Lysyl oxidase-like 2 as a therapeutic and preventive target in chronic kidney diseases

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Supervised by Professor Beom Jin Lim

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ABSTRACT

Lysyl oxidase-like 2 as a therapeutic and preventive target in chronic kidney diseases

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Tubulointerstitial fibrosis is a common end point of chronic kidney diseases, and preventing its progression is key to avoiding renal failure. Transforming growth factor-β (TGF-β) and its associated molecules promote tubulointerstitial fibrosis. However, effective therapies targeting these molecules are yet to be developed. Lysyl oxidase-like 2 (LOXL2), which is involved in invasive growth and metastasis of malignant neoplasms, has recently been reported to play a key role in hepatic and pulmonary fibrosis. However, currently, little is known regarding LOXL2 expression in the kidney and its involvement in tubulointerstitial fibrosis. The present study evaluated LOXL2 expression in human and mouse kidney tissues, as well as in cultured renal cells. The LOXL2-specific antibody AB0023 was administered to a mouse model of tubulointerstitial fibrosis, and the amount of fibrosis and levels of Smad-related molecules were analyzed. Molecules associated with epithelial-mesenchymal transition (EMT) were evaluated in LOXL2 knockdown tubular epithelial cells after TGF-β challenge. LOXL2 protein expression was detected in glomerular capillary loops and tubular epithelial cells in human and mouse kidneys. Glomerular LOXL2 was localized in the

cytoplasm of podocytes, as determined by double immunofluorescence microscopy using a podocyte marker (synaptopodin). This result was supported by western blot analysis, which demonstrated that LOXL2 protein expression is present in cultured human podocytes and HK-2 human proximal tubular cells. Furthermore, the mRNA and protein expression levels of LOXL2 were higher in the mouse model of tubulointerstitial fibrosis than in control mice. Additionally, immunohistochemistry results demonstrated that LOXL2 is present in the fibrous interstitium and infiltrating mononuclear cells in this model. The amount of fibrosis, as measured by trichrome and Sirius red staining, and the amount of total collagen, analyzed using a total collagen assay kit, decreased significantly in mice treated with AB0023 compared to those in the control group. There were no significant differences between the levels of Smad-related molecules. LOXL2 knockdown HK-2 cells showed significantly less increase in the expression of mesenchymal marker vimentin compared from that of the control cells after TGF-β challenge. The present study demonstrated that LOXL2 is expressed in various compartments of renal tissue. Association of LOXL2 with tubulointerstitial fibrosis was confirmed by increased LOXL2 protein expression in the renal fibrosis model and the amelioration of fibrosis in the AB0023-treated group. The association between LOXL2 and EMT markers observed following TGF-β challenge suggests that LOXL2 promotes fibrosis through the EMT/TGF-β signaling pathway. The present study was the first to demonstrate the relationship between renal tubulointerstitial fibrosis and LOXL2. Further studies are warranted to elucidate the regulatory role of LOXL2 on tubulointerstitial fibrosis.

Keywords: tubulointerstitial fibrosis, TGF-β, lysyl oxidase-like 2, epithelial-mesenchymal transition, podocytes, HK-2 cells, Smad

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I. INTRODUCTION

Fibrosis is defined as excessive deposition of collagen and other extracellular matrix (ECM) components within tissues. While fibrosis is a normal part of repair in wound healing that occurs after inflammation, it can also be a pathologic component of chronic disease, resulting in an abnormal deposition of collagen into internal organs stimulated with persistent injury.[1] The process of fibrosis consists of angiogenesis, migration and proliferation of fibroblasts, collagen synthesis, and connective tissue remodeling.[2] Various cytokines and growth factors regulate connective tissue deposition. Of these, transforming growth factor-β (TGF-β) is known to be the most important.[3] A wide range of cells, including alternatively activated macrophages, produce TGF-β.[4] The level of TGF-β produced is modulated by the activation of latent TGF-β, the rate of secretion of active TGF-β, and factors within the ECM, such as integrin.[5] TGF-β leads to the migration and proliferation of fibroblasts, synthesis of collagen and fibronectin by fibroblasts, and ECM deposition by reducing the degradation of

the ECM through the inhibition of metalloproteinases. A portion of these fibroblasts obtain the traits of smooth muscle cells, including actin filaments, transforming them into myofibroblasts.[3] In organs such as the lungs and kidneys, myofibroblasts synthesize and secrete collagen under TGF-β stimulation. Additionally, the matrix metalloproteinase (MMP) family orchestrates vascular rarefaction and the remodeling and deposition of connective tissue.[6]

Renal fibrogenesis is divided into four phases: priming, activation, execution, and progression.[7] Sustained injury leading to non-resolving inflammation brings about the fibrogenic stage into priming phase. Acute renal injury stimulates the secretion of proinflammatory chemokines, chemoattractants, leukocyte adhesion molecules, and other injurious molecules by tubules and peritubular capillaries.[8] Massive proteinuria, filtration of protein-bound cytokines into the tubular microenvironment, and physical stretching by hydrodynamic forces within the tubule also activate the tubular NF-κB signaling pathway. This pathway, in turn, leads to the production of multiple cytokines and growth factors, including plasminogen activator inhibitor-1 (PAI-1),[9] interleukin-1,[10] interleukin-6,[11] tumor necrosis factor-α,[12] monocyte chemoattractant protein-1/CCL2,[13,14] and RANTES/CCL5.[12,15] As these proinflammatory molecules recruit mononuclear cells, sustained profibrotic cytokine pressure within the local microenvironment activates fibroblasts and promotes the phenotypic transition of tubular epithelial cells, moving the process towards the

activation phase of fibrogenesis.[16-21]

The next stage is the execution phase, wherein activated fibroblasts synthesize, secrete, and assemble ECM components. A variety of extracellular cues, such as TGF-β1, platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), connective tissue growth factor (CTGF), and Angiotensin II (AngII) regulate activated fibroblasts.[22-27] In contrast, hepatocyte growth factor[28] and bone morphogenetic protein 7 (BMP-7)[29] inhibit the synthesis of matrix components by antagonizing TGF-β1. TGF-β1 and AngII act on corresponding receptors and activate the Smad2/3/4 complex, which in turn localizes into the nucleus and transcribes β1 integrin,[30,31] integrin-linked kinase (ILK),[32,33] particularly interesting new cysteine-histidine rich protein (PINCH),[34] and CTGF.[22,35] ILK acts both as a downstream effector of integrin[32] and the scaffolding protein that combines with PINCH and parvin to produce a multicomponent protein complex.[36] ILK also inhibits glycogen synthase kinase 3β (GSK3β), stabilizing Snail1 and β-catenin.[37,38] Snail1 is responsible for fibroblast motility and tubular epithelial-mesenchymal transition (EMT),[39] while β-catenin transcribes fibronectin and PAI-1.[40] In the early stage of fibrosis, the fibrosis is relatively reversible due to the susceptibility of the collagen matrix to proteolysis. However, as fibrosis progresses, the matrix undergoes modifications which cause it to stiffen, causing these changes to become irreversible. Enzymes, such as tissue transglutaminase and lysyl oxidase, are

responsible for these modifications.[41]

Fibrogenesis ends with the progression phase. The hallmarks of this phase are tubular injury and atrophy, microvascular rarefaction, and chronic hypoxia.[7]

Members of the lysyl oxidase (LOX) family [e.g., LOX and LOX-like (LOXL)1-4] are responsible for the cross-linking of collagen and elastin in the extracellular matrix through their copper-dependent amine oxidase activity. In addition, LOX family members exhibit various functions in cell proliferation, tumor invasion and metastasis, and organ development.[42] LOXL2 is the most thoroughly studied member of the LOX family. Its expression is associated with tumor cell differentiation in colon and esophageal carcinomas,[43] and with the proliferation, migration, and invasion of human hepatocellular carcinoma cells.[44] Increased LOXL2 expression is also associated with poor survival in squamous cell carcinoma of the larynx and lung,[45] and appears to serve a role in the metastatic potential of breast[46] and gastric carcinomas.[47] Potential mechanisms underlying the effects of LOXL2 include fibroblast activation in the tumor microenvironment,[48] induction of the EMT in tumor cells,[46] and matrix remodeling via regulation of tissue inhibitors of metalloproteinase (TIMP)-1 and MMP-9.[49]

