ECM) due to an imbalance between synthesis and degradation.[1] When a kidney injury progresses to chronic kidney disease or end-stage renal disease, renal tubulointerstitial fibrosis is a common final pathologic finding regardless of the initial cause.[1] Identifying initiating or aggravating factors associated with renal tubulointerstitial fibrosis is clinically relevant to the management of chronic kidney disease. IGFBP-3 has been investigated as a pro-fibrotic molecule for organ fibrosis in various fibrotic diseases.[2-8] It is the most abundant of the six members of the IGFBP family in the blood stream. IGFBP-3 has two distinctive functions, IGF–dependent and –independent functions. First, IGFBP-3 binds to circulating IGF-I or IGF-II, which prolongs the half-life of IGFs in the blood and facilitates their delivery to target cells.[2,3] In addition, IGFBP-3 has IGF–independent biological effects that involve neither binding of circulating IGFs nor modulation of the IGF receptor.[2,3] The secretion of IGFBP-3 has been observed in various cells and tissues, particularly those with a pathologic status such as fibro-proliferative conditions.[4-8] These cell– and tissue–specific effects suggest an

IGF–independent paracrine or autocrine function for IGFBP-3.[2-8] In fibroblasts from the skin of patients with systemic sclerosis, IGFBP-3 expression is higher.[6] An *ex vivo* human skin organ culture model demonstrated that IGFBP-3 increased dermal and collagen bundle thickness in human skin explants, leading to substantial dermal fibrosis.[6] Direct stimulation by recombinant IGFBP-3 induced production of ECM glycoprotein tenascin-C and type I collagen in primary lung fibroblasts from systemic sclerosis patients. Increased production of tenascin-C was reduced by silencing IGFBP-3.[8] In a study of patients with idiopathic pulmonary fibrosis (IPF), lung tissue and bronchoalveolar lavage fluid from IPF patients exhibited increased levels of IGFBP-3.[5] TGF-β increased secretion of IGFBP-3 in fibroblasts from IPF lungs, resulting in elevated expression of ECM components such as collagen and fibronectin.[4] In human airway smooth muscle cells, TGF-β1 treatment induced a 10- to 20-fold increase in levels of IGFBP-3 mRNA and protein,[7] and IGFBP-3 antisense oligomers blocked TGF-β1–induced cell proliferation.[7] These results suggest that IGFBP-3 is closely implicated in fibrosis.

The pathophysiologic role of IGFBP-3 in the kidney has not been fully explored.[9-11,15-17] Previous studies have shown higher concentrations of circulating or urinary IGFBP-3 in patients with dialysis,[17] diabetic nephropathy,[16] or glomerulonephritis[15] in association with modulation of IGF-I levels. In terms of IGF-I–independent effects in the kidney, the majority of studies deal with the association with cellular apoptosis.[9-11] Vasylyeva et al.[9] demonstrated that apoptosis occurred in rat mesangial cells exposed to recombinant IGFBP-3 under high ambient or standard glucose levels. Anti-sense IGFBP-3 oligos significantly inhibited apoptosis with enhanced phosphorylation of AKT at threonine 308.[9] In glomerular podocytes, IGFBP-3 mediated the development of podocyte apoptosis by modulating TGF-β1 and bone morphogenic protein-7 signals.[10] Recombinant IGFBP-3 induced podocyte apoptosis and a combination of IGFBP-3 and TGF-β1 enhanced podocyte apoptosis, suggesting IGFBP-3 has a direct effect on podocytes.[10] In a study with proximal tubular epithelial cells, high glucose increased IGFBP-3 expression and high glucose–induced apoptosis was attenuated by

IGFBP-3 siRNA,[11] which reduced levels of reactive oxygen species, indicating that IGFBP-3–mediated high glucose–induced apoptosis by oxidative stress.[11]

