

1 Inhibition of lysyl oxidase-like 2 ameliorates folic acid-induced renal tubulointerstitial fibrosis

2 Sung-Eun Choi¹, Nara Jeon¹, Hoon Young Choi^{2,3}, Hyeon Joo Jeong^{1,3}, *Beom Jin Lim^{1,3}

3 ¹Departments of Pathology and ²Internal Medicine, ³Institute of Kidney Disease Research, Yonsei

4 University College of Medicine, Seoul 03722, Republic of Korea

5 *Corresponding author: Dr. Beom Jin Lim, Department of Pathology, Yonsei University College of

6 Medicine, 50-1, Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

7 E-mail: bjlim@yuhs.ac

8

9 **Abstract**

10 Tubulointerstitial fibrosis is characterized by accumulation of the extracellular matrix in the interstitium.
11 Lysyl oxidase-like 2 (LOXL2), a member of the lysyl oxidase family, is known for promoting cancer
12 metastasis, invasion, and stromal fibrosis in various organs. Our previous study demonstrated
13 expression of LOXL2 in kidney podocytes and tubular epithelial cells, and the association between
14 elevated LOXL2 and tubulointerstitial fibrosis. The present study evaluated the effect of LOXL2
15 inhibition using an inhibitory monoclonal antibody (AB0023) on tubulointerstitial fibrosis in a folic
16 acid-induced tubulointerstitial fibrosis mouse model. We also evaluated the association of LOXL2 with
17 epithelial-mesenchymal transformation related molecules *in vitro* using HK-2 cells. Our data
18 demonstrate that AB0023 prevented the progression of tubulointerstitial fibrosis significantly, as
19 determined by trichrome and picro-sirius red staining, as well as the total collagen assay. The mean
20 expression of phosphorylated Smad2 and Smad4 was lower in the AB0023-treated group although it
21 was not statistically significant. Following transforming growth factor- β (TGF- β) challenge, LOXL2-
22 deficient HK-2 cells exhibited significantly lower expression of the mesenchymal markers vimentin
23 and fibronectin than control HK-2 cells. In conclusion, LOXL2 inhibition ameliorates renal fibrosis
24 through the TGF- β /Smad signalling pathway.

25

26 **Introduction**

27 As tubulointerstitial fibrosis is a common endpoint in renal disease with no effective treatment other
28 than dialysis, the need to understand the molecules and mechanisms involved is increasingly urgent.
29 Histologically, tubulointerstitial fibrosis is an accumulation of the extracellular matrix (ECM) in the
30 interstitium. ECM-producing cells are primarily activated fibroblasts [1]. Various cells such as pericytes,
31 endothelial cells, residual fibroblasts, and tubular epithelial cells are known to be the origin of

32 fibroblasts [2].

33 The epithelial-mesenchymal transformation (EMT) has been studied in cancer and benign
34 fibrotic diseases [3]. Once acute injury is imposed on the kidney, various chemokines and growth factors
35 cause inflammation, which in turn leads to the secretion of transforming growth factor- β (TGF- β) via
36 release of active TGF- β from latent TGF- β -binding protein via protease cleavage [4]. TGF- β is the
37 primary molecule responsible for EMT [5, 6], and the canonical and non-canonical pathways are the
38 downstream pathways of TGF- β [4, 7]. The hallmark of EMT is loss of epithelial phenotypes and
39 acquisition of mesenchymal phenotypes with activation of profibrotic genes to produce the ECM,
40 including fibronectin and types I and III collagen [3, 5, 8].

41 Lysyl oxidase-like 2 (LOXL2) is a member of the lysyl oxidase family, originally known as a
42 copper-dependent amine oxidase, that is involved in cross-linking collagen and elastin of the ECM [9].
43 Studies have also demonstrated additional functions for LOXL2 independent of its catalytic activity,
44 such as organ development [10], tumour invasion [11], and EMT [12, 13].

45 In our previous study, we found that LOXL2 is expressed in kidney podocytes and tubular
46 epithelial cells, and its expression is increased in the folic acid-induced murine fibrosis model [14]. In
47 this study, we evaluated the effect and therapeutic role of LOXL2 inhibitor AB0023 on the progression
48 of tubulointerstitial fibrosis in mice. In order to evaluate the contribution of LOXL2 in EMT, an *in vitro*
49 study using immortalized human proximal tubular epithelial cells (HK-2 cells) was also performed.

50

51 **Methods**

52

53 **Animal model of tubulointerstitial fibrosis and LOXL2 inhibition**

54 Male CD1 mice at 8 weeks of age (Orient Bio, Inc., Seongnam, South Korea) were used in this study.
55 The animals were housed in a facility maintained at 20°C and 12-h alternating light/dark cycles with

56 free access to rodent chow and water. Tubulointerstitial fibrosis was induced by intraperitoneal injection
57 of folic acid (240 µg/g body weight) [15, 16]. The folic acid solution was prepared by dissolving folic
58 acid powder (Sigma-Aldrich) in 0.3 M NaHCO₃. Control CD1 mice were injected intraperitoneally with
59 the same volume of vehicle (NaHCO₃). Urinary excretion of neutrophil gelatinase-associated lipocalin
60 (NGAL) was measured immediately before injection and at 3 days after injection using a Mouse
61 Lipocalin-2/NGAL Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems) to
62 ensure successful injection of folic acid, as manifested by a log scale increase in NGAL. The
63 concentration of urinary NGAL was normalized by the concentration of urinary creatinine as measured
64 by the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA). Mice without
65 an increase in NGAL at 3 days post-folic acid injection, indicating that folic acid was not successfully
66 injected, were omitted from the study. Ultimately, 16 mice injected with folic acid and six mice injected
67 with vehicle were examined in this study.

68 To inhibit LOXL2, AB0023 (Gilead Sciences, Foster City, CA, USA), an inhibitory
69 monoclonal antibody against LOXL2, was used. Nine of the 16 folic acid-injected mice were treated
70 with a dosage of 15 mg/kg body weight of AB0023 at 1 week before folic acid injection and twice a
71 week for 4 weeks afterwards. The remaining seven mice were injected with immunoglobulin G (IgG)
72 (GS-645864, Gilead Sciences) at the same dosage and on the same time schedule as the AB0023
73 treatment group. Mice were sacrificed via cervical dislocation 4 weeks after folic acid or vehicle
74 injection, and the right kidneys were harvested (Fig 1). Fresh frozen tissues were stored at -70°C for
75 western blot analysis and collagen measurement. Additional kidney tissues were fixed in 4%
76 formaldehyde for 24 hours at room temperature and embedded in paraffin overnight at 55-65°C using
77 an automatic tissue processor (EFTP-FAST 360; Intelsint, Turin, Italy).

78 The present study was approved by the Institutional Animal Care and Use Committee of the
79 Yonsei University Health System (Seoul, South Korea; approval number 2015-0247). All experiments
80 involving animals were carried out in accordance with the standards set forth by the Institutional Animal
81 Care and Use Committee of Yonsei University Health System.

82 **Fig 1. Injection protocol of folic acid, AB0023 (a monoclonal antibody against LOXL2), and**
83 **control IgG in the CD1 mouse model.**

84 The red arrow indicates AB0023 or control IgG intraperitoneal injection (15 mg/kg). AB0023 or control
85 IgG was injected 4 and 1 day before folic acid injection, and twice weekly until 4 weeks after folic acid
86 injection. Urinary neutrophil gelatinase-associated lipocalin (NGAL) was measured 3 days after folic
87 acid injection to ensure successful induction of renal fibrosis.