The contribution of LOXL2 to benign fibrosing diseases has been studied in several organs. Previous studies have indicated that LOXL2 expression is associated with hepatic fibrosis in Wilson's disease, primary biliary cirrhosis,[30]

and hepatocellular carcinoma.[50] Elevated serum LOXL2 levels are also associated with disease progression in idiopathic pulmonary fibrosis.[51] Additionally, LOXL2 upregulation is associated with scar formation following glaucoma surgery.[52]

Inhibitory monoclonal antibodies to LOXL2 have been developed. These include AB0023, which binds to human and mouse LOXL2, and AB0024 (simtuzumab), its humanized form. The antifibrotic effects of AB0023, AB0024, and other inhibitory antibodies have been determined in several organs. For example, AB0023 attenuated postoperative fibrosis in a rabbit model of glaucoma surgery.[52] In addition, AB0023 attenuated tetrachloride-induced hepatic fibrosis and decreased phosphorylated-Smad3 signaling in BALB/c mice. Further, in C57BL/6 mice, AB0023 attenuated high-dose bleomycin-induced pulmonary fibrosis, and this effect was found to be mediated by the inhibition of fibroblast recruitment and activation.[53] Based on the results of animal experiments, clinical trials of simtuzumab for human fibrosing diseases have been performed. The target diseases in these trials include advanced liver fibrosis due to human immunodeficiency virus and hepatitis C virus infection,[54] and idiopathic pulmonary fibrosis (clinicaltrials.gov/ct2/show/NCT01769196).

Although fibrosis is a clinically important pathological process in kidney disease, little is known regarding the expression of LOXL2 in renal tissue and

its contribution to the development of renal tubulointerstitial fibrosis. In the present study, the expression of LOXL2 in normal kidney was evaluated in tissues and cell lines. In addition, to evaluate its possible profibrotic role, LOXL2 expression in kidneys was evaluated in a mouse model of tubulointerstitial fibrosis. To confirm the role of LOXL2 in tubulointerstitial fibrosis and to evaluate the therapeutic applicability of LOXL2-targeted therapy for renal fibrosis, the anti-LOXL2 antibody AB0023 was administered to the mouse model of tubulointerstitial fibrosis. Finally, by using cultured tubular epithelial cells with or without LOXL2 knockdown, the mechanism by which LOXL2 mediates tubulointerstitial fibrosis was investigated.

II. MATERIALS AND METHODS

1. Renal cell culture

For the *in vitro* experiments, human podocytes and the most widely used proximal and distal tubular epithelial cell lines were selected. Immortalized human proximal tubular cells (HK-2 cells; cat. no. CRL-2190) were purchased from the American Type Culture Collection (Manassas, VA, USA) and canine tubular cells (MDCK cells; cat. no. 10034) were purchased from the Korean Cell Line Bank (Seoul, South Korea). HK-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM)/Nutrient Mixture F-12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 10% fetal bovine serum (FBS; Gibco). MDCK cells were cultured in DMEM (Gibco). Conditionally immortalized human podocytes were provided by Dr. Moin A. Saleem (University of Bristol, Bristol, UK) and Dr. Jun Oh (University Medical Center Hamburg-Eppendorf, Hamburg, Germany). Podocytes were grown in RPMI-1640 medium (Gibco), 10% FBS, and insulin-transferrin-selenium supplement (Gibco) at 33°C (in 5% CO_2) to activate the SV40 large T antigen. Upon reaching 70 to 80% confluence at 33°C, the cells were then cultured at 37°C (in 5% CO_2) for 2 weeks to induce differentiation,[55] which was confirmed by western blotting for synaptopodin. To silence LOXL2 expression at the cellular level, LOXL2 shRNA lentiviral particles (cat. no. sc-45222-v; Santa

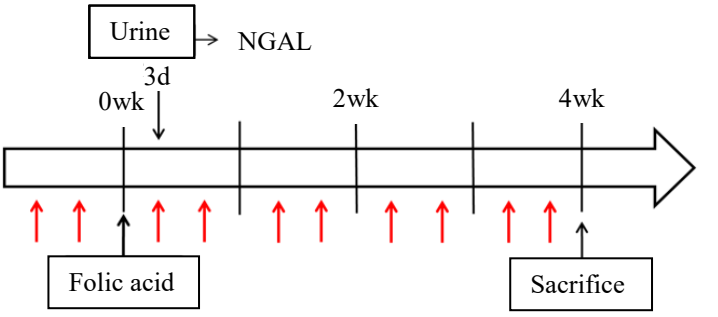
Cruz Biotechnology, Inc., Dallas, TX, USA) were transduced into HK-2 cells. HK-2 cells were plated on a 100-mm dish 24 hours prior to viral infection at a density of 2×10^5 cells/100-mm dish in DMEM/F-12. A mixture of complete medium with polybrene (cat. no. sc-134220; Santa Cruz Biotechnology, Inc.) at a final concentration of 5 μg/ml was prepared; 10 ml of this medium was added to each dish after removing the prior media. LOXL2 lentiviral particles were thawed at room temperature (range from 20℃ to 25℃) and mixed gently before use. HK-2 cells were infected by adding LOXL2-lentiviral particles to the culture at a multiplicity of infection (MOI) of 2, swirled gently for mixing, and incubated overnight. After 24 hours, the culture medium was removed and replaced with 10 ml of complete medium without polybrene. After overnight incubation, the culture medium was removed and replaced with 10 ml of complete medium with 2 μg/ml puromycin dihydrochloride (cat. no. sc-108071; Santa Cruz Biotechnology, Inc.). Medium was replaced with fresh puromycin (Santa Cruz Biotechnology)-containing medium every 2 to 3 days until identification of LOXL2 knockdown using real-time polymerase chain reaction (PCR) or western blot analysis was performed. Control shRNA lentiviral particles (cat. no. sc-108080; Santa Cruz Biotechnology, Inc.) were transduced to another line of HK-2 cells in the same manner as the LOXL2 shRNA particles.

2. Animal model of tubulointerstitial fibrosis

Male CD1 mice at 8 weeks of age (Orient Bio, Inc., Seongnam, South Korea) were used for animal experiments. Mice were housed at 20°C with a 12-hour light/dark cycle and free access to rodent chow and water. Tubulointerstitial fibrosis was induced in 4 mice (mean body weight: 42.5 g) by intraperitoneal injection of folic acid (240 μg/g body weight).[56,57] The folic acid solution was prepared by dissolving folic acid powder (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 0.3 M $NaHCO_3$. Control CD1 mice (n=4; mean body weight: 43.1 g) were intraperitoneally injected with the same amount of vehicle ($NaHCO_3$). Urinary excretion of neutrophil gelatinase-associated lipocalin (NGAL) was measured immediately before injection and at 3 days after injection using a Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) to ensure successful injection of folic acid, as manifested by a log scale increase in NGAL. Mice without an increase in NGAL 3 days after folic acid injection, indicating that folic acid was not successfully injected, were omitted from further experiments. After 4 weeks, the kidneys were harvested under anesthesia before euthanization. Fresh frozen tissues were stored at -70°C subsequent to instant freezing in liquid nitrogen. Additional kidney tissues were fixed in 4% formaldehyde for 24 h at room temperature and embedded in paraffin overnight at 55-65°C using an automatic tissue processer (EFTP-FAST 360; Intelsint, Turin, Italy).