In contrast to research on cellular apoptosis, the effect of IGFBP-3 on renal tubulointerstitial fibrosis has yet to be explored. Previous studies of a pro–fibrotic role for IGFBP-3 in various fibrotic diseases highlighted the need to evaluate IGFBP-3 as a therapeutic target in kidney fibrosis. In this study, the expression of IGFBP-3 was low under normal conditions, but increased under pro–fibrotic stimuli such as TGF-β1 and UUO. TGF-β1 induced cellular IGFBP-3 expression in dose– and time–dependent manners. In the kidneys of UUO mice, IGFBP-3 protein expression, as assessed by western blots and IHC staining, was significantly higher compared with mice subjected to sham operations. TGF-β1 significantly induced ECM protein expression of fibronectin and type I collagen in proximal tubular cells, and these increases were significantly abrogated by IGFBP-3 siRNA and IGFBP-3–specific binding aptamers. The anti–fibrotic effect of IGFBP-3 inhibition was consistent in animal models. In the kidneys of UUO mice, increased ECM protein expression was significantly reduced after treatment with IGFBP-3 aptamers, suggesting that IGFBP-3 is a significant mediator of renal tubulointerstitial fibrosis, both *in vitro* and *in vivo*. Emerging evidence indicates that IGFBP-3 is secreted by cells and can mediate its autocrine or paracrine action.[2,3] In the present study, high intensity of Cy3-IGFBP3 in the cytoplasm of TGF-β1–stimulated renal proximal tubular cells decreased after treatment with IGFBP-3 siRNA. When concentrations of IGFBP-3 were assessed by enzyme–linked immunosorbent assay (human IGFBP-3 Quantikine ELISA kit, #DGB300, R&D Systems, Minneapolis, MN, USA) in cell culture medium, IGFBP-3 concentrations were higher in the TGF-β1–treated cells compared with those in controls. IGFBP-3 siRNA treatment decreased the concentrations of IGFBP-3 in the cell culture medium (data not shown). This suggests that IGFBP-3 is produced and secreted by renal proximal tubular cells under stimulation of TGF-β. The mechanism by which secreted IGFBP-3 acts in the kidney has not been fully explored. IGFBP-3 interacts with other plasma membrane

receptors and intracellular proteins.[2,3] Among them, IGFBP-3 can directly associate with TGF-β receptors, such as TGF-β receptor types I, II, and V.[18] In this study, IGFBP-3–specific aptamers ssignificantly reduced phosphorylation of p38 MAP kinase in NRK-52E cells exposed to TGF-β1 and UUO mice. The p38 MAP kinase pathway is an important intracellular signal transduction pathway involved in pro-fibrotic processes, including UUO, and it is closely associated with the TGF-β signaling pathway.[19,20] In podocytes, recombinant IGFBP-3 induced podocyte apoptosis and phosphorylation of Smad2/3 and p38 MAP kinase.[10] Direct stimulation of primary lung fibroblasts with recombinant IGFBP-3 increased ECM proteins through the p38 MAP kinase–dependent signaling pathway.[8] Taken together, p38 MAP kinase plays an important role in the pathogenesis of IGFBP-3–induced renal tubulointerstitial fibrosis. This implies that autocrine effects between secreted IGFBP-3 and TGF-β receptors can explain the association with the p38 MAP kinase pathway. However, the possibility that the paracrine effects of secreted IGFBP-3 affect resident fibroblasts in the kidney cannot be excluded.

In the current study, DNA aptamers were used to inhibit the IGFBP-3 signaling pathway, due to a lack of chemical inhibitors for IGFBP-3. Aptamers are single-stranded DNA or RNA that bind a target molecule with high affinity and specificity.[12] Targets range from small molecules[21,22] to biomacromolecules,[23,24] infected cells,[25] stem cells,[26] and cancer cells.[27,28] In terms of biomedical applications, aptamers have been used as biomarkers, diagnostic imaging tools, drug screening agents, and therapeutics.[12] In particular, aptamers have been used in targeted therapy due to the specific target-binding abilities. Aptamers can also be used to screen for a wide array of molecular targets, including toxins or weak immunogenic targets that are difficult to generate antibodies against. Aptamer screening is efficient (with results available in a week in ideal conditions) and cost-effective. The relatively simple chemical structures of aptamers can achieve full conformational recovery even after thermal or chemical denaturation.[12] Because of these advantages, therapeutic aptamers have been investigated in various diseases, including HIV/AIDS, macular degeneration, and