88

89 **Evaluation of tubulointerstitial fibrosis**

90 **Semiquantitative analysis via histologic examination**

91 Paraffin-embedded samples of the AB0023-treated, IgG-injected, and vehicle-injected groups were cut
92 into 4- μ m sections. After deparaffinization and rehydration, sections were stained with Masson
93 trichrome and picro-sirius red. For picro-sirius red staining, sections were treated with Weigert's Iron
94 Hematoxylin for 8 min to stain nuclei and Direct Red 80 (Sigma-Aldrich; Merck KGaA, Darmstadt,
95 Germany) for 1 hour at room temperature to visualize collagen before washing with 0.5% glacial acid.
96 Slides were examined by light microscopy with or without polarisation for picro-sirius red or trichrome
97 staining, respectively. Photos were taken serially along the cortex at 200 \times magnification and the area
98 of interstitial fibrosis was measured using Image J software (version 1.50i; National Institutes of Health,
99 Bethesda, MD, USA).

100

101 **Quantitative analysis via total collagen analysis**

102 The content of collagen in fresh frozen cortex was evaluated by measuring hydroxyproline using the
103 Total Collagen Assay Kit (QuickZyme Biosciences, Leiden, Netherlands) according to the
104 manufacturer's guide. Briefly, samples were hydrolysed at 95°C in 6M HCl for 20 hours and then
105 centrifuged at 13,000 rpm for 10 min. The supernatant was collected and assayed by ELISA according
106 to the manufacturer's protocol. Total protein in the hydrolysed sample was also measured using the

107 Total Protein Assay Kit (QuickZyme) and the relative amount of collagen per protein was analysed.

108

109

110 **Renal cell culture and transfection**

111 HK-2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and
112 cultured in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 (Gibco; Thermo
113 Fisher Scientific, Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS; Gibco;
114 Thermo Fisher Scientific). To silence LOXL2 expression at the cellular level, LOXL2 shRNA lentiviral
115 particles (cat. no. sc-45222-v; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were transduced into
116 HK-2 cells cultured on collagen I (2 mg/ml, cat. no. 354236; Corning Inc., Corning, NY, USA)-coated
117 dishes[17]. HK-2 cells were treated with media containing 5 µg/ml of polybrene (cat. no. sc-134220;
118 Santa Cruz Biotechnology, Inc.), and then LOXL2 shRNA lentiviral particles and control shRNA
119 lentiviral particles of 1 and 2 multiplicity of infection (MOI) were added. Transfected cells were
120 selected by selection media containing 2 µg/ml puromycin dihydrochloride (cat. no. sc-108071; Santa
121 Cruz Biotechnology, Inc.). After confirming the decrease in LOXL2 expression by reverse
122 transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis (described
123 below), cells of 2 MOI were treated with serum-free media for 24 hours and then media containing
124 TGF-β (20 ng/ml, R&D Systems, Minneapolis, MN, USA) for 72 hours. Other LOXL2-deficient cells
125 (2 MOI) were treated with serum-free media for 24 hours and then media containing vehicle (0.1%
126 4mM HCl/BSA). Control shRNA lentiviral particles (cat. no. sc-108080; Santa Cruz Biotechnology,
127 Inc.) were transduced into another line of HK-2 cells in the same manner as that of LOXL2 shRNA
128 particles and were further incubated with media containing either TGF-β (20 ng/ml, R&D Systems) or
129 vehicle (0.1% 4mM HCl/BSA) after 24 hours of serum starvation.

130

131 **Western blot analysis for LOXL2, Smad-related molecules and**

132 **epithelial-mesenchymal transformation-related molecules**

133 Fresh frozen kidney tissues from mice and HK-2 cells were homogenised and western blotting was
134 performed following the same protocol of our previous work[14]. Radioimmunoprecipitation assay
135 buffer (Biosesang, Inc., Seongnam, Korea) with a protease inhibitor cocktail (Roche Diagnostics,
136 Indianapolis, IN, v USA) was prepared. After lysing the cells in buffer, the samples were centrifuged at
137 13,000 rpm for 30 min at 4°C. The protein concentration was measured through bicinchoninic acid
138 protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. When the protein
139 samples (50 µg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis
140 (SDS-PAGE) for 2 h at 100 V, they were transferred onto a polyvinylidene fluoride membrane and
141 blocked with 3% skim milk for 1 h at room temperature. Primary antibodies were incubated with the
142 membrane overnight at 4°C. The anti-mouse-specific primary antibodies purchased from Cell Signaling
143 Technology (Danvers, MA, USA) included anti-Smad2 (cat. no. 5339; 1:1000), anti-phosphorylated-
144 Smad2 (p-Smad2, Ser465/467) (cat. no. 3108; 1:500), anti-Smad3 (cat. no. 9523; 1:1000), anti-
145 phosphorylated-Smad3 (p-Smad3, Ser423/425) (cat. no. 9520; 1:1000), anti-Smad2/3 (cat. no. 8685;
146 1:1000), and anti-Smad4 (cat. no. 38454; 1:1000). The blocking solution used for the anti-phospho-
147 Smad2 and anti-phospho-Smad3 antibodies contained 5% bovine serum albumin (Sigma-Aldrich). The
148 primary antibodies applied to HK-2 cells were anti- α -smooth muscle actin (α -SMA, cat. no. A5228;
149 1:500; Sigma-Aldrich), anti-vimentin (cat. no. ab92547; 1:5000; Abcam, Cambridge, MA, USA), anti-
150 E-cadherin (cat. no. 610181; 1:500; BD Biosciences, San Jose, CA, USA), anti-zona occludens (ZO)-1
151 (cat. no. ab2272; 1:500; Sigma-Aldrich), anti-fibronectin (cat. no. sc8422; 1:1000; Santa Cruz
152 Biotechnology, Inc.), and anti-LOXL2 (cat. no. ab96233; 1:500; Abcam). The membrane was washed
153 with Tris-buffered saline which contains 0.1% Tween-20. It was then incubated with horseradish
154 peroxidase-labelled secondary antibodies (cat. no. sc-2020; 1:5,000; Santa Cruz Biotechnology, Inc.;
155 and cat. no. K4003; 1:5,000; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 1 hour at
156 room temperature. Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher

157 Scientific) was used to visualize protein bands. The membrane was stripped with Restore Western Blot
158 Stripping Buffer (Thermo Fisher Scientific) for 15 min at room temperature, and then it was incubated
159 with an anti- β -actin antibody (cat. no. sc-47778; 1:2,000; Santa Cruz Biotechnology, Inc.). The bands
160 were semi-quantified by densitometry using Image J software (version 1.50i; National Institutes of
161 Health, Bethesda, MD, USA).

162

163 **Statistical analysis**

164 Quantitative analysis was performed for the western blot and RT-qPCR results. Vehicle-injected mice,
165 folic acid-injected mice treated with AB0023, and folic acid-injected mice treated with control IgG were
166 compared. Data are expressed as the mean \pm standard deviation and compared using the Mann-Whitney
167 U test, one-way analysis of variance, Kruskal-Wallis test, and Wilcoxon signed rank test. The analyses
168 were performed using SPSS version 25 software (IBM-SPSS Inc., Armonk, NY, USA). Differences
169 with $p < 0.05$ were considered statistically significant.

170

171 **Results**

172 **LOXL2 inhibition prevented the progression of tubulointerstitial 173 fibrosis in the mouse model**

174 The amount of fibrosis measured by trichrome (Fig 2A) and picro-sirius red staining (Fig 2B) decreased
175 in mice treated with AB0023, compared to the control IgG-treated group (Fig 2C and D). Quantitative
176 measurement of fibrosis by total collagen analysis also showed that fibrosis decreased in mice treated
177 with AB0023 (Fig 2E).

178 **Fig 2. Effects of LOXL2 inhibition on the progression of tubulointerstitial fibrosis.**

179 Representative photos of renal cortex of folic acid-injected mice treated with control IgG or AB0023.
180 The amount of fibrosis was measured using Masson trichrome stain (A&C) and picro-sirius red stain
181 under polarized microscopy (B&D). The levels of fibrosis were significantly lower in the AB0023-
182 treated group compared to the control group. The total collagen assay showed similar result (E).
183 *p<0.017, **p<0.05. LOXL2, lysyl oxidase-like 2.

