



Farnesoid X receptor activation protects against renal fibrosis via modulation of β -catenin signaling

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ABSTRACT

Objective: Activation of farnesoid X receptor (FXR), a bile acid nuclear receptor, may be implicated in the pathophysiology of diabetic nephropathy. We explored a possible role for FXR activation in preventing renal fibrosis in high fat diet (HFD)-fed mice.

Methods: We investigated the effects of HFD on mouse kidney and renal tubular epithelial cells both *in vivo* and *in vitro*, and observed the changes of FXR and β -catenin pathway. FXR agonist was also used to alleviate this HFD-induced effect, and the interaction between FXR and β -catenin was further verified.

Results: Mice were fed by a 60% kcal fat diet for 20 weeks developed the typical traits of metabolic syndrome with subsequent renal lipid accumulation and renal injury. Treatment with the FXR agonist CDCA or GW4064 decreased body weight, renal lipid accumulation, as well as renal injury. Moreover, renal β -catenin signaling was activated and improved with FXR-agonist treatment in HFD-fed mice. To examine whether FXR affected β -catenin signaling, and was involved in tubulo-interstitial fibrosis, we explored the FXR expression and function in ox-LDL induced-renal tubular injury. In rat proximal tubular epithelial cells (NRK-52E) stimulated by ox-LDL, FXR protein was decreased compared to control group, and phosphorylated (Ser675) β -catenin was activated by ox-LDL in a dose- and time-dependent manner. Ox-LDL enhanced α -SMA and fibronectin expressions and reduced E-cadherin levels, whereas FXR agonism or FXR overexpression inhibited fibronectin and α -SMA expressions and restored E-cadherin. Moreover, FXR agonist treatment also decreased phosphorylated (Ser675) β -catenin, nuclear translocation and β -catenin-mediated transcription induced by ox-LDL in NRK-52E cells. We showed that FXR could bind with β -catenin via the AF1 domain, and disrupt the assembly of the core β -catenin/TCF4 complex.

Conclusion: These experimental data suggest that FXR activation, via modulating β -catenin signaling, may contribute to attenuating the development of lipid-mediated tubulo-interstitial fibrosis.

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Keywords Bile acid; FXR; β -Catenin; Tubular epithelial cells; Renal fibrosis

1. INTRODUCTION

Experimental evidence suggests that dyslipidemia may induce lipotoxicity, low-grade inflammation, and increased oxidative stress in renal tissues [1–4], thereby contributing to the development of both glomerulosclerosis and tubulo-interstitial fibrosis [5]. Dyslipidemia is

not only a cause of kidney injury, but also a consequence of chronic kidney disease (CKD), especially in nephrotic syndrome [6]. Consequently, treatment of lipid-mediated kidney injury is clinically important.

CKD causes major alterations in plasma lipoprotein profile, by inducing high levels of triglycerides, very low-density lipoprotein (VLDL) and

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oxidized low-density lipoprotein (ox-LDL), as well as low levels of high-density lipoprotein cholesterol [7]. Increased ox-LDL levels have been shown to cause renal injury through multiple mechanisms. Previous experimental studies have focused not only on the role of macrophage cells and endothelial dysfunction, but also on the role of ox-LDL in promoting glomerular sclerosis and tubulo-interstitial fibrosis [8,9]. It has been reported that ox-LDL may induce tubulo-interstitial fibrosis possibly through lysyl oxidase (LOX)-1-related oxidative stress via the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways [10].

Renal tubulo-interstitial fibrosis is typically characterized by accumulation of extracellular matrix (ECM) in the interstitial space, thus leading to destruction of renal parenchyma [11,12]. Studies showed that the tubular epithelial-to-mesenchymal transition is a fundamental process for driving renal fibrosis development [11]. Previous studies also showed that the β -catenin pathway is an evolutionarily conserved, developmental signaling pathway that regulates multiple biological processes. Among these, the activation of β -catenin signaling may induce tubular epithelial–mesenchymal transition and kidney fibrosis after injury [12].

Specifically, once initiated by its upstream activator, β -catenin is stabilized and binds to the T cell factor/lymphoid enhancer-binding factor to trigger transcription of β -catenin target genes, which are involved in regulating cell proliferation, stem cell maintenance, or targeting genes associated tubular epithelial–mesenchymal transition and fibrogenesis, such as fibronectin, E-cadherin and Snail [13]. In addition, it is also possible to hypothesize that inhibiting the activity of β -catenin signaling could become a new therapeutic option to delay CKD progression. However, to our knowledge, there is currently limited research on the β -catenin signaling pathway and the mechanisms of ox-LDL-induced injury in renal tubular epithelial cells, and it remains still unclear whether treatment can reduce this ox-LDL-induced kidney injury.

The farnesoid X receptor (FXR) is a nuclear bile acid receptor and transcription factor playing a key role in bile acid homeostasis, and lipid and glucose metabolism, with chenodeoxycholic acid (CDCA) being the most potent endogenous ligand. In addition, GW4064 is a highly effective and selective nonsteroidal agonist of FXR, which is usually used as a tool compound for investigating the physiological functions of FXR. Furthermore, obeticholic acid (OCA) is a semisynthetic derivative of CDCA, also known as INT-747, which was developed for the treatment of various liver diseases, including biliary atresia and non-alcoholic steatohepatitis [14,15]. FXR is mostly expressed in the liver and intestine, as well as widely in non-classical bile acid target tissues such as the kidneys [16]. However, it is becoming increasingly clear that FXR can also play a role in renal physiology and renal diseases [17]. FXR may be involved in the progression of many kidney diseases, including acute kidney injury and diabetic nephropathy, by regulating fatty acid oxidation, inflammation, apoptosis, fibrosis and other signaling pathways [18–20]. Our previous study also reported a possible molecular mechanism of FXR's involvement in lipid-induced kidney injury [4]. Experimentally, it has been reported that FXR activation reduces markers of extracellular matrix, i.e., TGF- β , Smad3, but the precise mechanisms for these effects are poorly known [21,22]. Furthermore, it has been reported that FXR is associated with β -catenin and may inhibit its activity in hepatocellular carcinoma [23]. Apart from its role in hepatocellular carcinoma development, whether FXR activation may also improve ox-LDL-induced kidney fibrosis, possibly through the β -catenin signaling pathway, is currently not known.

To test this experimental hypothesis, different strategies of FXR activation (either via GW4064, chenodeoxycholic acid [CDCA] and INT-747

[OCA], or via a FXR plasmid) were used both in proximal tubular epithelial cells (NRK-52E) and in high-fat diet (HFD)-fed mice.

2. MATERIALS AND METHODS

2.1. Cell culture and treatment

The NRK-52E cells were purchased from the China Center for Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE healthcare) with 5% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/mL penicillin/streptomycin (NCM Biotech, Suzhou, China) in a 5% CO₂ atmosphere at 37 °C. GW4064 (Cat. No. S2782, Selleck Chemicals LLC, Houston, USA) and CDCA (Cat. No. HY-76847, MedChemexpress, Monmouth Junction, USA) were dissolved in dimethyl sulfoxide (DMSO) and kept the final DMSO concentration below 0.1%. For lipid treatment, the NRK-52E cells were seeded at ~70% confluence in complete medium with 5% FBS and then serum-starved for 12 h, followed by ox-LDL treatment (Yiyuan Biotechnology, Guangzhou, China) for various times. For some experiments, after serum starvation, cells were either treated with FXR agonists or ICG-001 (β -catenin inhibitor, Cat. No. S2662, Selleck Chemicals LLC, Houston, USA) for another 24 h.

2.2. Plasmid transfection and luciferase assay

The construct pCMV3-C-flag- β -catenin (p β -catenin) was purchased from SinoBiological (Beijing, China). The plasmids of pcDNA3 and pcDNA3-flag-FXR (pFXR) were purchased from Zoonbio Biotechnology (Nanjing, China). The coding region of Rattus FXR (GI:11120689) gene was amplified and cloned into pcDNA3-flag vector. All plasmids were identified by sequencing. We also constructed the negative control siRNA, siRNA oligo duplexes targeting FXR and β -catenin (Tsingke Biotechnology Co., Ltd) for transient knocking down gene expression using Lipofectamine 8000.