3. Effect of AB0023 on an animal model of tubulointerstitial fibrosis

To inhibit LOXL2, AB0023 (Gilead Sciences, Foster City, CA, USA), an inhibitory monoclonal antibody of LOXL2, was used. CD1 mice were injected intraperitoneally with AB0023 1 week before folic acid injection, and twice a week for 4 weeks afterwards, at a dosage of 15 mg/kg body weight. Mice in the control group were injected with immunoglobulin G (IgG) (GS-645864, Gilead Sciences) at the same dosage and on the same schedule as the treatment group. Another group of mice were injected intraperitoneally with vehicle ($NaHCO_3$) instead of folic acid and no further injections of AB0023 or IgG. All mice were sacrificed 4 weeks after folic acid or vehicle injection. The right kidneys were harvested from all mice and coronally sectioned. For histologic examination, sections were stored in 4% paraformaldehyde for 24 hours at room temperature, and then embedded in paraffin. Tissue was also harvested from cortex and medulla and stored at -70°C for collagen measurement and western blot analysis.



↑ : AB0023 or control IgG (Gilead Sciences) IP injection (15 mg/kg), 4 days and 1 day before folic acid injection, and twice weekly until 4th week.

Figure 1. Injection protocol of AB0023, a monoclonal antibody of LOXL2, and control IgG in a CD1 mouse model.

The present study was approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (Seoul, South Korea). All experiments involving animals were carried out in accordance with the standards set forth by the Institutional Animal Care and Use Committee of Yonsei University Health System.

4. Mechanism of LOXL2-induced fibrosis

To identify the relationship of LOXL2 and EMT-associated molecules, TGF-β (R&D Systems, Minneapolis, MN, USA) was applied to LOXL2-deleted HK-2 cells and control shRNA (Santa Cruz Biotechnology, Inc.)-transduced cells, and the expression levels of EMT-associated molecules were comparatively analyzed. TGF-β was dissolved in 4 mM HCl containing 1 mg/ml human

bovine serum albumin to prepare a 20 μg/ml TGF-β stock solution. LOXL2-deleted and control cells were plated on 100-mm dishes at a density of 2×10^5 cells/100-mm dish. Six dishes each of LOXL2-deleted and control cells were designated as the experimental and control groups, respectively. Both groups were subjected to serum starvation overnight. TGF-β was mixed with serum-free DMEM at a concentration of 20 μg/ml and the mixture was applied to the experimental group. Vehicle (0.1% 4 mM HCl/BSA) was mixed with serum-free DMEM and the same amount of mixture was applied to the control group. After 72 hours, cells were lysed, and proteins were analyzed by western blotting.

5. Western blot analysis of LOXL2 and other molecules

HK-2 cells, MDCK cells, and differentiated human podocytes were lysed in radioimmunoprecipitation assay buffer (Biosesang, Inc., Seongnam, Korea) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The samples were centrifuged at 13,000 rpm for 30 min at 4°C, and protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Protein samples (50 μg) were separated by 10% SDS-PAGE for 2 hours at 100 volts and were then transferred to a polyvinylidene fluoride membrane. After blocking with 3% skim milk for 1 hour at room temperature, the membrane was

incubated with the following primary antibodies overnight at 4°C: anti-synaptopodin (cat. no. sc-21537; 1:2,000; Santa Cruz Biotechnology), and anti-LOXL2 (cat. no. ab96233; 1:500; Abcam, Cambridge, MA, USA). The membrane was then washed with Tris-buffered saline containing 0.1% Tween-20 and incubated with horseradish peroxidase-labeled secondary antibodies (cat. no. sc-2020; 1:5,000; Santa Cruz Biotechnology; and cat. no. K4003; 1:5,000; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 1 hour at room temperature. Protein bands were visualized using Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific). After stripping the membrane with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) for 15 minutes at room temperature, the membrane was incubated with an anti-β-actin antibody (cat. no. sc-47778; 1:2,000; Santa Cruz Biotechnology), which was used as a loading control. In addition, fresh frozen kidneys from mice were homogenized, and western blotting was performed in a similar manner.

The primary antibodies used for the mouse kidneys included anti-LOXL2 (Abcam), anti-Smad2 (cat. no. 5339; 1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Smad2 (Ser465/467) (cat. no. 3108; 1:500; Cell Signaling Technology), anti-Smad3 (cat. no. 9523; 1:1000; Cell Signaling Technology), anti-phospho-Smad3 (Ser423/425) (cat. no. 9520; 1:1000; Cell Signaling Technology), anti-Smad2/3 (cat. no. 8685; 1:1000; Cell Signaling

Technology), and anti-Smad4 (cat. no. 38454; 1:1000; Cell Signaling Technology).

LOXL2 knockdown and control cells were lysed, and proteins were separated in a similar manner to the above-mentioned cells. The primary antibodies used include: anti-vimentin (cat. no. ab92547; 1:5000; Abcam, Cambridge, MA, USA), anti-E-cadherin (cat. no. 610181; 1:500; BD Biosciences, San Jose, CA, USA), anti-ZO-1 (cat. no. ab2272; 1:500; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and anti-fibronectin (cat. no. sc8422; 1:1000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Semi-quantification of the bands was performed by densitometry using Image J software (version 1.50i; National Institutes of Health, Bethesda, MD, USA).

6. Immunohistochemistry and immunofluorescence analysis of LOXL2 in human and mouse kidneys

LOXL2 expression in human and mouse kidneys was evaluated by immunohistochemistry using an Olympus BX53 light microscope (Olympus Corporation, Tokyo, Japan). Paraffin-embedded human kidney tissues from 4 patients were obtained from the surgical pathology archive of the Department of Pathology, Yonsei University, Gangnam Severance Hospital (Seoul, South Korea). These tissues were obtained from a non-neoplastic portion of a nephrectomy specimen of a renal tumor. The use of archived human tissue was

approved by the institutional review board of Yonsei University, Gangnam Severance Hospital. The paraffin-embedded kidneys from the aforementioned vehicle- and folic acid-injected mice were also used for immunohistochemical analysis of LOXL2 expression.

Human and mouse kidney tissues were cut into 4-μm sections, deparaffinized, and rehydrated using xylene and ethanol. Antigen retrieval was conducted by microwaving the tissue sections in 0.01 M sodium citrate buffer (pH 6.0) for 10 minutes. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxidase for 10 minutes. The tissue sections were then incubated overnight with a primary antibody against LOXL2 (cat. no. ab96233; 1:1,000; Abcam) at 4°C, followed by incubation with a horseradish peroxidase-labeled secondary antibody (cat. no. K4003; prediluted; Dako; Agilent Technologies, Inc.) for 1 hour at room temperature. The protein was visualized using the chromogen diaminobenzidine. Double immunofluorescence staining for LOXL2 and synaptopodin was performed in a similar manner. Sections were incubated with the primary antibody against LOXL2 (1:100) for 4 hours at room temperature, followed by incubation with a Texas Red-conjugated anti-rabbit immunoglobulin G (cat. no. TI-1000; 1:50; Vector Laboratories, Inc., Burlingame, CA, USA) overnight at 4°C. Subsequently, the tissue sections were incubated with a primary antibody against synaptopodin (cat. no. 65294; 1:50; Progen Biotechnik GmbH, Heidelberg, Germany) for 4 hours at room

temperature, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (cat. no. FI-2000; 1:50; Vector Laboratories, Inc.) overnight at 4°C.

7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of LOXL2 expression

The mRNA expression levels of LOXL2 in renal cells and fresh frozen kidneys from the vehicle- or folic acid-injected mice were analyzed by RT-qPCR. RNA was extracted using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen GmbH) according to the manufacturers' protocols. PCR amplification was performed using TaqMan Gene Expression Master Mix and an ABI 7900 HT real-time PCR system (Applied Biosystems; Thermo Fisher Scientific) with the following thermal cycle: 2 min at 50°C for uracil DNA-glycosylase enzyme incubation and 10 min at 95°C for AmpiTaq Gold enzyme activation, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. TaqMan primer/probes for mouse LOXL2 (cat. no. Mm00804740_m1) and ribosomal 18S RNA (cat. no. Mm03928990_g1) were purchased from Applied Biosystems (Thermo Fisher Scientific). Expression was calculated using the $2^{-\Delta\Delta Cq}$ method.[58]

8. Evaluation of tubulointerstitial fibrosis

A. Semiquantitative analysis via histologic examination

Paraffin-embedded samples of the AB0023-treated group and the control group were cut into 4-μm sections. After deparaffinization and rehydration, sections were stained with Masson's trichrome and Sirius red. For Sirius red staining, sections were treated with Weigert's Iron Hematoxylin for 8 minutes to stain nuclei and Direct Red 80 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 hour at room temperature to visualize collagen, and then washed with 0.5% glacial acetic acid. Slides were examined by light microscopy with (for Sirius red) or without (for trichrome) polarization. Photos were serially taken along the cortex under 200× magnification and the area of interstitial fibrosis was measured using Image J software (version 1.50i; National Institutes of Health, Bethesda, MD, USA).