184

185 **LOXL2 inhibition may influence the canonical TGF- β /Smad
186 signalling pathway**

187 Smad signaling pathway molecules, including p-Smad3, p-Smad2, and Smad4 exhibited no significant
188 difference with LOXL2 inhibition (Fig. 3). However, the amounts of p-Smad2 and Smad4 tended to
189 decrease in the AB0023-treated group relative to the control group.

190 **Fig 3. Effects of LOXL2 inhibition on the TGF- β /Smad pathway.**

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

195

196 **LOXL2 knockdown in HK-2 cells reduced the expression of some
197 EMT-associated molecules**

198 Transfection of HK-2 cells with LOXL2 shRNA resulted in LOXL2 knockdown (see S1 Fig). In control
199 HK-2 cells transfected with control shRNA, TGF- β treatment (72 hours) reduced the levels of epithelial
200 marker E-cadherin, and increased the levels of mesenchymal markers vimentin and fibronectin. Multiple

201 comparison analysis revealed that vimentin level was significantly lower in LOXL2 knockdown cells
202 than in control shRNA-transduced cells after TGF- β treatment (Fig 4). Considering that the level of
203 vimentin increases after TGF- β challenge in control cells, the decreasing trend of the vimentin level in
204 LOXL2 knockdown cells after TGF- β challenge is more meaningful. The epithelial markers ZO-1 and
205 E-cadherin did not show a significant difference after TGF- β treatment in LOXL2 knockdown and
206 control cells, while the level of E-cadherin was markedly decreased by TGF- β challenge.

207 **Fig 4. Expression of EMT-associated molecules after TGF- β challenge in LOXL2 knockdown**
208 **HK-2 cells.**

209 HK-2 cells were transfected with either control shRNA virus or LOXL2 shRNA lentivirus to
210 knockdown LOXL2 expression. After 24 hours of serum starvation, both the LOXL2-deficient cells
211 and control cells were incubated with either TGF- β or vehicle for 72 hours. Compared to control HK-2
212 cells, LOXL2-deficient HK-2 cells showed decreased expression of vimentin after TGF- β challenge.
213 *p<0.05. N=6 for all four groups. LOXL2(+), HK-2 cells transfected with control shRNA; LOXL2(-),
214 LOXL2-deficient HK-2 cells by LOXL2 shRNA transfection; LOXL2, lysyl oxidase-like 2; ZO-1, zona
215 occludens-1.

216
217

218 **Discussion**

219 Inhibition of LOXL2 via AB0023 has been shown to reduce fibrosis in various organs. For instance,
220 AB0023 attenuated postoperative fibrosis in a rabbit model of glaucoma surgery [18]. AB0023 also
221 attenuated tetrachloride-induced hepatic fibrosis in BALB/c mice, and high-dose bleomycin-induced
222 pulmonary fibrosis in C57BL/6 mice [19]. Although a clinical trial of simtuzumab, a humanized form
223 of AB0023, resulted in no significant changes in fibrosis in human immunodeficiency virus- and

224 hepatitis C virus-infected adults, serum samples suggested up-regulation of TGF- β 3 and interleukin-10
225 pathways with treatment, suggesting the future evaluation for clinical trials with simtuzumab after the
226 modulation of TGF- β 3 [20]. We previously observed that LOXL2 is expressed in tubular epithelial cells,
227 and presumed a role for LOXL2 in TGF- β -mediated tubulointerstitial fibrosis. This hypothesis is
228 supported by the increased LOXL2 mRNA and protein levels detected in the kidneys of mice with folic
229 acid-induced tubulointerstitial fibrosis [11]. The positive regulatory role of LOXL2 in tubulointerstitial
230 fibrosis is confirmed by the reduction of fibrosis in this folic acid-induced fibrosis mouse model with
231 the inhibition of LOXL2 by the LOXL2-specific antibody AB0023. To the best of our knowledge, this
232 is the first study demonstrating that LOXL2 inhibition leads to attenuated fibrosis in the murine kidney
233 injury model..

234 In addition to fibrosis, TGF- β is involved in various biological activities, such as cell
235 proliferation, apoptosis, differentiation, autophagy, and the immune response [21]. Thus, it is critical to
236 investigate therapeutic strategies related to the downstream pathways of TGF- β due to the possible
237 adverse effects of directly targeting this cytokine [22].

238 EMT is a major mechanism that contributes to renal fibrosis in response to multiple molecules,
239 including TGF- β 1 [1], connective tissue growth factor [23], and angiotensin II [11], in tubular epithelial
240 cells. However, TGF- β 1 is the most potent inducer of EMT [5, 8]. As mentioned above, EMT is a well-
241 described process characterized by a loss of epithelial cell adhesion molecules, such as E-cadherin and
242 ZO-1, *de novo* α -SMA expression and actin filament reorganization, transformation of myofibroblastic
243 morphology, tubular basement membrane disruption, and cell migration/infiltration into the interstitium
244 [5, 8]. However, conflicting results have been reported from *in vivo* studies as tubular cells that have
245 undergone partial EMT relay proinflammatory and profibrogenic signals to the interstitium without
246 directly contributing to the myofibroblast population [24]. Accordingly, the relationship between
247 LOXL2 and the TGF- β pathway *in vivo*, and the TGF- β -mediated relationship between LOXL2 and
248 EMT *in vitro*, were investigated in this study. Thus, canonical pathway-related molecules were studied
249 *in vivo* and markers expressed by tubular cells during EMT were studied *in vitro*. The lack of significant

250 differences in the level of Smad molecules after LOXL2 inhibition in this study may be due to the lapse
251 of time between folic acid injection and analysis. Murine kidneys were harvested at 4 weeks after this
252 injury, by which time fibrogenesis could have already been completed. Stallons et al. reported that TGF-
253 β 1 and α -SMA levels increased until 6 days after folic acid injection, and gradually decreased afterwards
254 in a similar experiment where a 250 mg/kg dose of folic acid was injected intraperitoneally [16]. Tang
255 et al. reported that after the administration of high glucose doses, the expression of p-Smad2 and p-
256 Smad3 increased in HK-2 cells for 30 to 60 min and 30 to 120 min, respectively, before decreasing
257 gradually [25]. Our study differed from this experiment; in particular, the time between intervention
258 and injury was substantially longer than that in previous studies. A more rapid analysis of Smad
259 molecules after folic acid injection may have revealed a more pronounced change in their expression in
260 this study.

261 Experiments on HK-2 cells *in vitro* after TGF- β challenge revealed less increase in the
262 myofibroblast marker vimentin in LOXL2 knockdown cells. These data indicate that LOXL2 plays a
263 regulatory role in EMT. Other studies have shown no reduction in epithelial markers, such as E-cadherin,
264 with LOXL2 inhibition after TGF- β challenge, while a significant reduction of E-cadherin was observed
265 here. However, cell types and experimental methods used in this study differ from those reported
266 previously [12, 13, 26]. Although E-cadhrin and ZO-1 level remained insignificant, it can be inferred
267 that LOXL2 might be related to EMT pathway based on significant change of vimentin. Further studies
268 are warranted to elucidate the mechanisms underlying the LOXL2-EMT-related pathway.

269 In conclusion, inhibition of LOXL2 ameliorates renal fibrosis. LOXL2 is associated with TGF-
270 β -mediated tubulointerstitial fibrosis and EMT. Improved understanding of the role of LOXL2 in the
271 kidney may illuminate the pathophysiology of tubulointerstitial fibrosis and glomerulosclerosis, and
272 potentially lead to the discovery of novel therapeutic targets for treating these conditions.

273

274 **References**

275 1. Meran S, Steadman R. Fibroblasts and myofibroblasts in renal fibrosis. *Int J Exp Pathol.* 2011;92(3):158-67. Epub 2011/03/02. doi: 10.1111/j.1365-2613.2011.00764.x. PubMed PMID: 21355940; PubMed Central PMCID: PMCPMC3101489.