diabetes.[25,29,30] In this study, increased expression of IGFBP-3 in TGF-β1–treated cells was significantly reduced by treatment with IGFBP-3 siRNA accompanied by reduced fluorescence of IGFBP-3 aptamers, suggesting specific binding between IGFBP-3 and aptamers. Although a previous study showed that the liver and the kidney were major clearance organs of aptamers,[31] *in vivo* bio-distribution of IGFBP-3 aptamers was not investigated in the present study. This limited the investigation of the interaction between IGFBP-3 and IGFBP-3 aptamers *in vivo*. Nevertheless, administration of IGFBP-3 aptamer significantly reduced fibrosis-related protein expression in TGF-β1–treated renal proximal tubular cells and UUO mice. Masson's trichrome staining demonstrated that renal tubulointerstitial fibrosis was significantly attenuated in UUO mice treated with IGFBP-3 aptamers compared with sham-operation control mice. These results suggest that IGFBP-3–specific binding aptamers can serve as anti-fibrotic therapeutics for kidney fibrosis.

In summary, this study showed for the first time that IGFBP-3–specific binding aptamers can alleviate renal tubulointerstitial fibrosis both *in vitro* and *in vivo* by inhibiting p38 MAP kinase activation. IGFBP-3 may therefore be an important mediator in the pathogenesis of renal tubulointerstitial fibrosis, and the anti-fibrotic effect of IGFBP-3–specific binding aptamers can be attributed to inhibition of the p38 MAP kinase signaling pathway. Inhibiting IGFBP-3 may be an intriguing target for chronic kidney diseases associated with renal tubulointerstitial fibrosis.

## V. CONCLUSION

To clarify the role of IGFBP-3 and the consequences of its inhibition by IGFBP-3–specific aptamers in renal tubulointerstitial fibrosis, the therapeutic effect of IGFBP-3 aptamers on TGF-β1–induced tubular epithelial cell injury *in vitro* and UUO-induced renal fibrosis *in vivo* was investigated.

1. TGF-β1 induced IGFBP-3 expression in dose– and time–dependent manners. TGF-β1 significantly induced the protein expression of fibronectin and type I collagen in cultured NRK-52E cells, and these increases were significantly abrogated by treatment with IGFBP-3 siRNA and IGFBP-3–specific binding aptamer.
2. The protein expression of phospho-p38 was significantly increased in cultured NRK-52E cells exposed to TGF-β1, and IGFBP-3 aptamer treatment significantly alleviated p38 MAP kinase activation.
3. IGFBP-3 protein expression in the kidneys, assessed by western blots and IHC staining, was significantly increased in UUO mice compared with sham-operated mice.
4. Compared with sham–operated control mice, the protein expressions of fibronectin and type I collagen were significantly increased in UUO mice, and these increases were significantly ameliorated by treatment with IGFBP-3–specific binding aptamers.
5. Masson's trichrome staining showed that renal tubulointerstitial fibrosis was significantly more severe in the left kidneys of UUO mice compared with control mice, and this excessive fibrosis was significantly attenuated in IGFBP-3 aptamer–treated UUO mice.
6. Compared with sham–operated mice, phospho-p38 protein expression was increased in the kidneys of UUO mice, and IGFBP3 aptamer treatment significantly abrogated p38 MAP kinase activation in UUO mice.

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<ABSTRACT (IN KOREAN)>

# 일측성 요관 폐쇄로 유발된 신섬유화 모델에서 insulin-like growth factor binding protein-3 특이적 압타머의 신보호 효과

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이 미 정

배경: 세뇨관-간질성 섬유화는 만성 신질환의 전형적인 병리학적 소견으로, 만성 신질환의 진행과 밀접한 연관이 있다. 최근의 연구들에서 insulin-like growth factor binding protein-3 (IGFBP-3)는 다양한 세포들과 장기에서, 각 세포의 종류와 조직의 종류에 따라 특이적인 역할을 갖는 것이 보고되고 있으며, 특히 섬유화에 주요한 매개체로 작용하는 것이 보고되고 있다. 하지만 신장 섬유화에 대한 IGFBP-3의 병태생리적 역할에 대해서는 연구가 부족한 실정이다. 따라서 본 연구에서는 TGF-β1으로 자극된 신장 세뇨관 상피세포 손상 모델과 일측성 요관 폐쇄로 유발된 신섬유화 동물 모델을 이용하여 신장 섬유화에 있어서의 IGFBP-3의 역할을 규명하고자 하였다. 나아가 IGFBP-3 신호전달 경로 차단을 위해 IGFBP-3에 특이적으로 결합하는 DNA 압타머를 이용하여 신장 섬유화가 억제되는지 연구하였다.