B. Quantitative analysis via total collagen assay

The content of collagen in fresh frozen cortex was evaluated by measuring hydroxyproline using the Total Collagen Assay Kit (QuickZyme Biosciences, Leiden, Netherlands) according to the manufacturer's guide. Samples were hydrolyzed in 95°C 6M HCl for 20 hours and then centrifuged at 13,000 rpm for 10 minutes. The supernatants were collected and assayed by an enzyme-linked immunosorbent assay, according to the manufacturer's guide.

Total protein in hydrolyzed samples was also measured using a Total Protein Assay Kit (QuickZyme). The relative amount of collagen per protein was analyzed by dividing the amount of collagen measured by Total Collagen Assay by the amount of total protein using Total Protein Assay Kit.

9. Urinary NGAL as a marker of kidney injury

NGAL, also known as lipocalin 2, was quantified using the Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems). A log-scale increase of urinary NGAL relative to the baseline was used as an indication of the successful injection of folic acid. Mice without a significant increase in NGAL 3 days after folic acid injection, indicating that folic acid was not successfully injected, were omitted from further experiments. The concentration of urinary NGAL was normalized to the urinary creatinine concentration measured by the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA).

10. Statistical analysis

Quantitative analysis was performed for the western blot and RT-qPCR results. Folic acid-injected and vehicle-treated control groups, and folic acid-injected mice with AB0023 treatment, folic acid-injected mice with control IgG treatment, and vehicle-injected mice were compared. Data are expressed as the mean ± standard deviation and were compared using the Mann-Whitney U test,

one-way analysis of variance, Kruskal-Wallis test, and Wilcoxon signed rank test. Analyses were performed using SPSS version 25 (IBM-SPSS Inc., Armonk, NY, USA). $p<0.05$ was considered to indicate a statistically significant difference.

III. RESULTS

1. LOXL2 protein expression in human and mouse kidneys

Immunohistochemistry results demonstrated LOXL2 protein expression in glomeruli and tubular epithelial cells of human kidneys (Fig. 2A). In glomeruli, LOXL2 staining was observed along the outer surface of capillary loops. In tubular epithelial cells, LOXL2 staining was cytoplasmic, with no nuclear or membranous staining observed (Fig. 2A). LOXL2 expression was detected in proximal and distal tubules; however, more prominent staining was detected in distal tubular epithelial cells. In mouse kidneys, LOXL2 staining was also observed in glomeruli and tubular epithelial cells (Fig. 2B).

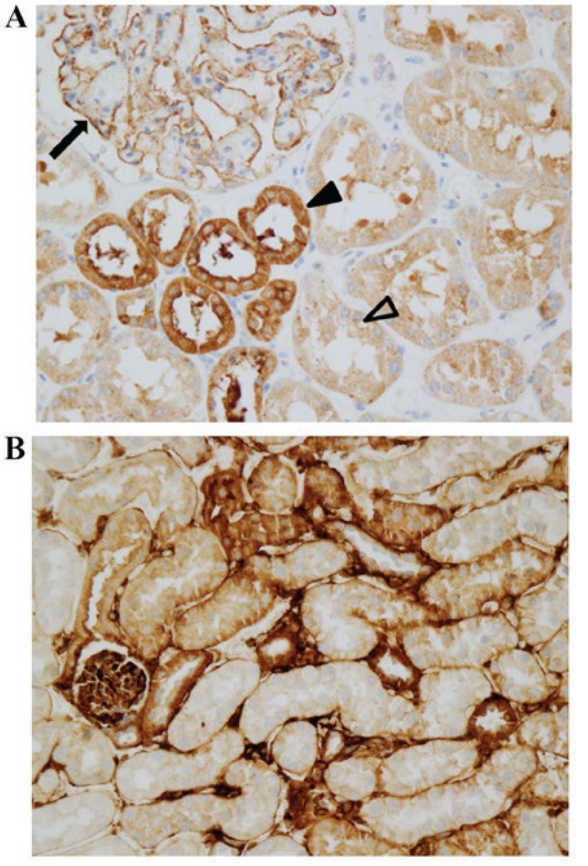


Figure 2. LOXL2 expression in human and mouse kidneys. Immunohistochemistry revealed LOXL2 expression in glomeruli and tubular epithelial cells in (A) human and (B) mouse kidney samples. In glomeruli, LOXL2 staining was observed along glomerular capillary loops (arrow). In tubular epithelial cells, LOXL2 staining was observed in the cytoplasm (arrow heads). The staining was more prominent in distal tubules (black arrow head) than in proximal tubules (open arrow head). Magnification, 400×. LOXL2, lysyl oxidase-like 2.

To determine the precise location of LOXL2 expression, double immunofluorescence microscopy, using the podocyte marker synaptopodin, was performed in human kidneys. LOXL2 expression was detected in the cytoplasm of podocytes, where synaptopodin expression was also observed (Fig. 3).

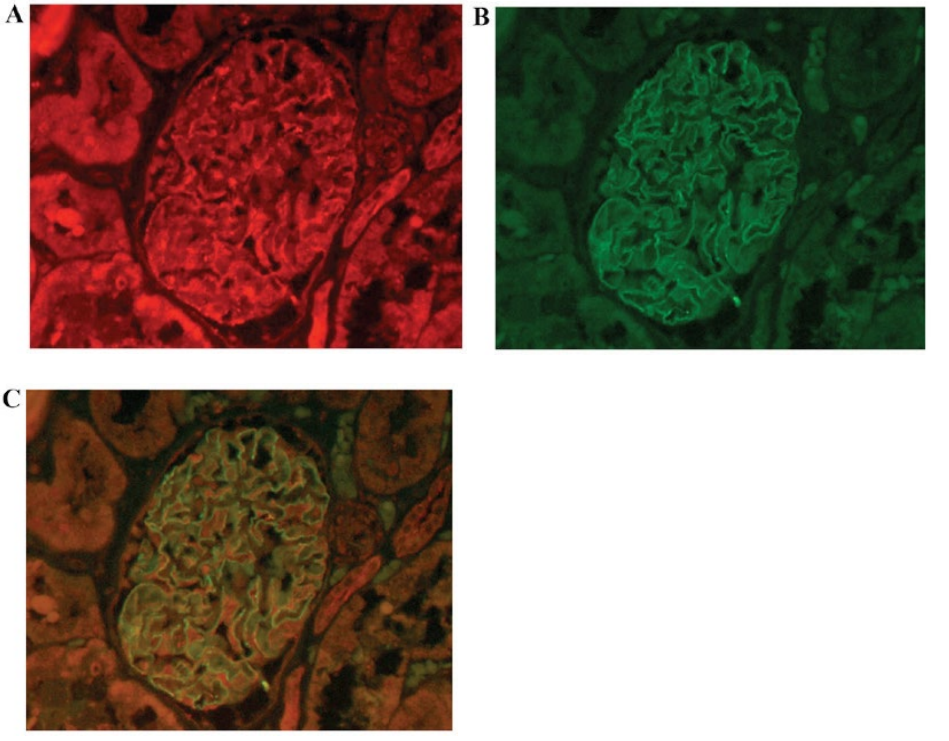


Figure 3. Double immunofluorescence microscopy analysis of LOXL2 in human podocytes. In glomeruli, (A) LOXL2 and (B) the podocyte-specific marker synaptopodin were (C) colocalized in podocyte cytoplasm. Magnification, 400×. LOXL2, lysyl oxidase-like 2.