276 2. Liu Y. Cellular and molecular mechanisms of renal fibrosis. *Nature Reviews Nephrology.* 2011;7:684. doi: 10.1038/nrneph.2011.149.

277 3. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol.* 2014;15(3):178-96. Epub 2014/02/22. doi: 10.1038/nrm3758. PubMed PMID: 24556840; PubMed Central PMCID: PMCPMC4240281.

278 4. Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-beta: the master regulator of fibrosis. *Nat Rev Nephrol.* 2016;12(6):325-38. Epub 2016/04/26. doi: 10.1038/nrneph.2016.48. PubMed PMID: 27108839.

279 5. Yang J, Liu Y. Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. *Am J Pathol.* 2001;159(4):1465-75. Epub 2001/10/05. doi: 10.1016/S0002-9440(10)62533-3. PubMed PMID: 11583974; PubMed Central PMCID: PMCPMC1850509.

280 6. Friedman SL, Sheppard D, Duffield JS, Violette S. Therapy for fibrotic diseases: nearing the starting line. *Sci Transl Med.* 2013;5(167):167sr1. Epub 2013/01/11. doi: 10.1126/scitranslmed.3004700. PubMed PMID: 23303606.

281 7. Sutariya B, Jhonsa D, Saraf MN. TGF-beta: the connecting link between nephropathy and fibrosis. *Immunopharmacol Immunotoxicol.* 2016;38(1):39-49. Epub 2016/02/07. doi: 10.3109/08923973.2015.1127382. PubMed PMID: 26849902.

282 8. Hills CE, Squires PE. The role of TGF-beta and epithelial-to mesenchymal transition in diabetic nephropathy. *Cytokine Growth Factor Rev.* 2011;22(3):131-9. Epub 2011/07/16. doi: 10.1016/j.cytogfr.2011.06.002. PubMed PMID: 21757394.

283 9. Nishioka T, Eustace A, West C. Lysyl oxidase: from basic science to future cancer treatment. *Cell Struct Funct.* 2012;37(1):75-80. Epub 2012/03/29. PubMed PMID: 22453058.

284 10. Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. *Am J Pathol.* 2005;167(4):927-36. Epub 2005/09/30. doi: 10.1016/S0002-9440(10)61183-2. PubMed PMID: 16192629; PubMed Central PMCID: PMCPMC1603668.

285 11. Ahn SG, Dong SM, Oshima A, Kim WH, Lee HM, Lee SA, et al. LOXL2 expression is associated with invasiveness and negatively influences survival in breast cancer patients. *Breast Cancer Res Treat.* 2013;141(1):89-99. Epub 2013/08/13. doi: 10.1007/s10549-013-2662-3. PubMed PMID: 23933800.

286 12. Cuevas EP, Moreno-Bueno G, Canesin G, Santos V, Portillo F, Cano A. LOXL2 catalytically inactive mutants mediate epithelial-to-mesenchymal transition. *Biol Open.* 2014;3(2):129-37. Epub

312 2014/01/15. doi: 10.1242/bio.20146841. PubMed PMID: 24414204; PubMed Central PMCID: 313 PMCPMC3925316.

314 13. Cuevas EP, Eraso P, Mazon MJ, Santos V, Moreno-Bueno G, Cano A, et al. LOXL2 drives 315 epithelial-mesenchymal transition via activation of IRE1-XBP1 signalling pathway. *Sci Rep.* 316 2017;7:44988. Epub 2017/03/24. doi: 10.1038/srep44988. PubMed PMID: 28332555; PubMed Central 317 PMCID: PMCPMC5362953.

318 14. Choi SE, Jeon N, Choi HY, Shin JI, Jeong HJ, Lim BJ. Lysyl oxidaselike 2 is expressed in 319 kidney tissue and is associated with the progression of tubulointerstitial fibrosis. *Mol Med Rep.* 320 2017;16(3):2477-82. Epub 2017/07/06. doi: 10.3892/mmr.2017.6918. PubMed PMID: 28677767; 321 PubMed Central PMCID: PMCPMC5548064.

322 15. Long DA, Woolf AS, Suda T, Yuan HT. Increased renal angiopoietin-1 expression in folic 323 acid-induced nephrotoxicity in mice. *J Am Soc Nephrol.* 2001;12(12):2721-31. Epub 2001/12/01. 324 PubMed PMID: 11729241.

325 16. Stallons LJ, Whitaker RM, Schnellmann RG. Suppressed mitochondrial biogenesis in folic 326 acid-induced acute kidney injury and early fibrosis. *Toxicol Lett.* 2014;224(3):326-32. Epub 327 2013/11/28. doi: 10.1016/j.toxlet.2013.11.014. PubMed PMID: 24275386; PubMed Central PMCID: 328 PMCPMC3987699.

329 17. Yen CL, Li YJ, Wu HH, Weng CH, Lee CC, Chen YC, et al. Stimulation of transforming growth 330 factor-beta-1 and contact with type I collagen cooperatively facilitate irreversible transdifferentiation 331 in proximal tubular cells. *Biomed J.* 2016;39(1):39-49. Epub 2016/04/24. doi: 10.1016/j.bj.2015.08.004. 332 PubMed PMID: 27105597; PubMed Central PMCID: PMCPMC6138427.

333 18. Van Bergen T, Marshall D, Van de Veire S, Vandewalle E, Moons L, Herman J, et al. The role 334 of LOX and LOXL2 in scar formation after glaucoma surgery. *Invest Ophthalmol Vis Sci.* 335 2013;54(8):5788-96. Epub 2013/07/04. doi: 10.1167/iovs.13-11696. PubMed PMID: 23821193.

336 19. Barry-Hamilton V, Spangler R, Marshall D, McCauley S, Rodriguez HM, Oyasu M, et al. 337 Allosteric inhibition of lysyl oxidase-like-2 impedes the development of a pathologic 338 microenvironment. *Nat Med.* 2010;16(9):1009-17. Epub 2010/09/08. doi: 10.1038/nm.2208. PubMed 339 PMID: 20818376.

340 20. Meissner EG, McLaughlin M, Matthews L, Gharib AM, Wood BJ, Levy E, et al. Sintuzumab 341 treatment of advanced liver fibrosis in HIV and HCV-infected adults: results of a 6-month open- 342 label safety trial. *Liver Int.* 2016;36(12):1783-92. Epub 2016/05/28. doi: 10.1111/liv.13177. PubMed 343 PMID: 27232579; PubMed Central PMCID: PMCPMC5116256.

344 21. Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. *J Am Soc Nephrol.* 345 2002;13(10):2600-10. Epub 2002/09/20. doi: 10.1097/01.asn.0000033611.79556.ae. PubMed PMID: 346 12239251.

347 22. Pohlers D, Brenmoehl J, Loffler I, Muller CK, Leipner C, Schultze-Mosgau S, et al. TGF-beta 348 and fibrosis in different organs - molecular pathway imprints. *Biochim Biophys Acta.*

349 2009;1792(8):746-56. Epub 2009/06/23. doi: 10.1016/j.bbadi.2009.06.004. PubMed PMID: 19539753.
350 23. Cheng M, Liu F, Peng Y, Chen J, Chen G, Xiao L, et al. Construction of a CTGF and RFP-
351 coexpressed renal tubular epithelial cell and its application on evaluation of CTGF-specific siRNAs
352 on epithelial-mesenchymal transition. *Urology*. 2014;83(6):1443 e1-8. Epub 2014/04/08. doi:
353 10.1016/j.urology.2013.12.053. PubMed PMID: 24703458.
354 24. Grande MT, Sanchez-Laorden B, Lopez-Blau C, De Frutos CA, Boutet A, Arevalo M, et al.
355 Snail1-induced partial epithelial-to-mesenchymal transition drives renal fibrosis in mice and can be
356 targeted to reverse established disease. *Nat Med*. 2015;21(9):989-97. Epub 2015/08/04. doi:
357 10.1038/nm.3901. PubMed PMID: 26236989.
358 25. Tang WB, Ling GH, Sun L, Zhang K, Zhu X, Zhou X, et al. Smad Anchor for Receptor
359 Activation Regulates High Glucose-Induced EMT via Modulation of Smad2 and Smad3 Activities in
360 Renal Tubular Epithelial Cells. *Nephron*. 2015;130(3):213-20. Epub 2015/07/15. doi:
361 10.1159/000431105. PubMed PMID: 26159183.
362 26. Peinado H, Del Carmen Iglesias-de la Cruz M, Olmeda D, Csiszar K, Fong KS, Vega S, et al.
363 A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. *EMBO J*.
364 2005;24(19):3446-58. Epub 2005/08/13. doi: 10.1038/sj.emboj.7600781. PubMed PMID: 16096638;
365 PubMed Central PMCID: PMC1276164.