For transfection, NRK-52E cells were transfected with pFXR and p β -catenin construct or FXR siRNA with or without β -catenin siRNA using lipofectamine 8000 (Invitrogen; Thermo Fisher Scientific, Inc.) and Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.). The pcDNA3 construct (Invitrogen) or control siRNA was transfected as a vehicle control. Dual luciferase reporter assay was performed using a dual-luciferase reporter assay system kit (Promega) according to the instruction manuals. The β -catenin transcriptional activity was determined using the TOPflash reporter system, which contains the thymidine kinase (TK) minimal promoter and two sets of triplicate TCF binding sites upstream of the luciferase open reading frame (Millipore). NRK-52E cells were co-transfected with the TOPflash plasmid (1 μ g) and the FXR-expression vector or the empty vector pcDNA3 or p β -catenin or FXR siRNA or β -catenin siRNA, and together with the internal control reporter plasmid (*Renilla reniformis* luciferase, 0.1 μ g). After 6 h, ox-LDL was treated for 24 h. Then, the cells were lysed for luciferase assay.

2.3. Western blot analysis

RIPA lysis buffer (Beyotime Institute of Biotechnology) containing PMSF (Beyotime Institute of Biotechnology) and phosphatase inhibitors (NCM Biotech) was used for cell lysis and protein extraction. Protein was determined by NanoDrop (Thermo Fisher Scientific, Inc.) and mixed with SDS-PAGE sample buffer and separated by SDS-PAGE. The following antibodies were used in the study: anti-E-cadherin (Cat. No. 14472; Cell signaling), anti-phospho- β -catenin (Ser675) (Cat. No. 9567; Cell signaling), anti-phospho- β -catenin (Ser 33/37/Thr41) (Cat. No. 9561; Cell signaling), anti- β -catenin (Cat. No. 610154; BD Transduction Laboratories, San Jose, CA), anti-TATA-binding protein

(TBP) (AB181-100, Abcam), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (abs132004; Absin Bioscience), anti-FXR (sc-25309, Santa Cruze; Cat No: 25055-1-AP, Proteintech), anti-Snail (ab17732, Abcam), anti-Fibronectin (ab2413, Abcam), and anti- α -SMA (A5228, Sigma Aldrich), respectively.

2.4. Nuclear and cytoplasmic fractionation

NRK-52E cells were washed twice with pre-chilled PBS, and nuclear proteins were extracted using nuclear and cytoplasmic extraction reagents according to the manufacturer's procedures (Thermo Scientific, Rockford, IL, USA). After centrifugation, the cell pellets were resuspended in buffer A and lysed with homogenizer. The cell nuclei were collected by centrifugation at 5000 rpm for 15 min and washed with buffer B. The nuclei were lysed in SDS sample buffer. TBP was used as loading control.

2.5. Co-immunoprecipitation

NRK-52E cells were washed twice with pre-chilled PBS and then lysed on ice by adding 1 mL of native lysis buffer (1% Triton X-100, 1% protease inhibitors cocktail and 1% phosphatase inhibitors cocktail I and II (NCM Biotech)). After pre-washing with IgG, cell lysates were incubated with 4 μ g anti- β -catenin or anti-FXR for 2 h at room temperature and then precipitated with 30 μ L protein A/G plus agarose beads. Precipitated complexes were analyzed by Western blotting for FXR and β -catenin detection.

2.6. Animal model

Twenty-four male C57 BL/6J mice (5 weeks, weight 20 g) were purchased from Beijing Vital River Laboratory Animal Technology Company. These mice were maintained in the animal center of Shanghai General Hospital under standard conditions (temperature: 24 °C; humidity: 40–70%; 12/12 h of light/dark cycle and air exchange). After 3 weeks, the mice were randomly divided into 4 groups: a 10% fat diet group (Control group, n = 6) (Cat. MD12031, medicience. Ltd); a 60% high fat diet (HFD) group (Cat. MD12033, medicience. Ltd) (n = 6); a HFD group with 0.1% CDCA (n = 6) (Cat. No. HY-76847, MedChemExpress) (Cat. MDDZ515, medicience. Ltd); and a 30 mg/kg INT-747 group (n = 6) (Dalian Meilun Biotechnology Co., Ltd) (Cat. MDDZ516, medicience. Ltd). A high fat diet (HFD) usually includes 60 kcal % fat, 20 kcal % protein, and 20 kcal % carbohydrate, respectively [24]. At 15 weeks of age, one mouse in the HFD group was found dead in a cage. The cause of death was uncertain, but the hair on the head and back of several mice in the cage was gone. The dead mouse was more seriously affected. We speculated that the mouse in the cage had been involved in a violent incident.

After a 12-week treatment, all mice were euthanized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) and samples were harvested for further determinations, including Oil Red O staining, immunohistochemistry and Western blot analyses. Urine specimens were collected on Monday morning of week 20. Urinary albumin-to-creatinine ratio (ACR) was measured using a fully automatic biochemical analyzer (Rayto Life and Analytical Sciences Co. Lta). Biochemical measurements including blood urea nitrogen (BUN), serum creatinine, albumin, alanine aminotransferase (ALT), total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-c) were measured using an automated analyzer (Abbott AxSYM), according to the standard laboratory methods. All mice were treated in accordance with animal protection guidelines and experiment procedures were approved by the guidelines of the ethics committee of the Shanghai General Hospital.

2.7. Immunofluorescence staining

Kidney cryosections (3 μ M) were fixed with 4% paraformaldehyde for 15 min, then blocked with blocking buffer for 1 h at room temperature followed by β -catenin (#37447S, Cell signaling) incubation for 10–12 h at 4 °C.

For the immunocytofluorescence, NRK-52E cells were seeded on coverslips, fixed with methanol and acetone (1:1) at –20 °C for 10 min, blocked with 20% normal donkey serum for 30 min at room temperature, and then mixed with anti-E-cadherin. Primary antibody incubation was made for fibronectin, α -SMA, FXR or β -catenin. Afterwards, NRK-52E cells were stained with Cy3-or Cy2-conjugated secondary antibodies for 30 min. Cells were also double-stained with 4',6-diamino-2-phenylindole-hydrochloric acid (DAPI, P0131, Beyotime, Shanghai, China) to visualize nuclei. Finally, cells were observed under a Leica fluorescence microscope.

2.8. Histology and immunohistochemical staining

Renal specimens were fixed overnight with 4% paraformaldehyde at 4 °C, then paraffin-embedded and 3-micron sections were prepared. Masson staining and Sirius red staining were performed according to manufacturer's instructions. In each group, each slice was randomly selected with at least three 100-fold visual fields to be photographed, and calculated the positive areas of Masson staining and Sirius red staining. For immunohistochemical staining, antigen retrieval was performed at 96 °C for 10 min in nitrate solution. Then, sections were blocked with 3% bovine serum albumin (Cat. No. A8020; Solarbio Science & Technology) for 30 min at room temperature, followed by anti- β -catenin, α -SMA or fibronectin Connexin primary antibody incubation overnight at 4 °C. Finally, slides were incubated with secondary antibody, washed and developed with DAB (Cat. No. K5007; Dako; Agilent Technologies).

2.9. Statistical analysis

All statistical analyses were performed using SPSS statistics version 22.0 (IBM SPSS Statistics. Armonk, NY, USA). One-way analysis of variance (1-way ANOVA) was used for multiple group comparisons. The Student-Newman-Keuls post-hoc test was used for post-hoc analyses. A p-value <0.05 was deemed to indicate statistical significance.

3. RESULTS

3.1. Ox-LDL inhibits FXR expression and induces β -catenin signaling in tubular epithelial cells

FXR protein expression was decreased in NRK-52E cells after 50 μ g/mL ox-LDL treatment, as confirmed by Western blot analyses (Figure 1A and B). Immunofluorescence staining showed nuclear localization of FXR in basal conditions, whereas the staining for FXR stimulated by ox-LDL decreased after 6 h (Figure 1C). Immunofluorescence staining also showed nuclear localization of β -catenin incubated with ox-LDL (50 μ g/mL) for 6 h, whereas β -catenin was located in cell–cell junction and cytoplasm under basal circumstance (Figure 1D). It has been reported that phosphorylation β -catenin at Ser33/37 promotes its degradation while phosphorylation at Ser675 induces β -catenin accumulation in the nucleus and increases its transcriptional activity [25]. Figure 1A and B showed that an ox-LDL-stimulated β -catenin activation in NRK-52E cells, as revealed by up-regulation of phosphorylated (Ser675) β -catenin and down-regulation of phosphorylated (Ser33/37) β -catenin in a dose and time dependent manner.