2. LOXL2 expression in cultured cells

In cultured cell lines, LOXL2 expression was detected in human podocytes and HK-2 cells, as determined by western blot analysis, which supports the aforementioned results. However, MDCK cells, which are tubular cells derived from canine kidney, did not express LOXL2 (Fig. 4).

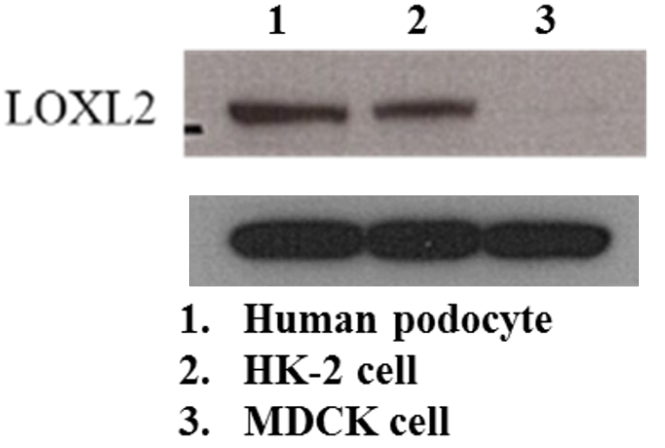


Figure 4. Western blot analysis of LOXL2 expression in cultured proximal tubular cells and podocytes. LOXL2 protein levels in cultured human proximal tubular cells (HK-2), canine tubular cells (MDCK), and immortalized human podocytes were evaluated by western blotting. The results demonstrated that LOXL2 is expressed in HK-2 cells and podocytes, but not in MDCK cells. Lane 1, human podocytes; lane 2, HK-2 cells; lane 3, MDCK cells. LOXL2, lysyl oxidase-like 2.

3. LOXL2 expression in a mouse model of tubulointerstitial fibrosis

Folic acid injection successfully induced diffuse renal tubulointerstitial fibrosis in mice (Fig. 5A and B). Immunohistochemistry analysis of LOXL2 demonstrated strong immunoreactivity in infiltrating inflammatory cells and the interstitium, in addition to glomerular and tubular expression (Fig. 5C). RT-qPCR analysis indicated that the mRNA expression levels of LOXL2 were significantly increased in folic acid-injected mice compared with vehicle-injected controls ($p=0.029$; Fig. 5D). In addition, as determined by western blotting, the protein expression levels of LOXL2 were increased in folic acid-injected mice compared with vehicle-injected mice ($p=0.023$; Fig. 5E).

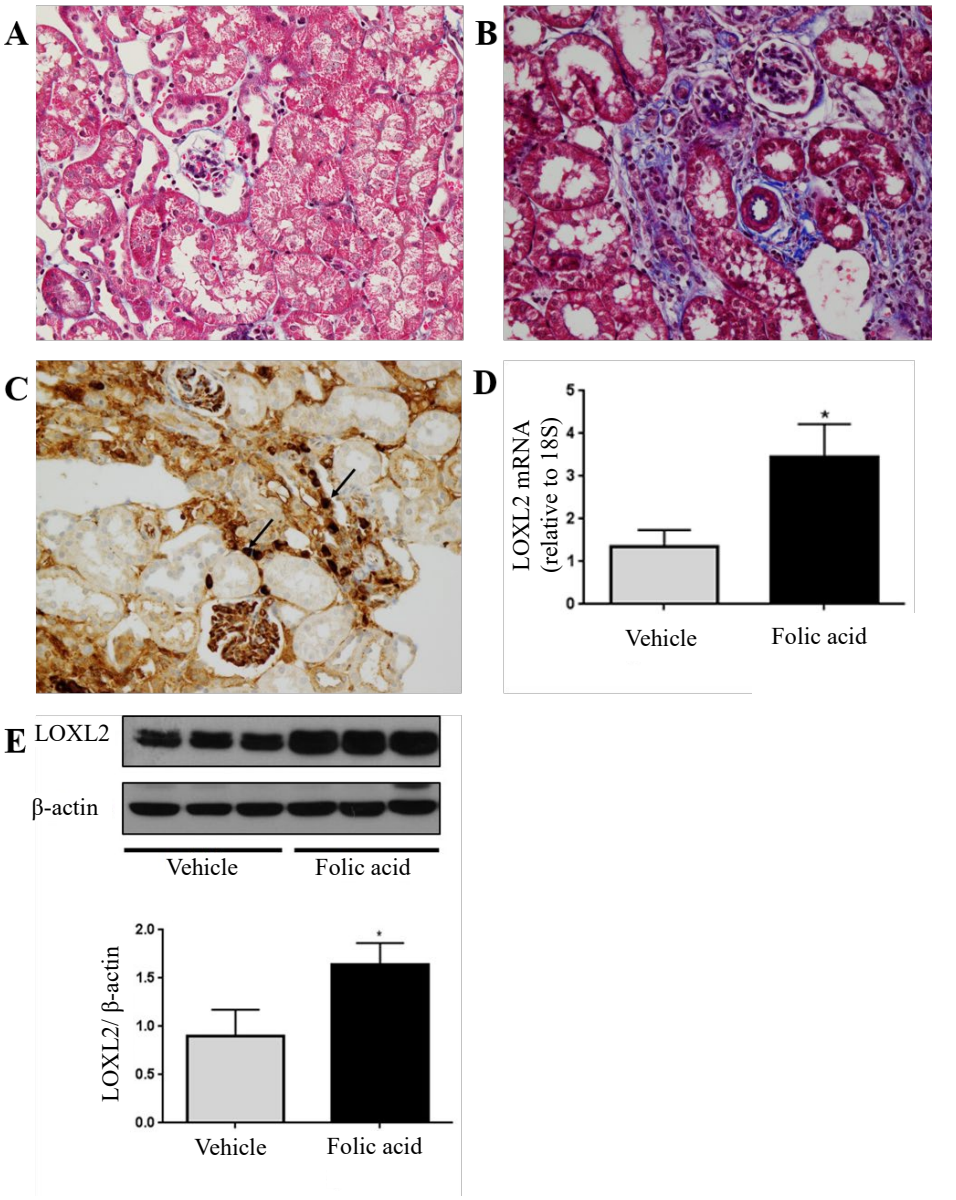


Figure 5. LOXL2 expression in a mouse model of tubulointerstitial fibrosis. Compared with in (A) vehicle-treated control mice, (B) diffuse tubulointerstitial fibrosis was induced 4 weeks after intraperitoneal injection of folic acid in CD1 mice. (C) Immunohistochemistry analysis detected LOXL2 protein expression

in the fibrous interstitium and infiltrating mononuclear cells (arrows) in folic acid-injected mice. (D) mRNA expression levels of LOXL2 were markedly increased in folic acid-injected mice, compared with vehicle-treated mice. (E) Western blotting revealed a significant increase in LOXL2 protein expression in folic acid-injected mice. Magnification, 400×. *$p<0.05$. LOXL2, lysyl oxidase-like 2.

4. LOXL2 inhibition prevented the progression of tubulointerstitial fibrosis in a mouse model

The amount of fibrosis measured by trichrome (Fig. 6A) and Sirius red staining (Fig. 6B) decreased in mice treated with AB0023, compared to the control IgG-injected group. Quantitative measurement of fibrosis by total collagen analysis also showed that the fibrosis decreased in mice treated with AB0023 (Fig. 6C).