366

367 **Supporting information**

368 **S1 Fig. Transfection of HK-2 cells with LOXL2 shRNA resulted in LOXL2 knockdown.**

369 HK-2 cells were transfected with either LOXL2 shRNA lentivirus to knockdown LOXL2 expression,
370 or control shRNA virus. After 24hours of serum starvation, both the LOXL2- deficient cells and control
371 cells were incubated with either TGF- β or vehicle for 72 hours. Whereas LOXL2 increased in control
372 HK-2 cells after TGF- β challenge, LOXL2-deficient cells showed no significant difference in LOXL2
373 level. *p<0.05. LOXL2(+), HK-2 cells transfected with control shRNA; LOXL2(-), LOXL2-deficient
374 HK-2 cells by LOXL2 shRNA transfection; LOXL2, lysyl oxidase-like 2.

375

Urine → Neutrophil gelatinase-associated lipocalin

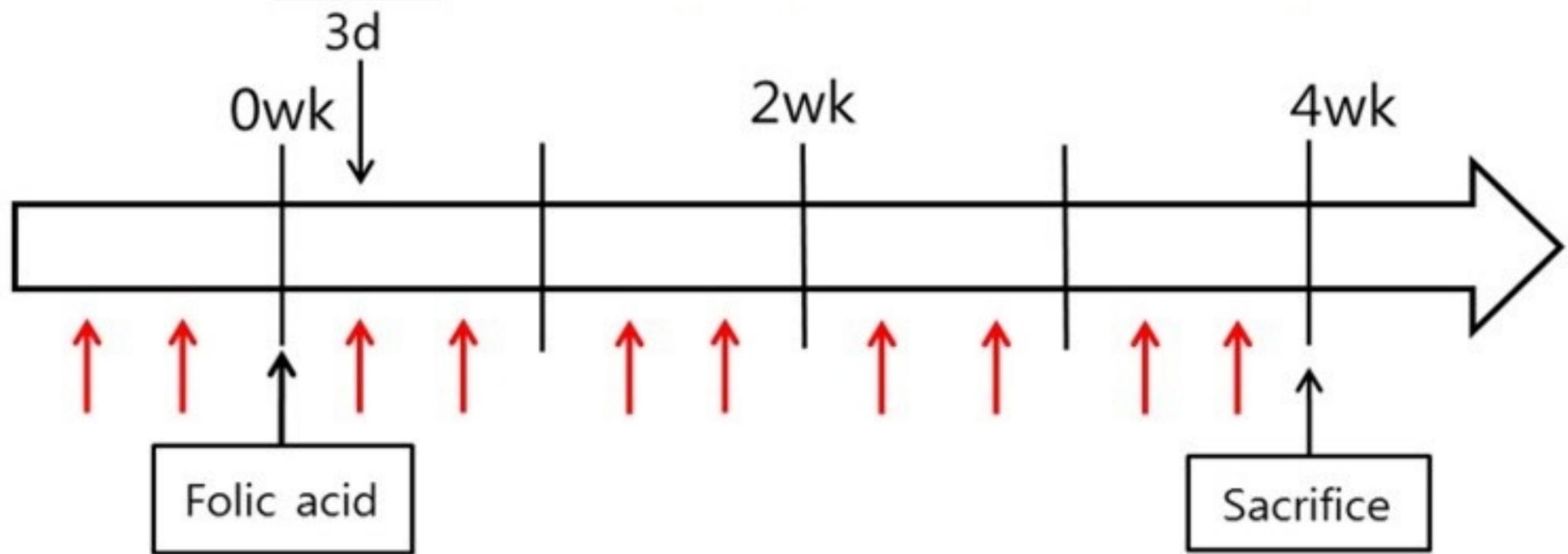
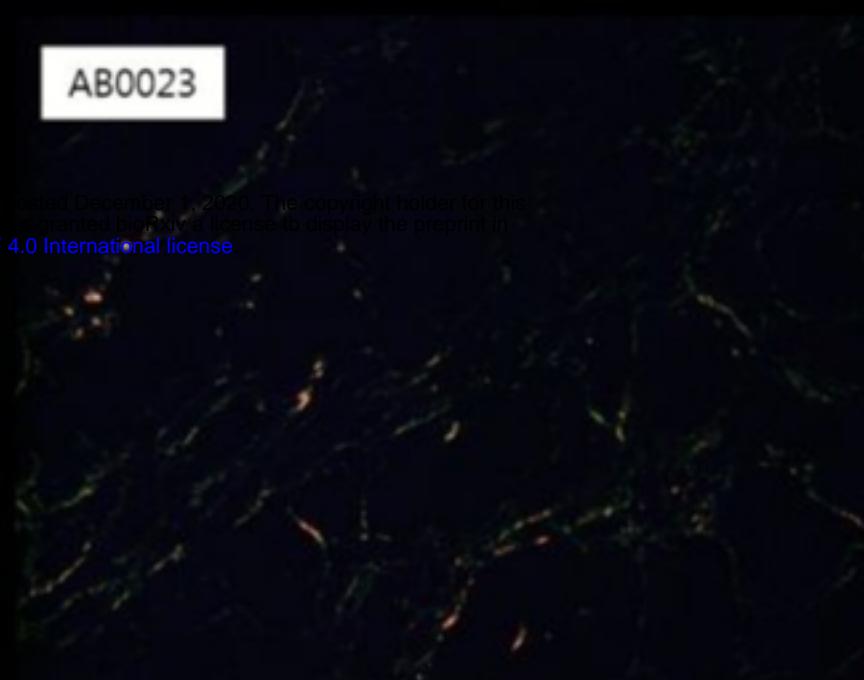
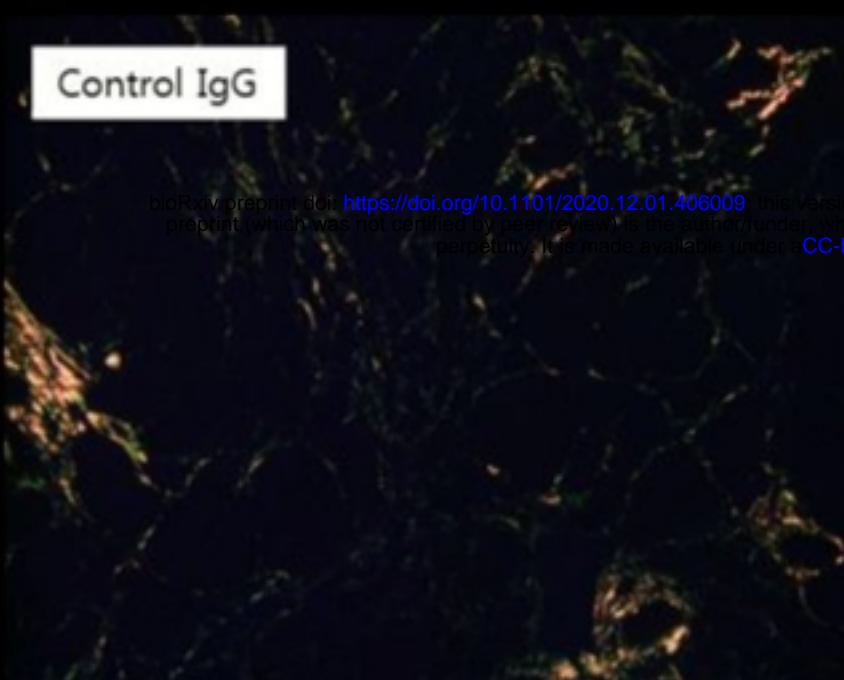
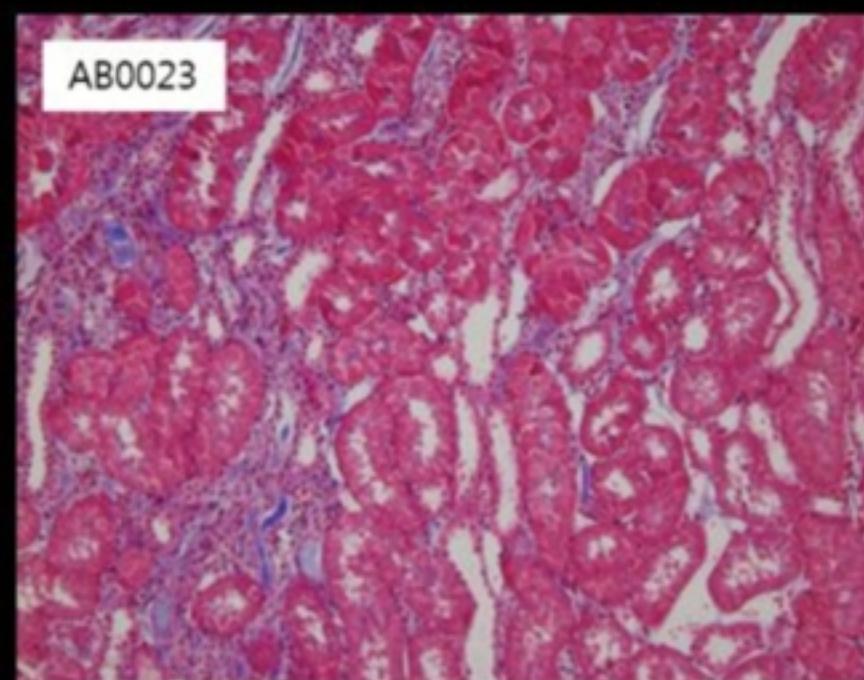
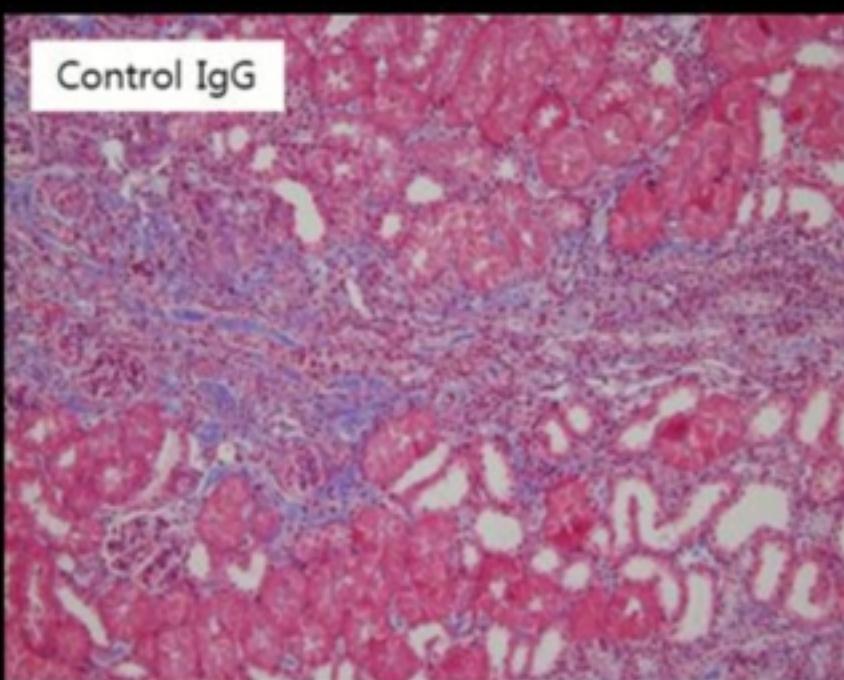