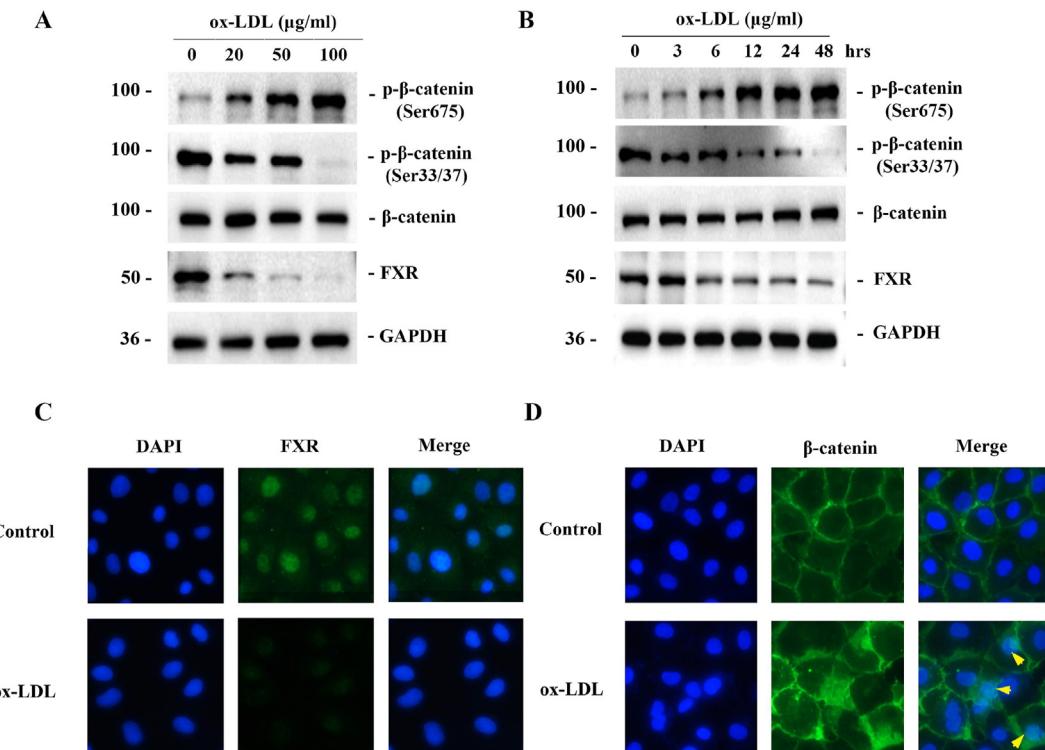


Figure 1: Ox-LDL decreases FXR protein expression and activates β -catenin signaling in tubular epithelial cells. (A and B) ox-LDL decreases FXR expression in NRK-52E cells. The NRK-52E cells were treated with 50 μ g/mL ox-LDL for 24 h and the cell lysates were immunoblotted with antibodies against FXR and GAPDH, respectively. NRK-52E cells were also treated either with various concentrations of ox-LDL for 24 h, or with 50 μ g/mL for various periods of time as indicated. Whole cell lysates were immunoblotted with specific antibodies against phosphor- β -catenin (Ser675, Ser33/37) and total β -catenin, respectively. (C) Immunofluorescence staining shows the FXR expression and localization in NRK-52E cells incubated with or without ox-LDL. (D) Immunofluorescence staining shows β -catenin expression in NRK-52E cells after treatment with or without 50 μ g/mL ox-LDL, for 6 h. Arrowheads denote nuclear location of β -catenin. Abbreviations: NRK-52E, rat proximal tubular epithelial cell; ox-LDL, oxidized low-density lipoprotein; FXR, farnesoid X receptor.

3.2. FXR activation improves ox-LDL-mediated tubulo-interstitial fibrosis

Figure 2A and B showed that ox-LDL (50 μ g/mL) induced the biomarkers of myofibroblasts (such as fibronectin, α -SMA and Snail), and suppressed the expression of E-cadherin after 24 h. Then, treatment with FXR agonists (either GW4064 or CDCA) attenuated the ox-LDL-induced expression of α -SMA and Snail at different dosages. Quantitative determination revealed a 1.3-fold induction of fibronectin protein stimulated by ox-LDL, then treatment with CDCA attenuated the induction in different dosages (Figure 2D). Moreover, for determining the function of FXR induction, FXR was overexpressed by transient transfection with the expression vector of flag-tagged FXR in NRK-52E cells. We found that FXR overexpression inhibited fibronectin expression and improved E-cadherin expression induced by ox-LDL (Figure 2C and E). Immunofluorescence staining showed that ox-LDL led to a weakening of E-cadherin expression in plasma membranes and an increase in fibronectin and α -SMA expression (Figure 2F–H). Treatment with FXR agonists (either CDCA or GW4064) also improved the ox-LDL-induced tubular fibrosis. These observations suggest that ox-LDL was able to trigger tubulo-interstitial fibrosis, but activation of FXR expression ameliorated the fibrosis in tubular epithelial cells *in vitro*.

In order to assess the requirement for inhibition of β -catenin signaling to decrease fibronectin induction by ox-LDL, we inhibited β -catenin function with the antagonist ICG-001. The NRK-52E cells were treated with ICG-001 (10 μ M), followed by ox-LDL incubation. As shown in Figure 3A and B, ICG-001 induced inhibition of β -catenin signaling and

attenuated 1.6-fold fibronectin production by ox-LDL, which was similar to the effect of inhibition of this pathway by FXR activation.

3.3. Activation of FXR expression represses ox-LDL-induced fibrosis in tubular epithelial cells via inhibiting β -catenin signaling

To determine the possibility that activation of FXR expression repressed ox-LDL-induced fibrosis in NRK-52E via inhibiting β -catenin signaling, we first investigated whether FXR activation inhibited the phosphorylation (Ser 675) of β -catenin. In these experiments, we found that FXR agonist inhibited the phosphorylation (Ser 675) of β -catenin in a dose-dependent manner (Figure 3C). Overexpression of FXR also inhibited the expression of phosphorylation (Ser 675) of β -catenin (Figure 3D). Moreover, we also investigated the intracellular distribution of β -catenin in tubular epithelial cells, by scrutinizing β -catenin subcellular localization incubated with ox-LDL after treatment with FXR agonists. The alteration of FXR activation did not affect cellular β -catenin levels, but ox-LDL promoted β -catenin translocation into the nuclei and GW4064 decreased β -catenin expression in the nuclei. (Figure 3E and F). We also explored the β -catenin-mediated transcriptional activity in a TOPflash luciferase reporter system, which demonstrated that exogenous FXR inhibited the 1.7-fold transcriptional activity induced by ox-LDL (Figure 3G). Then, NRK-52E cells were cotransfected with TOPflash reporter plasmid and either FXR siRNA with or without β -catenin siRNA, which confirmed that FXR siRNA could increase 1.3-fold β -catenin transcriptional activity; then, cotransfected with β -catenin siRNA, FXR siRNA did not increase the β -catenin transcriptional activity (Figure 3H–J). These data suggest that