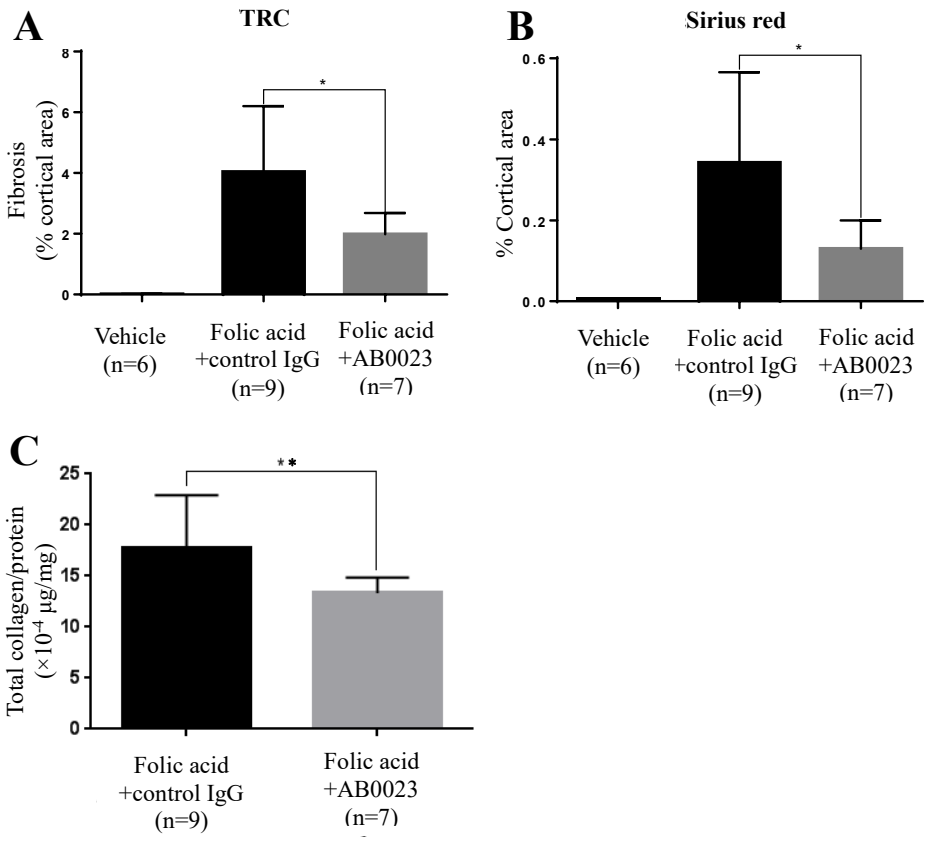


Figure 6. Collagen analysis of renal cortex using trichrome (TRC, A) and Sirius red (B) staining, and using the Total Collagen Assay Kit (C). The levels of fibrosis and total collagen were significantly decreased in the LOXL-2 antibody-treated group compared to the control group. *$p<0.017$. **$p<0.05$. LOXL2, lysyl oxidase-like 2.

5. LOXL2 inhibition may influence the canonical TGF-β/Smad signaling pathway

Smad signaling pathway molecules, including phosphorylated Smad3 (p-Smad3), phosphorylated Smad2 (p-Smad2), and Smad4 exhibited no significant difference with LOXL2 inhibition (Fig. 7). However, the amounts of p-Smad2 and Smad4 tended to decrease in the AB0023-treated group relative to the control group.

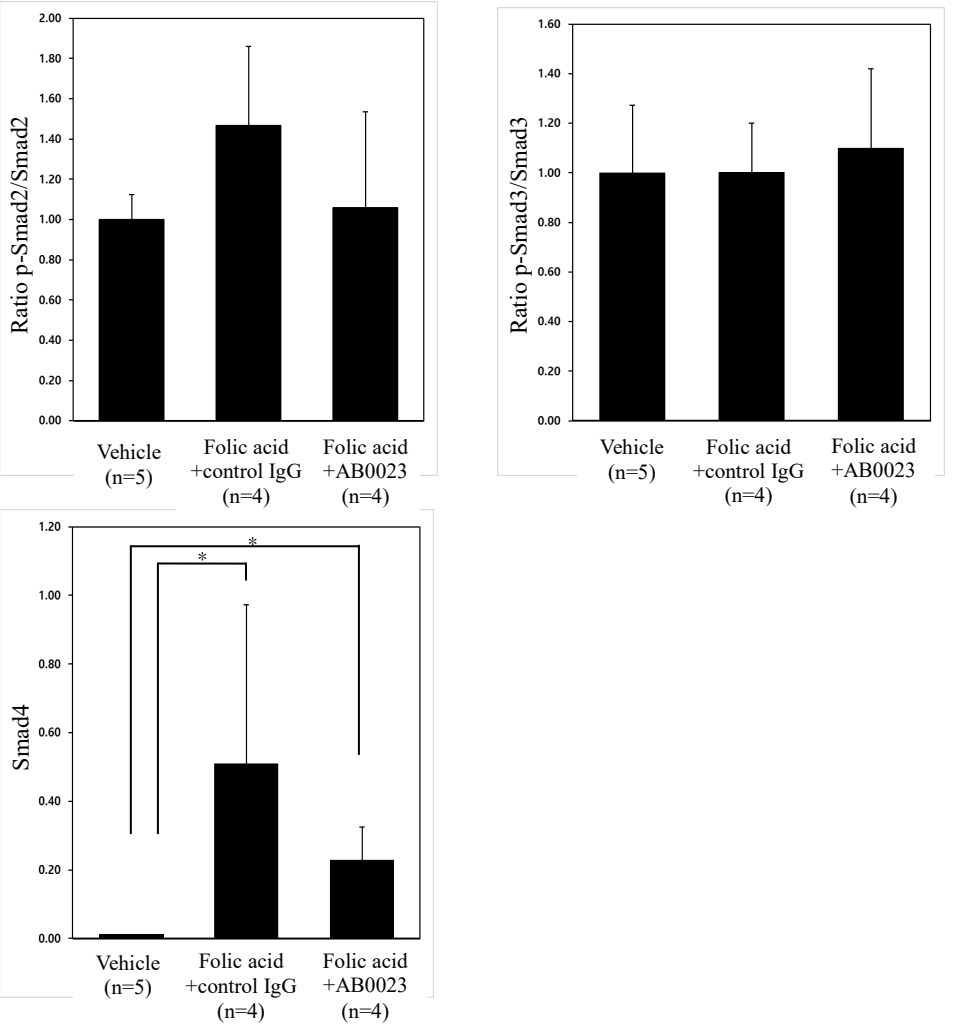


Figure 7. Effects of LOXL2 inhibition on the TGF-β/Smad pathway. There was no significant difference in the level of Smad pathway-related molecules, but the amount of p-Smad2 and Smad4 tended to be lower after LOXL2 inhibition. p values: 0.376, 0.784, and 0.010 for p-Smad2/total Smad2, p-Smad3/total Smad3, and Smad4, respectively. *p<0.017. LOXL2, lysyl oxidase-like 2.

6. LOXL2 knockdown HK-2 cells show reduced expression of EMT-associated molecules

TGF-β treatment caused a decrease in the level of the epithelial marker E-cadherin and an increase in vimentin, a mesenchymal marker, and fibronectin in control HK-2 cells. On multiple comparison analysis, vimentin level in LOXL2 knockdown cells was significantly lower than that of the control shRNA-transduced cells after TGF-β treatment (Fig. 8). Considering that the level of vimentin increases after TGF-β challenge in control cells, the decreasing trend of the vimentin level in LOXL2 knockdown cells after TGF-β challenge is more meaningful. The epithelial markers zona occludens (ZO)-1 and E-cadherin did not show a significant difference after TGF-β treatment in LOXL2 knockdown and control cells, while the level of E-cadherin was markedly decreased by TGF-β challenge.