Fig 1



bioRxiv preprint doi: <https://doi.org/10.1101/2020.12.01.406009>; this version posted December 1, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

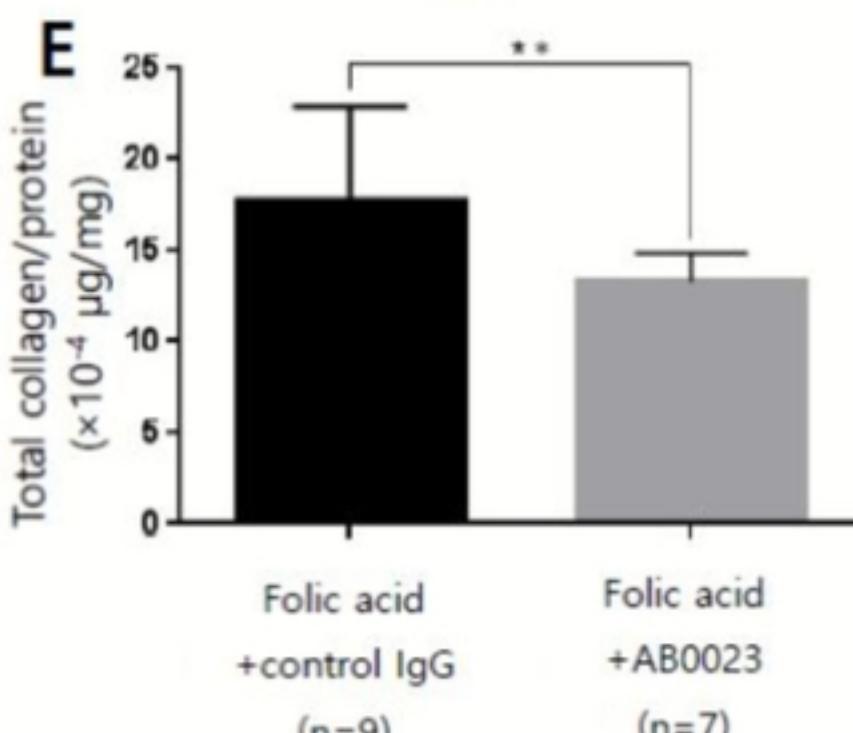
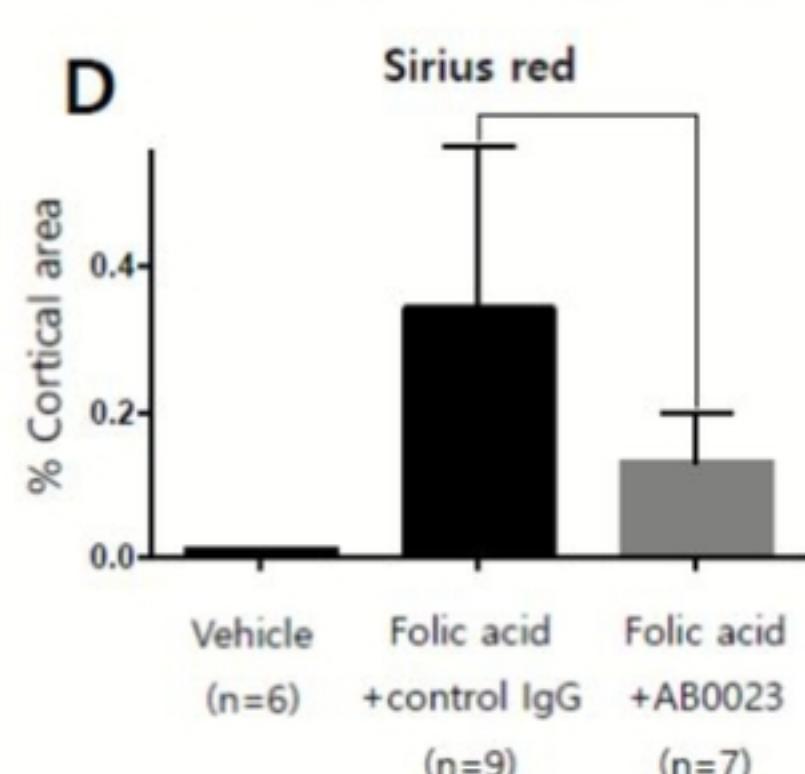
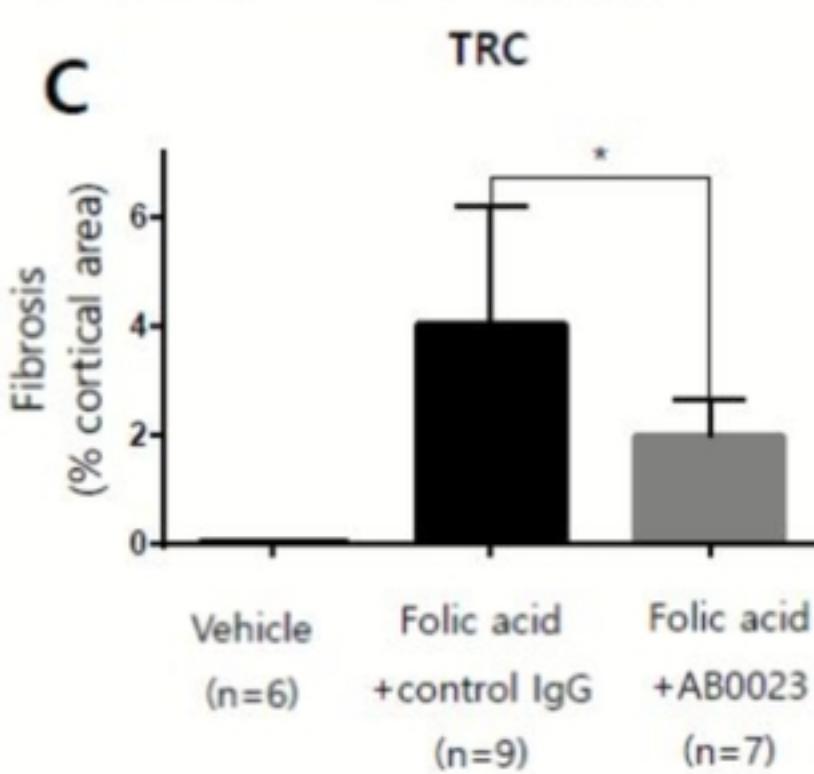