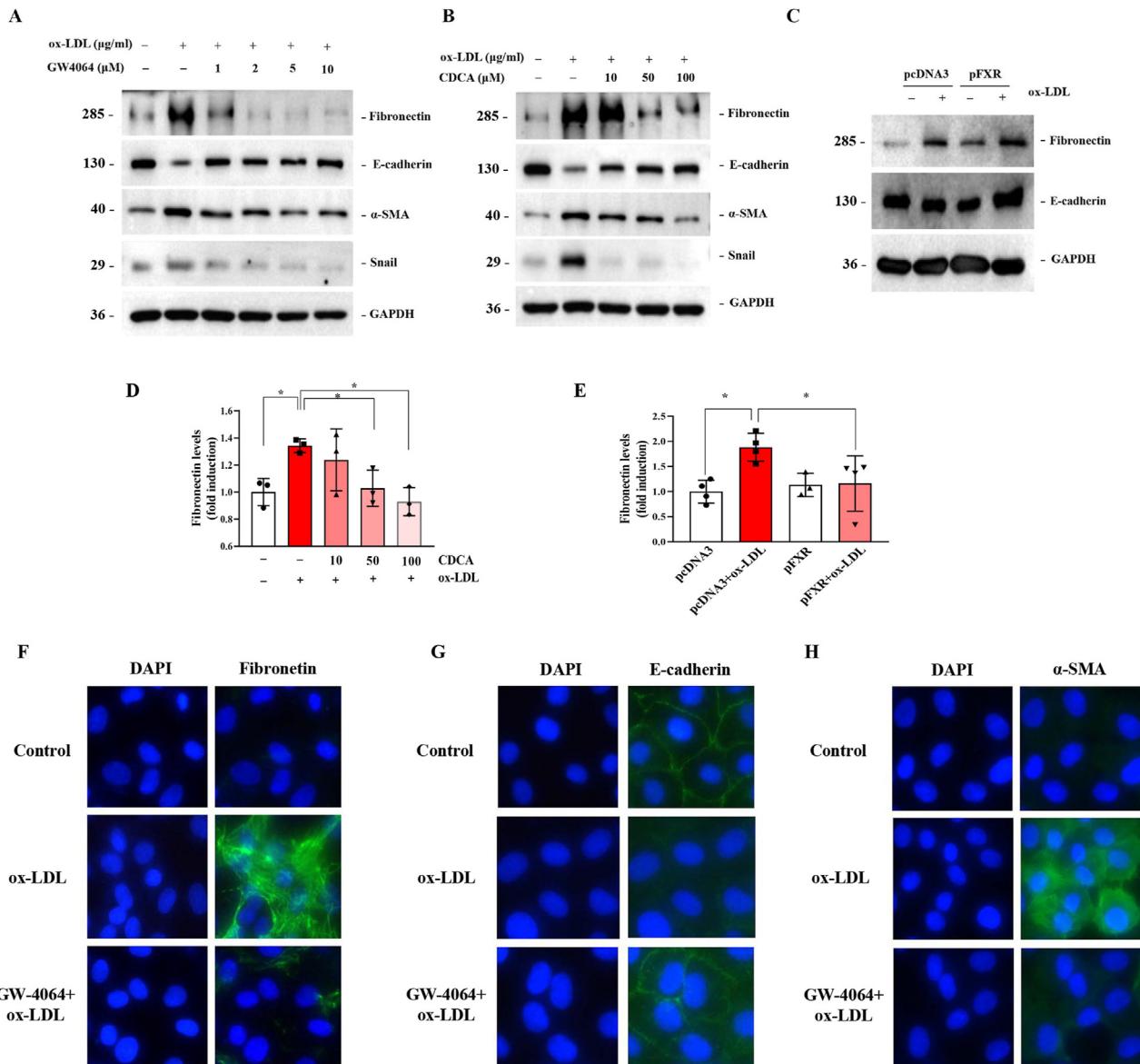


Figure 2: Treatment with FXR agonists or FXR overexpression suppress fibronectin, α -SMA and Snail and recover E-cadherin induced by ox-LDL. After pretreatment with different concentrations of GW4064 (A), chenodeoxycholic acid (CDCA) (B), or vehicle control DMSO for 30 min, NRK-52E cells were incubated with 50 μ g/mL ox-LDL for 24 h. The protein levels of fibronectin, α -SMA, Snail and E-cadherin were examined by Western blot analysis. (C) Overexpression of FXR in NRK-52E cells is confirmed by Western blotting with anti-Flag antibody. NRK-52E cells were transiently transfected with either the expression vector of Flag-tagged FXR (pFXR) or empty vector (pcDNA3). Ox-LDL induces fibronectin and suppresses E-cadherin in NRK-52E cells and treatment with forced expression of FXR suppresses fibronectin and increases E-cadherin expression. (D–E) The quantitative data of fibronectin level is presented. Values normalized to GAPDH and reported as mean \pm S.E.M. * p < 0.05. Immunofluorescence staining (F–H) shows that ox-LDL suppresses epithelial marker E-cadherin and induces fibronectin and α -SMA expression in NRK-52E cells. Then, treatment with GW4064 suppresses fibronectin and α -SMA expression and recovers E-cadherin.

FXR is a key factor for the stabilization and nuclear translocation of β -catenin mainly via modulating β -catenin phosphorylation. Since the nuclear translocation of β -catenin is a fundamental process for controlling its target gene transcription in the nucleus that causes tubular fibrosis, our results also suggest that activation of FXR expression can repress ox-LDL-mediated fibrosis in tubular epithelial cells mainly via inhibiting β -catenin signaling.

3.4. FXR/ β -catenin interaction is induced in ox-LDL stimulated tubular epithelial cells

Using immunofluorescent staining, we showed that FXR and β -catenin were co-localized in the nucleus in ox-LDL-stimulated NRK-52E cells

(Figure 4A). The interaction between FXR and β -catenin was confirmed by co-immunoprecipitation. In NRK-52E cells, we examined the interaction of FXR with β -catenin in the basal state, after treatment with GW4064, and also after treatment with GW4064 incubated with ox-LDL (Figure 4B and C). Expression of FXR, pDEL-FXR and p β -catenin were confirmed by Western blotting with anti-flag (Figure 4D and E). To explore the role of FXR interaction with β -catenin in ox-LDL-mediated β -catenin transcription activity, the indicated constructs were transfected and the TOPflash signal was determined (Figure 4F). Indeed, β -catenin overexpression restores the decrease in signaling caused by FXR under ox-LDL treatment. Notably, FXR overexpression is sufficient to reduce the TOPflash signal while this could be partially