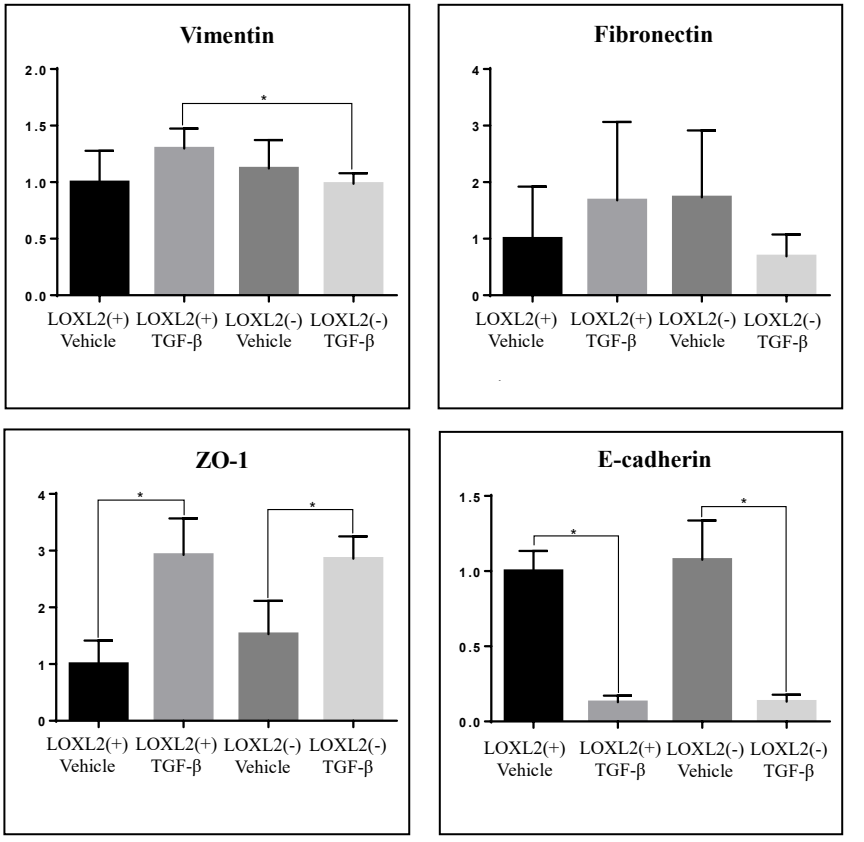


Figure 8. Expression of EMT-associated molecules after TGF-β challenge in LOXL2 knockdown HK-2 cells. LOXL2 knockdown HK-2 cells showed decreased expression of vimentin after TGF-β challenge. *$p<0.05$. LOXL2, lysyl oxidase-like 2. N=6 for all four groups.

IV. DISCUSSION

Tubulointerstitial fibrosis occurs during the progression of all chronic kidney diseases. Preventing tubulointerstitial fibrosis, regardless of its etiology, is important to the preservation of renal function. TGF-β is a key molecule in the development and progression of tubulointerstitial fibrosis. Activators of latent TGF-β include integrin αvβ6, downstream signal transduction molecules of the TGF-β signaling pathway, such as members of the Smad family and mitogen-activated protein kinases (MAPK), and upstream signal transduction molecules. Crosstalk signal transduction pathways, molecules, and mechanisms that interact with TGF-β signaling include the Wnt/β-catenin pathway, the epidermal growth factor receptor signaling, mTOR pathway and p53.[59] Recently, knowledge regarding the mechanisms underlying the pathogenesis of tubulointerstitial fibrosis has increased, and an animal study regarding its prevention has produced promising results.[60] However, few therapeutic agents for clinical use have been developed. Therefore, a better understanding of the molecular mechanisms underlying the development of tubulointerstitial fibrosis is required for the development of novel treatment strategies.

LOXL2, which plays a role in cancer metastasis, is also involved in organ fibrosis. Previous studies have demonstrated the involvement of LOXL2 in hepatic and pulmonary fibrosis,[30] and clinical trials have evaluated a LOXL2-specific inhibitor.[51-53] Although the clinical implications of kidney

fibrosis are substantial, little is currently known regarding LOXL2 expression in this organ, and the role of LOXL2 in tubulointerstitial fibrosis remains to be elucidated. Therefore, the present study evaluated LOXL2 expression in cellular compartments of the kidney and its possible contribution to tubulointerstitial fibrosis.

The association between LOXL2 and TGF-β has been investigated in numerous studies. Sethi *et al* demonstrated that the expression of LOX family genes, including LOXL2, is induced by TGF-β1, TGF-β2, and TGF-β3, and is mediated by canonical Smad signaling and noncanonical signaling pathways.[61] Voloshenyuk *et al* reported that TGF-β1 upregulates LOX expression in cardiac fibroblasts, and this phenomenon may be prevented by inhibiting Smad3.[62] Conversely, an inhibitory monoclonal antibody against LOXL2 has been reported to decrease fibroblast activation and TGF-β signaling, suggesting that LOXL2 plays a role in activating TGF-β.[53] Direct suppression of TGF-β was not successful in preventing renal fibrosis due to the diversity of TGF-β isoforms and signaling pathways.[63] Therefore, it may be possible that LOXL2, in addition to other molecules involved in TGF-β signaling pathways, should be considered as therapeutic targets. A previous study reported that LOXL2 expression in HK-2 cells was increased by hypoxia and hyperglycemia, and this alteration was associated with hypoxia inducible factor-1α.[64] The present study revealed that LOXL2 is primarily expressed in tubular cells in the kidney,

particularly in distal tubular cells. There are numerous mechanisms by which tubular epithelial cells initiate or contribute to the progression of tubulointerstitial fibrosis. Following hypoxic, toxic, or immunological insult-induced injury, tubular cells secrete chemoattractants to induce interstitial inflammation.[65] TGF-β1 and the type II TGF-β1 receptor play critical roles in the link between inflammation and fibrosis via the TGF-β/Smad3 signaling pathway.[66] EMT may also contribute to interstitial fibrosis by providing a source of fibrogenic myofibroblasts.[67] Tubular epithelial cells lose cell-cell adhesion and acquire myofibroblast properties through the induction of TGF-β. Other signaling pathways such as Wnt/β-catenin signaling[68] and cellular components including autophagy through upregulation of ILK[69] are also involved in this process. Although the mechanisms underlying this phenomenon remain unclear, it is highly probable that tubular epithelial cells play a critical role in the progression of tubulointerstitial fibrosis.

The present observation that LOXL2 is expressed in tubular epithelial cells suggests a role for LOXL2 in TGF-β-mediated tubulointerstitial fibrosis. This hypothesis is supported by the increased LOXL2 mRNA and protein levels detected in the kidneys of mice with folic acid-induced tubulointerstitial fibrosis. The positive regulatory role of LOXL2 in tubulointerstitial fibrosis is confirmed by the reduction of fibrosis in this folic acid-induced fibrosis mouse model with the inhibition of LOXL2 by the LOXL2-specific antibody AB0023.

In addition to fibrosis, TGF-β demonstrates various biological activities, such as cell proliferation, apoptosis, differentiation, autophagy, and the immune response.[70] Thus, it is more desirable to investigate therapeutic strategies related to downstream pathways of TGF-β due to the possible adverse effects of directly targeting TGF-β.[71] TGF-β acts on multiple cell types, including podocytes, tubular epithelial cells, mesangial cells, and endothelial cells, through a well-documented canonical signaling pathway that involves phosphorylation/activation of Smad2 and Smad3 by the kinase activity of TGF-β receptor type I (TβR-I).[72] In this pathway, the common Smad, Smad4, is recruited to form a Smad2/3/4 complex that translocates to the nucleus[59] and transcribes profibrotic molecules, such as collagen type I[73] and TIMP.[73] Inhibitory Smads, such as Smad6 and Smad7, inhibit the activation of Smad2 and Smad3 through their interaction with TβR-I.[35] Another group of Smads (Smad1, 5, and 8) respond to receptor other than TβR-I, a downstream effector of the BMP subfamily.[35,74] The common role of Smad4 in facilitating the nuclear translocation of the Smad1/5/8 and Smad2/3/4 complexes in response to BMPs and TGF-β, respectively, confers the designation “common” to Smad4.[75] In addition to the Smad-dependent pathway, TβR-I activated by TGF-β acts on a variety of Smad-independent noncanonical signaling pathways that modify the TGF-β/Smad signaling pathway, including the MAPK pathway, phosphatidylinositol-3 kinase (PI3K) cascades, and the Wnt/β-catenin

pathway.[59,72]