Fig 2

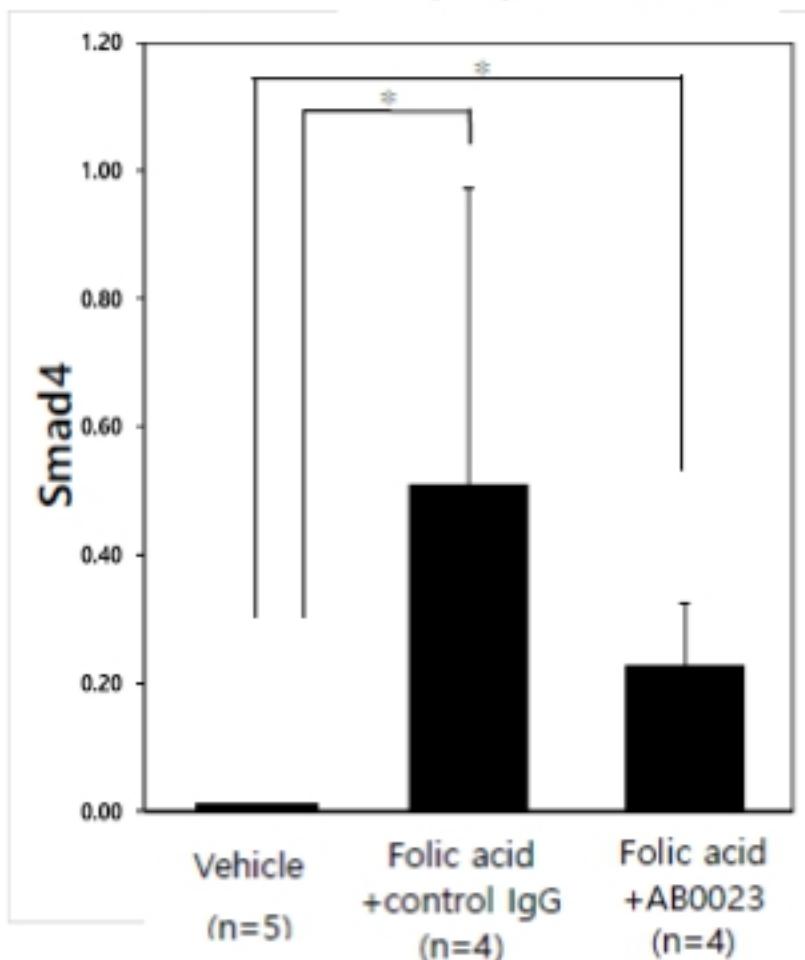
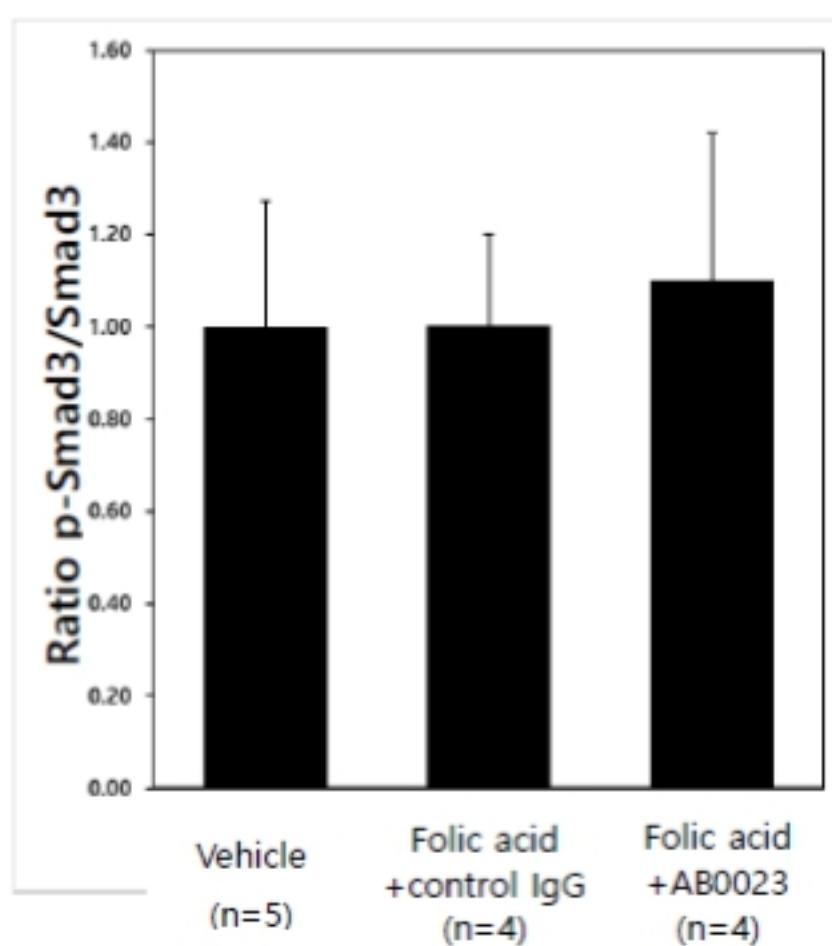
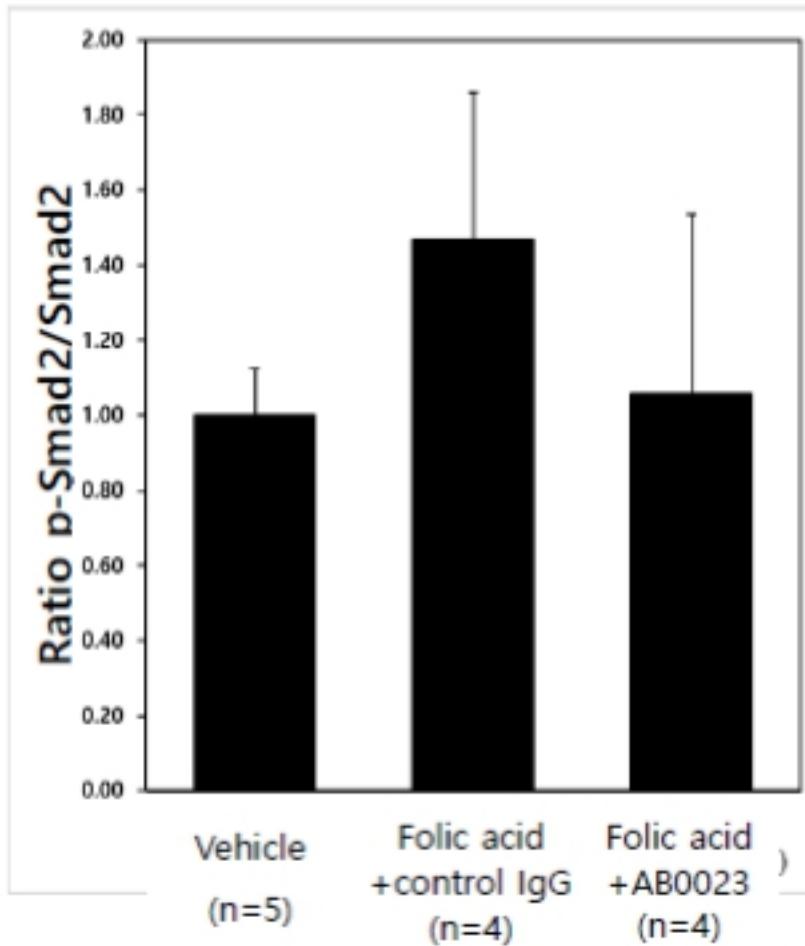
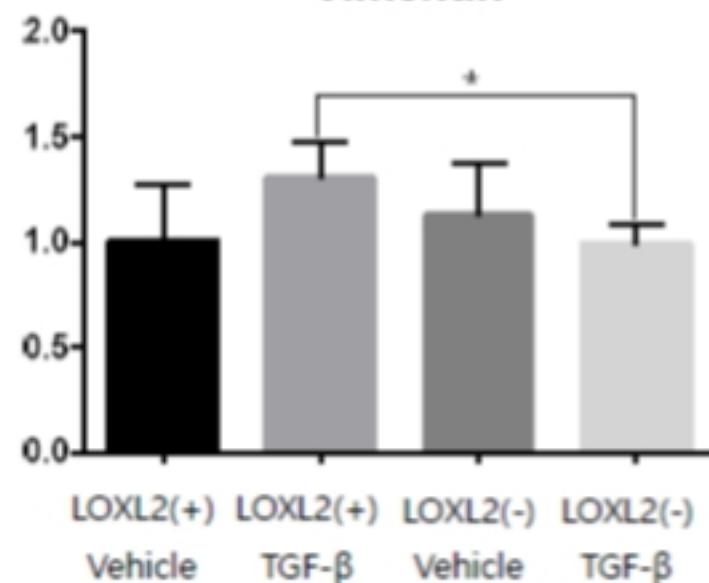