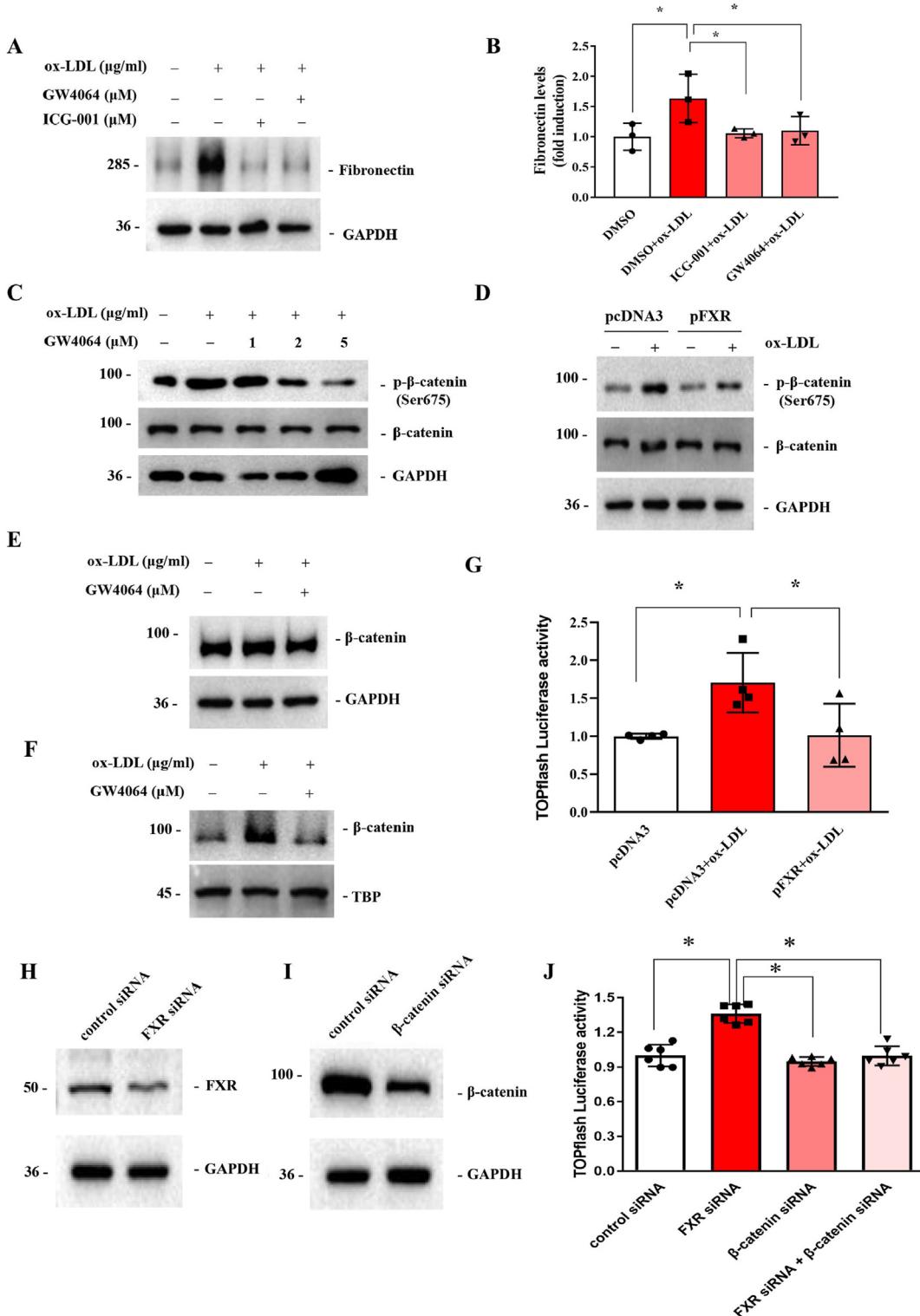


Figure 3: Overexpression of FXR inhibits activation of β-catenin signaling in tubular epithelial cells. (A–B) ICG-001 and GW4064 inhibit fibronectin expression induced by ox-LDL. NRK-52E cells were pretreated with ICG-001 10 μM or GW4064 5 μM for 30 min, then incubated with ox-LDL (50 μg/mL) for 24 h. (C) After pretreatment with various concentrations of GW4064 for 30 min, NRK-52E cells were incubated with 50 μg/mL ox-LDL for 24 h. The protein level of phosphor-β-catenin (Ser675) was examined by Western blot analysis. (D) Ectopic expression of FXR decreases phosphor-β-catenin (Ser675) induced by ox-LDL. (E–F) Ox-LDL promotes β-catenin translocation into the nuclei and GW4064 decreases β-catenin expression in the nuclei. (G) NRK-52E cells were co-transfected with TOPflash reporter plasmid and either pcDNA3 or pFXR vector, followed by incubation with or without 50 μg/mL ox-LDL. (H–I) Specific knockdown of FXR and β-catenin with siRNA oligo duplexes was confirmed by western blot in NRK-52E. (J) NRK-52E cells were co-transfected with TOPflash reporter plasmid and either control siRNA, FXR siRNA or β-catenin siRNA. Down-regulation of FXR in NRK-52E cells promotes β-catenin mediated transcriptional activity. Relative luciferase is reported ($n = 6$) and values shown are means \pm S.E.M, * $p < 0.05$.

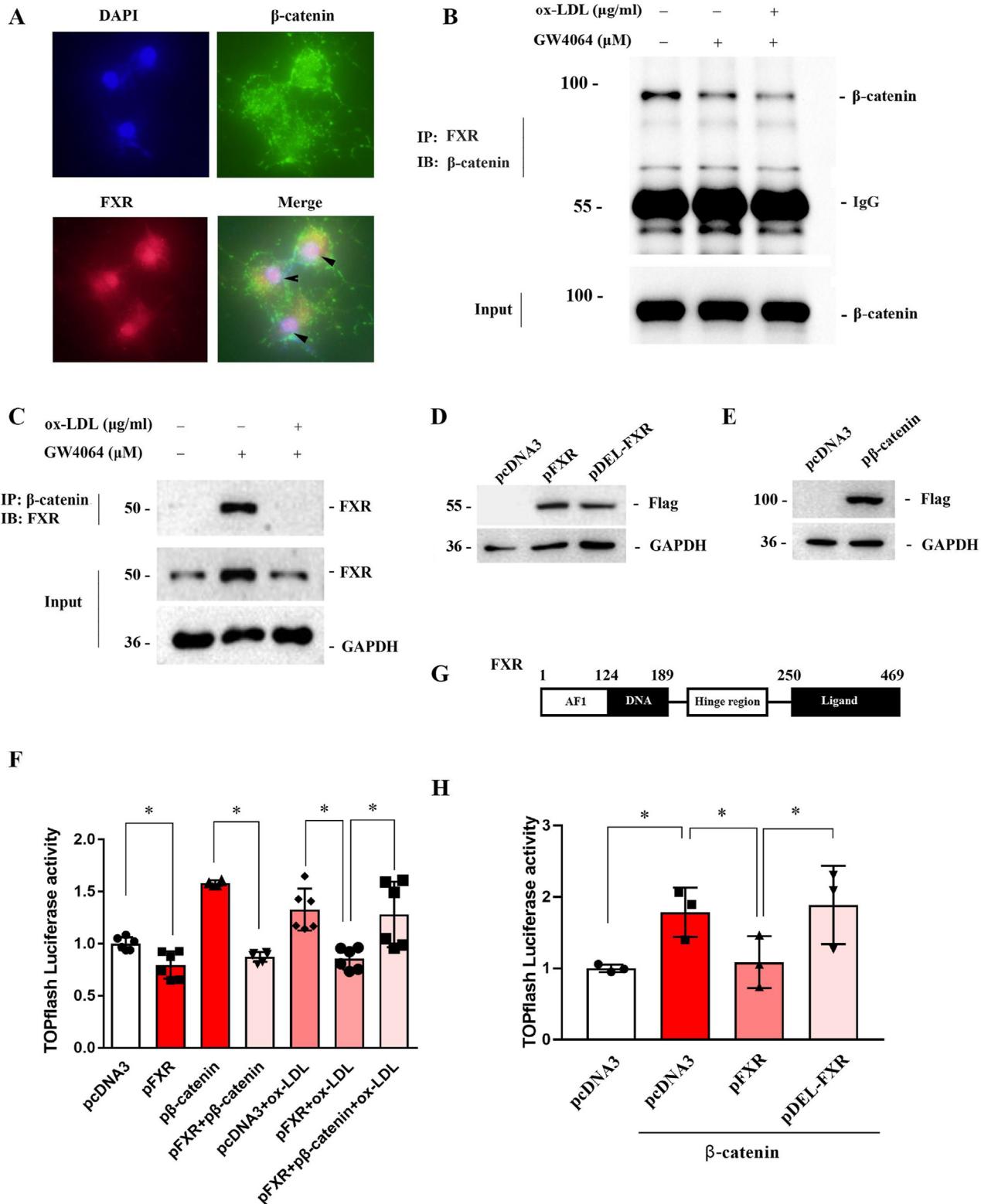


Figure 4: FXR/β-catenin interaction is induced in ox-LDL stimulated tubular epithelial cells. (A) Co-localization of FXR and β-catenin in NRK-52E cells. NRK-52E cells were double stained with anti-FXR antibody and β-catenin antibody. (B–C) Association of FXR with β-catenin was confirmed by *in vitro* co-immunoprecipitation. Immunoprecipitation was carried out in NRK-52E cell lysates using anti-FXR antibody or anti-β-catenin, followed by IgG agarose incubation. After washing, bound β-catenin or FXR was detected by Western blot. (D–E) Overexpression of pFXR, pDEL-FXR and pβ-catenin in NRK-52E cells were confirmed by Western blotting with anti-Flag antibody. (F) NRK-52E cells were cotransfected with TOPflash reporter plasmid and either pcDNA3, pFXR vector or pβ-catenin, followed by incubation with or without 50 μg/ml ox-LDL. (G) Diagram shows FXR functional domains, including the AF1 domain, the DNA binding domain (DBD), the hinge domain, the ligand binding domain (LBD), and the AF-2 domain. (H) The AF1 domain of FXR was required for suppression of β-catenin mediated transcriptional activity. NRK-52E cells were transfected with TOPflash reporter, β-catenin and pFXR, pDEL-FXR. Luciferase activity was measured using cell lysates 24 h after transfection. Relative luciferase is reported ($n = 3$) and values shown are means \pm S.E.M, * $p < 0.05$.

restored by β -catenin overexpression. These data indicate that FXR interacts with β -catenin, being implicated in the regulation of β -catenin transcription activity in ox-LDL treated conditions.

The FXR structure is characterized by a N-terminal AF1 domain, a DNA binding domain (DBD), a hinge domain, a ligand binding domain (LBD), and a C-terminal AF2 domain, respectively (Figure 4G). To identify the specific domain, which could mediate the interaction with β -catenin and is potentially involved in its inhibitory effect on β -catenin transcriptional activity, we constructed a Flag tagged full length FXR, a AF1 domain deletion mutant defined as pDEL-FXR (124–469), and p β -catenin pFXR and FXR deletion mutants were co-transfected with p β -catenin and TOPflash reporter in NRK-52E cells. We found that the full length FXR could inhibit β -catenin mediated transcription activity, while the pDEL-FXR without the N-terminal AF1 domain did not have any effect on β -catenin mediated TOPflash reporter activity (Figure 4H). These results indicate that the N-terminal AF1 domain in FXR is required for its inhibitory activity.

3.5. FXR activation protects against renal fibrosis in HFD mice

Compared to mice treated with control diet, HFD-fed mice had higher body weight (49.01 ± 1.89 g vs. 27.59 ± 1.62 g), higher total cholesterol (5.73 ± 0.65 vs. 2.55 ± 0.64 mmol/L), higher LDL-cholesterol (0.46 ± 0.11 vs. 0.19 ± 0.07 mmol/L), and had more severe renal injury, with a remarkable increase in albuminuria (37.1 ± 3.79 vs. 23.24 ± 4.77 mg/g), serum creatinine (4.8 ± 1.3 vs. 1.11 μ mol/L) and BUN levels (10.7 ± 1.81 vs. 7.5 ± 1.39 mmol/L). Treatment with CDCA or INT-747 improved albuminuria and decreased levels of body weight, serum ALT, BUN, creatinine and lipids (Figure 5). In addition, Oil red O staining showed that HFD-fed mice also had greater kidney lipid accumulation in the glomeruli and tubulo-interstitium, which was improved by treatment with either CDCA or INT-747 (Figure 6A).