방법: 생체 외 실험으로는 신장 근위 세뇨관 상피세포 (NRK-52E cell)를 대조군과 TGF-β1 (10 ng/mL) 투여군으로 나누어 배양하였으며, IGFBP-3의 차단을 위해 IGFBP-3 small interfering RNA (siRNA) (50 nM) 와 IGFBP-3 특이적 DNA 압타머 (200 nM) 을 48시간 처리하였다. 생체 내 실험은 C57BL/6

마우스를 4 그룹 (sham 수술+위약 투여군 (대조군), sham 수술+IGFBP-3 압타머 투여군, 일측성 요관 폐쇄 수술+위약 투여군, 일측성 요관 폐쇄 수술+IGFBP-3 압타머 투여군, 각 그룹당 6마리) 으로 나누어 진행하였다. IGFBP-3 압타머 (1 mg/kg) 와 위약은 수술 전 1시간 그리고 수술 후 48시간마다 복강 내로 주사로 시행하였다. 수술 7일 후 마우스를 희생하여 좌측 신장을 적출하였다. 섬유화-관련 단백과 mitogen-activated protein (MAP) kinase 신호전달체계와 연관된 단백의 발현은 western blot을 이용하여 분석하였으며, 신섬유화 정도는 면역 조직화학적 염색과 Masson's trichrome 염색으로 확인하였다.

결과: TGF-β1으로 자극한 신장 근위세뇨관 세포에서 IGFBP-3의 mRNA와 단백 발현이 용량, 시간에 비례하여 증가되었다. TGF-β1은 섬유화 관련 단백인 fibronectin과 type I collagen의 발현을 증가시켰고, IGFBP-3 siRNA를 처리했을 때 섬유화 관련 단백의 발현은 감소하였다. TGF-β1 자극에 의해 증가한 신장 세뇨관 세포의 섬유화 관련 단백 발현은 IGFBP-3 특이적 압타머를 투여하였을 때 유의하게 감소하였다. 일측성 요관 폐쇄 수술을 받은 마우스는 대조군에 비해 IGFBP-3 의 발현과 섬유화 관련 단백 발현이 증가되어있었다. IGFBP-3 특이적 압타머를 투여한 일측성 요관 폐쇄 수술을 받은 마우스에서는 요관 폐쇄로 인해 증가되었던 섬유화 관련 단백 발현이 감소하였다. 또한 면역 조직화학적 염색과 Masson's trichrome 염색을 통해 일측성 요관 폐쇄 수술을 받은 마우스에서 IGFBP-3 압타머를 투여한 경우 신장 세뇨관-간질 섬유화의 정도가 감소되었음을 확인하였다. 세포 실험과 동물 실험에서 모두 IGFBP-3 압타머 투여에 의해 p38 MAP kinase 신호 전달 경로의 활성화가 억제되었다.

결론: 이상의 결과를 종합하여 볼 때, IGFBP-3가 신장 세뇨관 세포와 동물 모델에서 신 섬유화를 매개하는 역할을 한다는 것을 알 수 있었다. 더욱이 IGFBP-3에 특이적으로 결합하는 압타머를 투여하여 신호전달 경로를 억제했을 때 신장 세뇨관 세포와 동물의 신 섬유화가 억제되는 결과를 통해, IGFBP-3를 향후 신섬유화와 관련된 다양한 신질환의 가능성 있는 치료 타겟으로 고려할 수 있을 것으로 생각된다.

핵심되는 말: 압타머, insulin-like growth factor binding protein-3, p38 mitogen-activated proteinkinase, 일측성 요관 폐쇄, 신섬유화