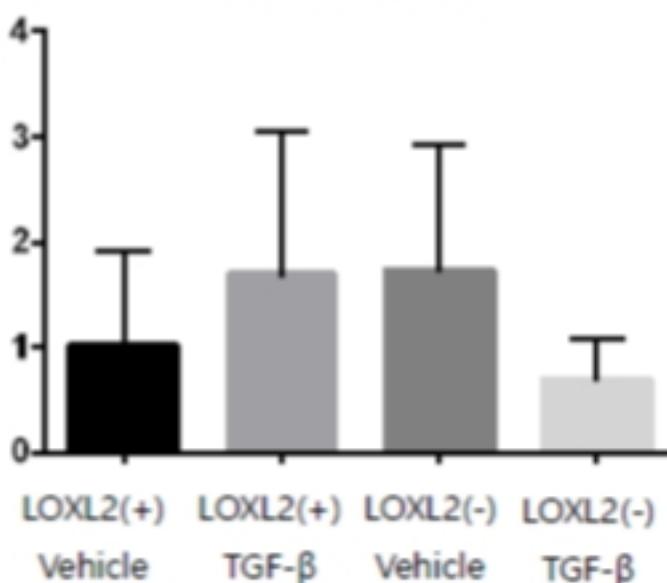
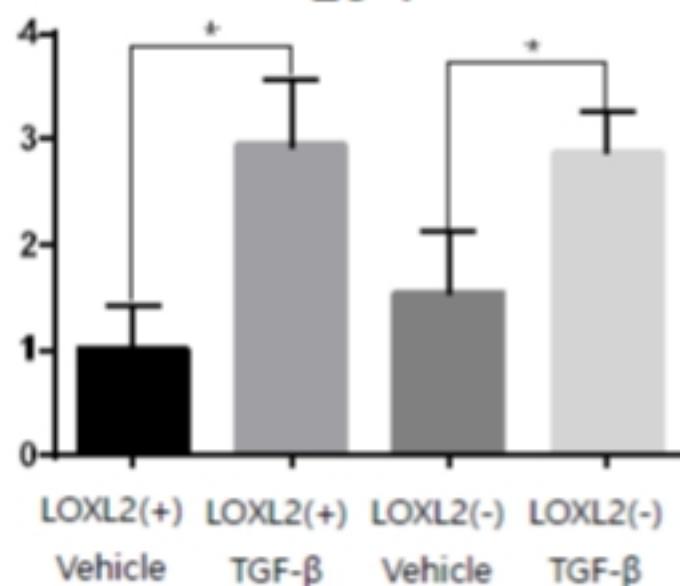
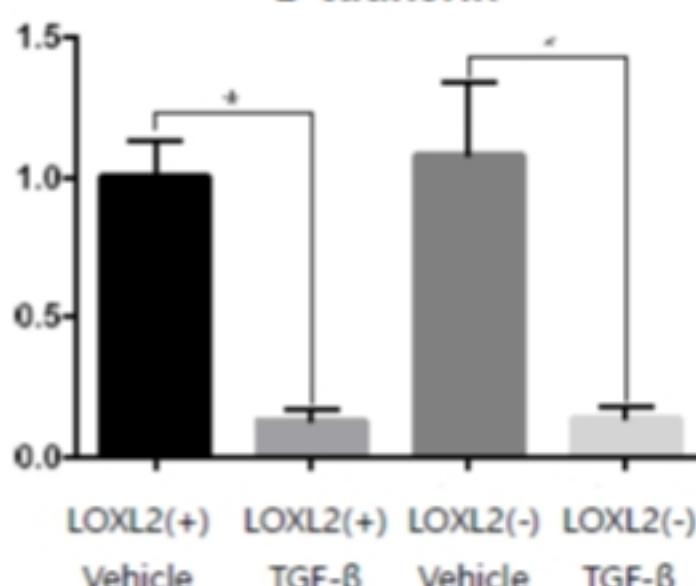


Fig 3

Vimentin**Fibronectin****ZO-1****E-cadherin****Fig 4**