Furthermore, we also examined the expression of β -catenin, fibronectin and α -SMA in the kidneys of HFD-fed mice treated by either CDCA or INT-747 in order to examine the FXR-mediated inhibition of β -catenin in renal fibrosis (Figure 6B–C). The Masson's staining and Sirius red staining revealed that treatment with either CDCA or INT-747 decreased tissue fibrosis in HFD-fed mice (Figure 6D–F). As shown in Figure 7A–C, we examined β -catenin protein expression and localization in 20-week mice. Immunohistochemical staining analysis showed that positive β -catenin staining was markedly increased in renal tubular cells of HFD-fed mice compared to the control mice, and β -catenin was predominantly localized in the nuclei of tubular cells in HFD-fed mice (Figure 7D–E, arrowheads). Western blot analysis also showed an increase in phosphorylated β -catenin (Ser675) expression (Figure 8A). Treatment with either CDCA or INT-747 decreased positive β -catenin levels in immunohistochemical staining, and the expression of phosphorylated β -catenin (Ser675) was decreased in CDCA or INT-747 treatment groups. In addition, immunohistochemical staining and Western blot analyses showed that FXR activation led to a reduction in fibronectin and α -SMA expressions. Compared to control mice, quantitative determination revealed a ~5-fold induction of fibronectin protein, ~4-fold induction of the relative abundance of phosphorylated β -catenin (Ser 675) protein, and 1.3-fold induction of α -SMA protein in HFD mice (Figure 8B–D). Moreover, treatment with FXR agonists can attenuate these protein inductions. These data indicated that FXR activation may protect

against renal fibrosis in HFD-fed mice possibly by inhibiting the β -catenin pathway.

4. DISCUSSION

Our experimental data show that ox-LDL represses the expression of the epithelia marker E-cadherin, and induces the expression of fibronectin and α -SMA, as markers of myofibroblasts. Conversely, FXR activation leads to induction of E-cadherin and repression of fibronectin and α -SMA levels stimulated by ox-LDL, thereby confirming that ox-LDL is able to trigger tubulo-interstitial fibrosis, but activation of FXR expression ameliorates the fibrosis in tubular epithelial cells *in vitro*. In addition, ox-LDL decreases FXR abundance in the nuclei and increases β -catenin phosphorylation and nuclear localization, thus activating the β -catenin signaling pathway. FXR activation inhibits the β -catenin phosphorylation and nuclear localization, thereby reducing the activation of β -catenin signaling and its target gene expression (Figure 8E–F). In a mouse model of HFD-induced nephropathy, we have also shown that treatment with FXR agonists reduces body weight, improves plasma lipid profile and albuminuria, and represses the expression of fibronectin, α -SMA and β -catenin phosphorylation. Overall, these results suggest that FXR may interact with β -catenin, regulating β -catenin signaling activity, and thereby participating in the development of renal fibrosis.

FXR is a ligand activated transcription factor that is involved in the regulation of bile acid metabolism [26]. It has been reported that FXR agonists may also improve proteinuria, as well as podocyte injury, mesangial expansion and tubulo-interstitial fibrosis in both diabetic DBA/2J and db/db mice [27]. However, the precise mechanisms by which FXR activation may improve renal fibrosis require better understanding. In our experimental study, we showed that FXR expression was downregulated in ox-LDL-mediated injury of tubular cells, and that FXR over-expression inhibited ox-LDL-mediated renal injury. Consistently, a previous study reported that FXR activation suppressed renal fibrosis in a unilateral ureteral occlusion mouse model via regulating Smad3 [28]. Another experimental study also reported that FXR activation suppressed the β -catenin-mediated activation of Wnt target genes, which are involved in spontaneous hepatocellular carcinoma [23]. Recently, one study also reported that FXR may exert a tumor-suppressor role in colorectal cancer by antagonizing β -catenin signaling [29].

To date, growing evidence supports a role of dysregulated lipid metabolism in the pathogenesis of renal disease [30–32]. Increased lipid metabolites may induce the release of multiple pro-inflammatory and pro-fibrotic cytokines and chemokines, and promote the expression of renal extracellular matrix, inflammation and fibrosis, thereby contributing to the development and progression of CKD [33]. Lipotoxicity may also adversely affect renal lipid metabolism, leading to increased production of proteins involved in lipid synthesis, while decreasing those involved in lipid oxidation. When excess free fatty acids are not oxidized, they are esterified with glycerol and become intra-renal lipid droplets, which may induce inflammation and cell apoptosis, thus further aggravating CKD [34].

Although FXR activation can exert important hepato-protective effects against various stress conditions [35,36], very few studies have examined the possible renal protective effects of FXR activation in HFD-fed mice. FXR activation has been reported to improve metabolic derangement and insulin resistance, all of which may reduce the risk of developing diabetic nephropathy [27]. In our experimental study, the FXR agonists CDCA or INT-747, both ameliorated lipid dysfunction and albuminuria in HFD-fed mice, associated with regulation of β -catenin

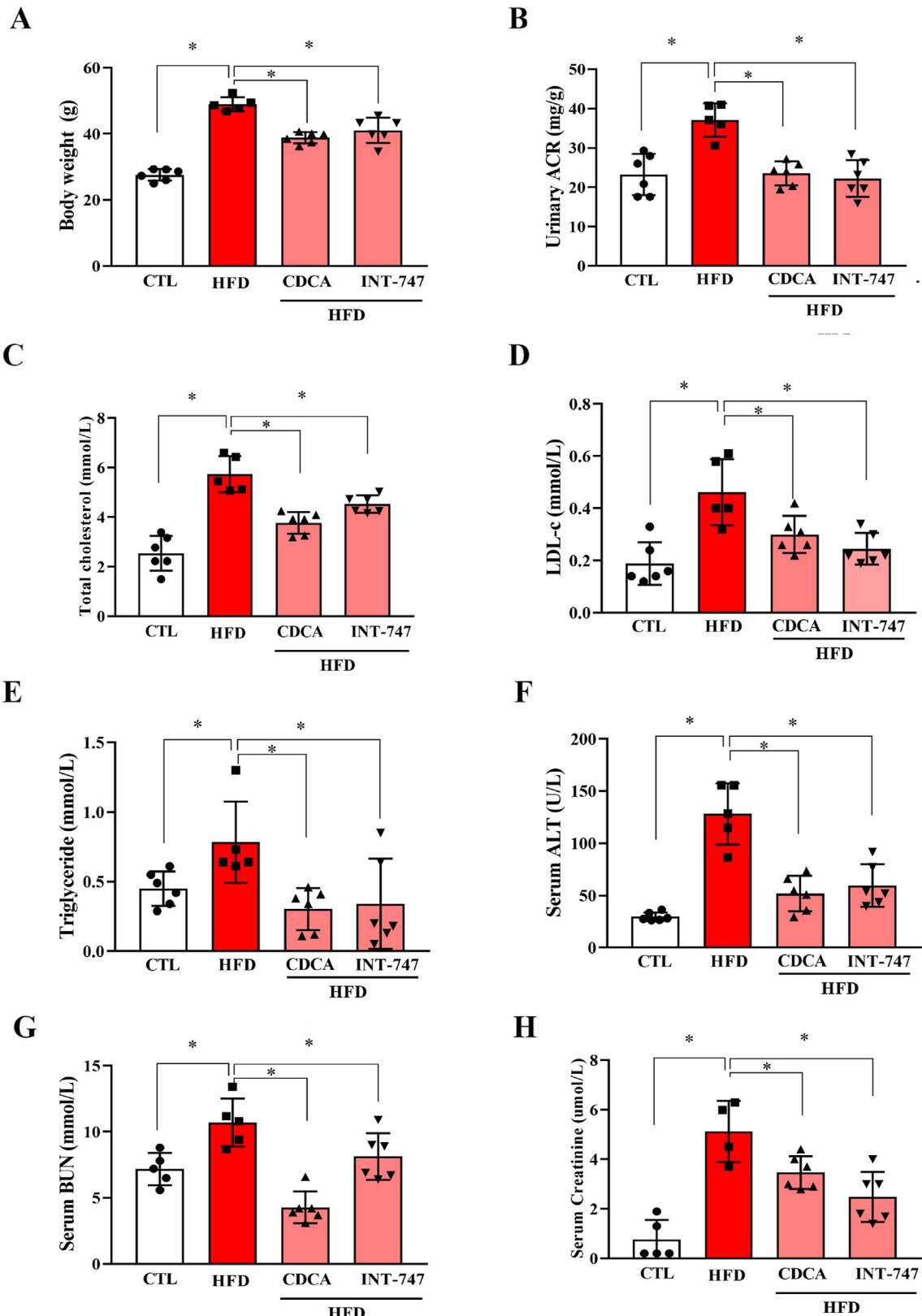


Figure 5: Characteristics of mice in the different groups after treatment. FXR agonist (CDCA or INT-747) treatment improves body weight (A), albuminuria, expressed as urinary albumin to creatinine ratio (ACR) (B), serum total cholesterol (C), LDL-c (D), triglycerides (E), ALT levels (F), BUN (G) and creatinine (H) at 20 weeks in 60% HFD-treated mice. Values shown are means \pm S.E.M., * $p < 0.05$.