EMT is a major mechanism that contributes to renal fibrosis in response to multiple molecules, including TGF-β1,[76] CTGF,[77] and AngII[77] in the tubular epithelium. However, TGF-β1 is the most potent inducer of EMT.[78,79] As mentioned above, EMT is a well described process accompanied by a loss of epithelial cell adhesion molecules, such as E-cadherin and ZO-1, *de novo* α-smooth muscle actin (α-SMA) expression and actin filament reorganization, transformation of myofibroblastic morphology, tubular basement membrane (TBM) disruption, and cell migration/infiltration into the interstitium.[79,80] However, conflicting results have been reported *in vivo*, as tubular cells that have undergone partial EMT relay proinflammatory and profibrogenic signals to the interstitium without directly contributing to the myofibroblast population.[81] Accordingly, the relationship between LOXL2 and the TGF-β pathway *in vivo,* and the TGF-β-mediated relationship between LOXL2 and EMT *in vitro* were investigated. Thus, canonical pathway-related molecules were studied *in vivo* and markers expressed by tubular cells during EMT were studied *in vitro*. The lack of significant differences in the amount of Smad molecules after LOXL2 inhibition in this study might be due to the delay in time between folic acid injection and analysis. Murine kidneys were harvested 4 weeks after this injury, by which time fibrogenesis could have already been completed. Stallons et al. reported that TGF-β1 and α-SMA levels increased until 6 days after folic acid

injection, and gradually decreased afterwards in a similar experiment where a 250 mg/kg dose of folic acid was injected intraperitoneally.[57] Tang et al. reported that after the administration of high doses of glucose, the p-Smad2 and p-Smad3 expression increased in HK-2 cells for 30 to 90 minutes and for 30 to 60 minutes, respectively, and gradually decreased thereafter.[82] Although this experiment is not identical to that of this research, the time between intervention and injury is substantially longer than that in previous studies. A more rapid analysis of Smad molecules after folic acid injection may have revealed a more pronounced change in their expression in this study.

Experiments on HK-2 cells *in vitro* after TGF-β challenge revealed no increases in the myofibroblast markers vimentin in LOXL2 knockdown cells. This indicates that LOXL2 plays a regulatory role in EMT. Other studies have shown no reduction in epithelial markers, such as E-cadherin, with LOXL2 inhibition after TGF-β challenge, while a significant reduction of E-cadherin was observed here. However, cell types and experimental methods used in this study differ from those reported previously.[83-85] Further studies are warranted to elucidate the mechanisms underlying the LOXL2-EMT-related pathway.

The expression of LOXL2 in infiltrating inflammatory cells detected in the present study is interesting considering that infiltrating macrophages also express TGF-β.[86] This finding suggests that the potential mechanisms underlying the effects of LOXL2 in tubulointerstitial fibrosis are complex.

The present study also detected LOXL2 expression in podocytes, a finding that may have clinical significance. Considerable evidence supports the role of podocyte injury as a key factor in the pathogenesis of focal segmental glomerulosclerosis.[87-89] Podocyte detachment is also an important pathogenic mechanism in the progression of diabetic nephropathy,[90] which is characterized by nodular glomerulosclerosis. Considering the profibrogenic function of LOXL2 in other organs, it is reasonable to hypothesize that LOXL2 expression in podocytes may contribute to the progression of glomerulosclerosis.

It is notable that LOXL2 immunostaining was stronger in distal tubular cells than in proximal tubular cells, but that LOXL2 protein was not detected in MDCK cells, which have been used in many experiments as a surrogate for human distal tubular cells. However, studies have also shown that MDCK cells exhibit the properties of cells other than distal tubular cells, depending on the purpose of the study and the type of experiment. [91-93]

In conclusion, LOXL2, which is a protein involved in extracellular matrix remodeling and organ fibrosis, is expressed in renal tubular epithelial cells and podocytes. It is associated with TGF-β-mediated tubulointerstitial fibrosis and EMT. Improved understanding of the role of LOXL2 in the kidney may illuminate the pathophysiology of tubulointerstitial fibrosis and glomerulosclerosis, and potentially lead to the discovery of novel therapeutic targets.

V. CONCLUSION

LOXL2 is expressed in normal mouse and human kidneys. LOXL2 expression is upregulated in renal fibrosis, and the inhibition of LOXL2 ameliorates renal fibrosis. LOXL2 is related to TGF-β/Smad signaling and EMT both directly and indirectly. Thus, LOXL2 may serve as a potential preventive or therapeutic target in chronic kidney disease due to its beneficial effects against tubulointerstitial fibrosis.

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만성신질환 섬유화의 예방 및 치료 물질로서의 lysyl oxidase-like 2

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요세관간질섬유화는 만성신질환의 공통되는 소견으로, 이것의 진행을 막는 것은 신부전예방의 핵심이라고 할 수 있다. Transforming growth factor-β (TGF-β)를 비롯한 여러 종류의 분자들이 요세관간질섬유화를 일으키는 것으로 알려져 있으나 효과적인 표적치료제는 아직 개발되어 있지 않다. Lysyl oxidase-like 2 (LOXL2)는 악성종양의 침윤과 전이에 관련된 것으로 알려졌으나, 최근 간섬유화와 폐섬유화와도 관련이 있는 것이 밝혀졌다. 아직까지 신장에서의 LOXL2 발현과 LOXL2가 요세관간질섬유화에 미치는 영향에 대해서는 거의 알려진 바가 없다. 본 연구에서는 인체 및 쥐의 신장조직과 세포에서 LOXL2 발현을 규명하고자 했다. 이후 LOXL2 특이적 항체인 AB0023을

쥐 요세관간질섬유화 모델에 투여하여 섬유화의 변화 및 Smad관련 분자들의 변화를 조사하였다. 또 LOXL2 유전자 녹다운 HK-2 요세관상피세포에 TGF-를 처리한 이후 상피중간엽이행 (epithelial-mesenchymal transition, EMT)에 관련된 분자들의 변화를 관찰하였다. 그 결과 인체와 쥐의 신장 내 사구체혈관고리와 요세관상피세포에서 LOXL2 단백질이 발현하는 것을 관찰하였다. 족돌기세포 표지자인 synaptopodin을 사용한 이중 면역형광현미경검사에서 LOXL2가 족돌기세포의 세포질 내에 발현되는 것을 확인하였다. 또한 족돌기세포와 HK-2 세포를 배양한 후 western blot 분석을 시행하여 LOXL2가 발현되는 것을 관찰하였다. 쥐 요세관간질섬유화 모델에서는 LOXL2의 mRNA와 단백질 발현이 대조군에 비해 증가하였고, LOXL2 면역조직화학 염색 상에서, 섬유화된 간질과, 간질에 침윤하고 있는 단핵구성 면역세포에서 LOXL2가 발현되었다. 쥐 요세관간질섬유화 모델에 LOXL2의 단클론항체인 AB0023을 처리했을 때 섬유화의 진행이 억제되는 것을 trichrome, Sirius red 염색과 총 콜라겐 측정을 통해 확인하였다. Smad 관련 분자는 통계적으로 의미 있는 변화를 보이지는 않았다. LOXL2 유전자 녹다운 HK-2 세포에 TGF-β를 처리한 이후 증가한 중간엽형질표지자 vimentin의 양이 대조군에 TGF-β를 처리한 이후 증가한 vimeintin 양보다 적었다. 요세관간질섬유화 모델에서 LOXL2 발현이 증가했고, 그 모델에 AB0023을 처리하자 섬유화가 감소한 것을 통해 LOXL2가 요세관간질섬유화에 기여하는 것을 알 수 있었다. 이러한

LOXL2와 요세관간질섬유화의 관련성은 EMT 및 TGF-β/Smad 경로를 통한 것일 가능성이 있음을 확인하였다. 본 연구는 요세관간질섬유화와 LOXL2의 관련성에 대해서 처음으로 조사 했다는 것에 의의가 있다. LOXL2가 요세관간질섬유화에 어떠한 경로를 거쳐 영향을 미치는지에 대하여 후속 연구가 필요하다.

핵심되는 말 : 요세관간질섬유화, TGF-β, lysyl oxidase-like 2, 상피중간엽이행, 족돌기세포, HK-2 근위세뇨관세포, Smad