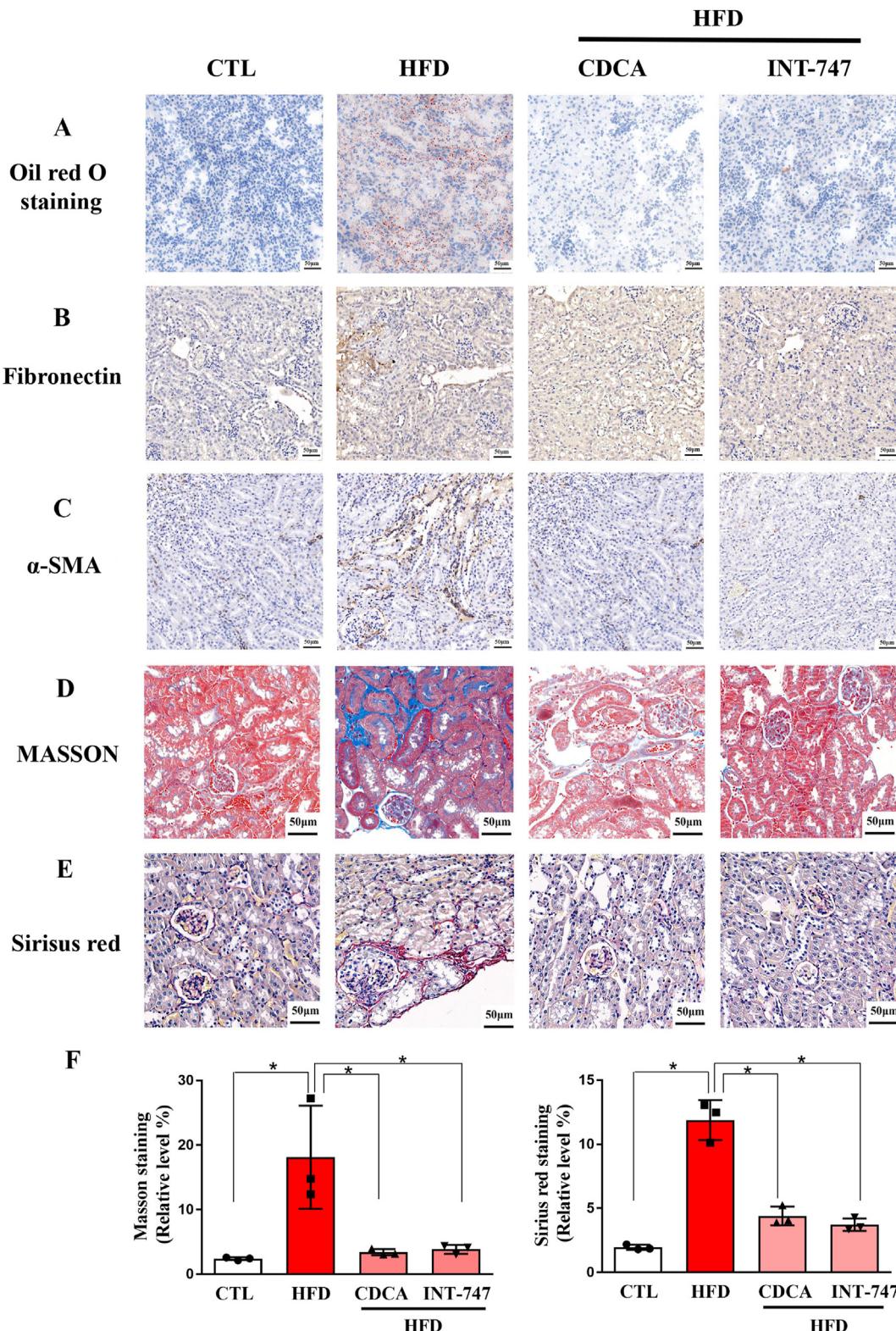


Figure 6: FXR activation prevents renal lipid accumulation and protects against renal fibrosis in HFD mice. (A) Oil red O staining of kidney sections. (B–C) Representative micrographs display the induction of localization of fibronectin and α -SMA in HFD mice. Kidneys from control, HFD, CDCA and INT-747 group for 20 weeks were stained immunohistochemically for fibronectin and α -SMA protein. Representative images of Masson staining (D) and Sirius red staining (E). The positive area of Masson and Sirius red were calculated (F) ($n = 5$). Values shown are means \pm S.E.M. * $p < 0.05$.

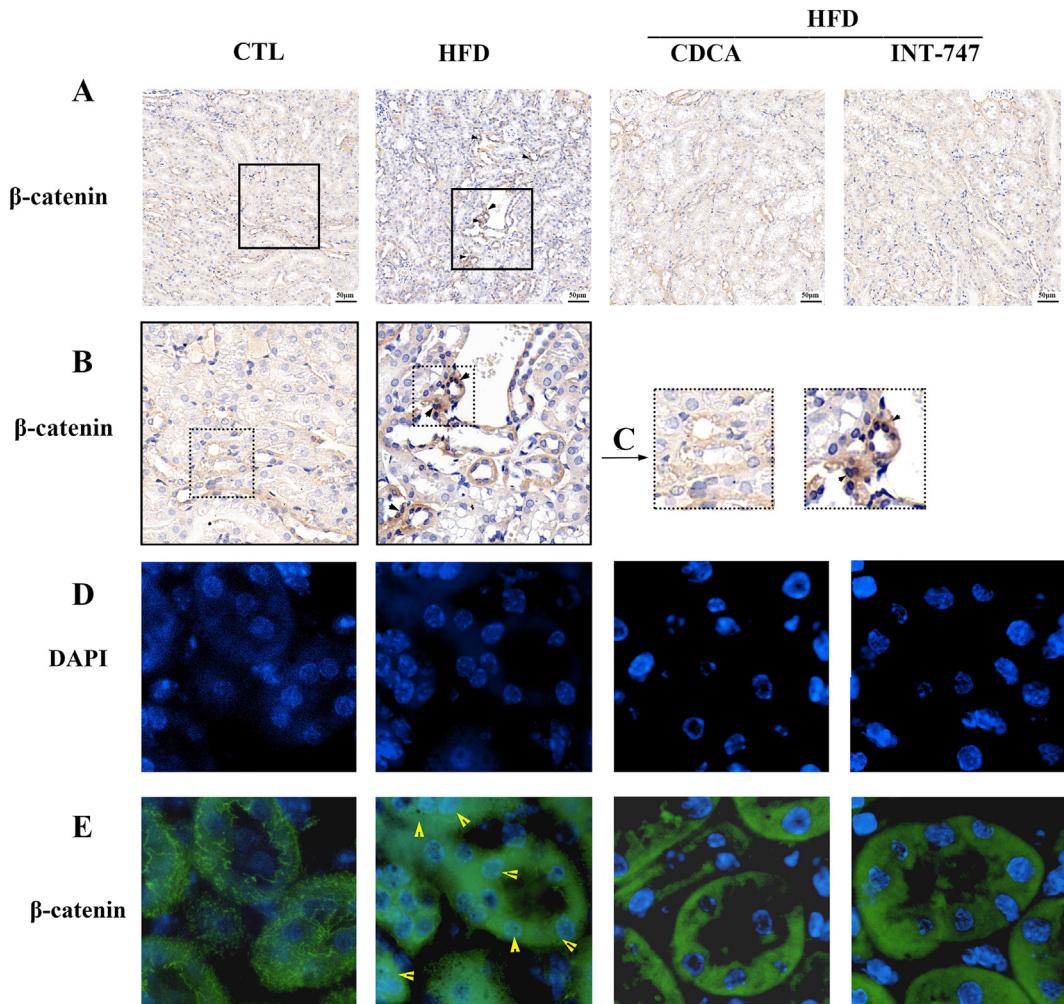


Figure 7: FXR activation inhibits activation of β -catenin signaling in HFD mice. Representative micrographs display the induction of localization of β -catenin in HFD mice. Kidneys from control, HFD, CDCA and INT-747 group for 20 weeks were stained immunohistochemically for β -catenin protein (A–B). Arrowheads indicate β -catenin-positive tubular epithelial cells. Enlarged image from the boxed area in B are shown in C. Immunofluorescence staining (D–E) shows β -catenin expression in kidney ($\times 100$). Arrowheads denote nuclear location of β -catenin.

activity. Collectively, these data support a possible renal protective effect of FXR activation to inhibit tubulo-interstitial fibrogenesis in lipid-induced nephropathy.

One of the main findings in our study is that the possible beneficial effects of FXR activation on lipid-induced tubulo-interstitial fibrosis might be, at least partly, mediated by modulation of the β -catenin signaling activity. Our study shows that FXR activation suppresses the β -catenin-mediated activation of the Wnt target genes, which are involved in tubulo-interstitial fibrogenesis. First, after ox-LDL treatment, the FXR abundance was decreased in the nuclei, and FXR interacted with β -catenin, especially in the nuclei. Second, FXR inhibition induced β -catenin signaling activation, whereas increased expression/activity of FXR decreased β -catenin signaling activity. Third, after ox-LDL stimulation, the abundance of β -catenin translocated into the nucleus, β -catenin transcriptional activity and its target gene expression were increased (whilst the total β -catenin abundance did not change). A recent study showed that in HEK293 cells, the full length FXR and its mutants containing the N-terminal AF1 domain were

able to bind with β -catenin; conversely, other FXR mutants, which did not have the N-terminal AF1 domain, lost their β -catenin binding ability [23]. Moreover, to identify the possible underlying mechanisms, in our study we showed that FXR interacted with β -catenin signaling using the co-immunoprecipitation assay, and tested the effects of FXR on β -catenin mediated transcriptional activity using a cell-based TOPflash reporter assay in ox-LDL-exposed NRK-52E cells. We also showed that the AF1 domain of FXR was required for the suppression of β -catenin-mediated transcriptional activity.

In conclusion, our experimental study shows that FXR activation may be implicated in decreasing β -catenin signaling activation and protecting against renal fibrosis development. Our study also shows that through interaction with β -catenin, FXR may act as a mediator for ox-LDL regulation β -catenin signaling in tubular epithelial cells. However, further investigation of the regulation and function of the FXR- β -catenin interaction in the development and amelioration of renal fibrosis *in vivo* in man is required to better elucidate whether FXR agonists can be used to treat or protect against tubulo-interstitial fibrosis.

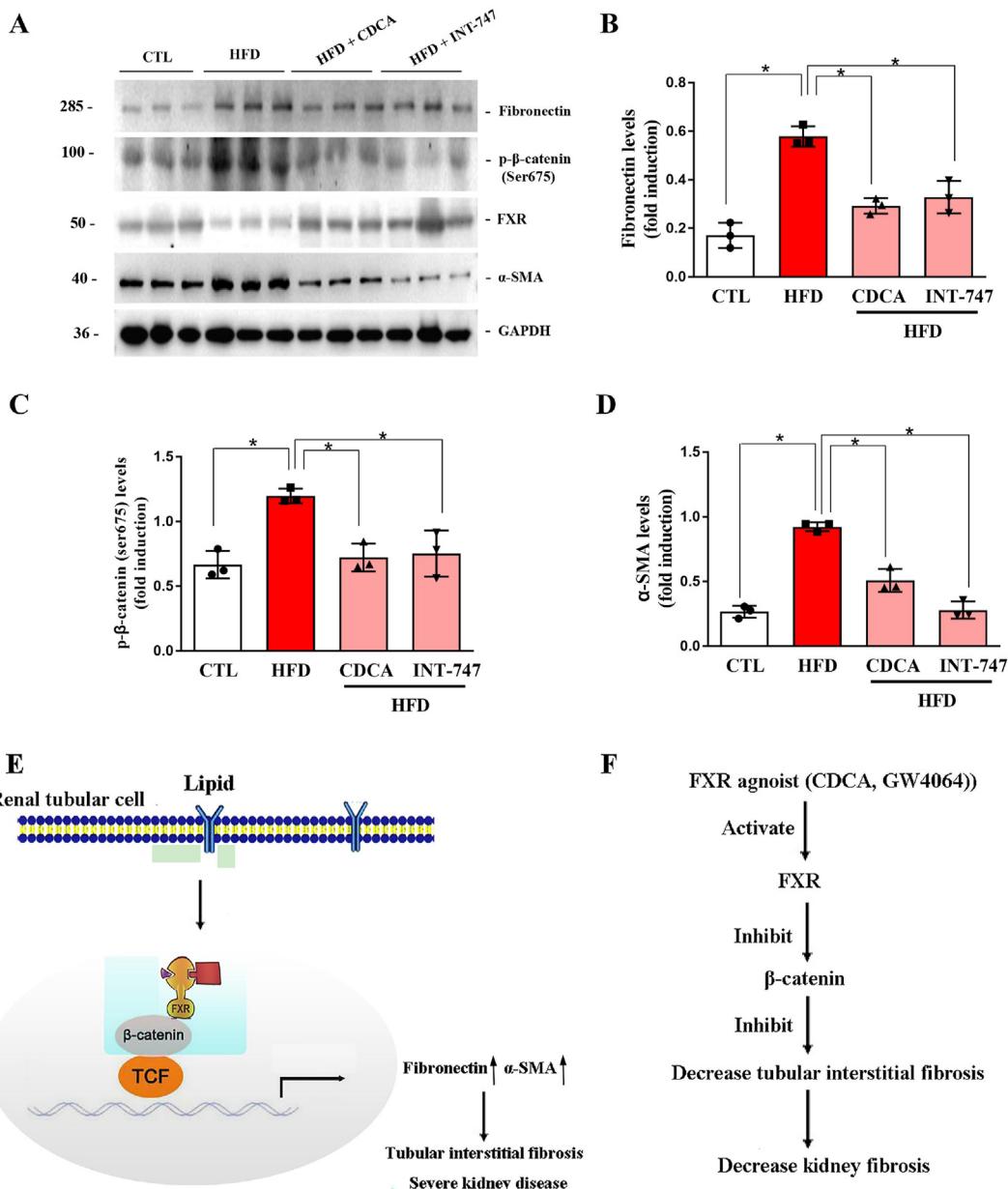


Figure 8: FXR activation protects against β-catenin signaling in HFD mice. Representative Western blot analysis (A) and quantitative data (B–D) show the activation of β-catenin and induction of fibronectin, α-SMA and reduction of FXR protein in HFD mice. (E–F) Schematic Figure illustrating the molecular mechanisms by which FXR and β-catenin may act to regulate the development of renal tubulo-interstitial fibrosis. Ox-LDL-exposed tubular epithelial cells, activates tubular markers of extracellular matrix (fibronectin, α-SMA) and promotes the development of tubulo-interstitial fibrosis via activity of the FXR/β-catenin complex (E). In contrast, up-regulation of FXR activity negatively regulates the FXR/β-catenin complex and inhibits tubular markers of extracellular matrix towards the ox-LDL-induced tubular interstitial fibrosis (F).

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AUTHOR CONTRIBUTIONS

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Ming-Hua Zheng: Conceptualization, Methodology.
Meng-Zhu Fu: Formal analysis, Resources,
Shi-Liang Zhang and Meng-Yang Zhong: Resources.
Giovanni Targher and Christopher D. Byrne: Writing – Review & Editing.
Fuqiang Yuan and Yan Lu: Resources.
Wei-Jie Yuan: Supervision.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request.

ABBREVIATION LIST

FXR	farnesoid X receptor
NRK-52E	rat proximal tubular epithelial cells
CKD	chronic kidney disease
ox-LDL	oxidized low-density lipoprotein
LOX	lysyl oxidase
ERK	extracellular signal-regulated kinase
MAPK	mitogen-activated protein kinase
ECM	extracellular matrix
CDCA	chenodeoxycholic acid
HFD	high-fat diet

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