Supplemental Information

Materials and Methods

Chemicals and Reagents

GW4064, chenodeoxycholic acid (CDCA), cell culture media and supplements were purchased from Sigma (St Louis, MO, USA). The mouse antibody against farnesoid X receptor (FXR) was from R&D, and rabbit antibody against AQP2 was from Millipore (AB3066). Antibodies against caspase-3, cleaved caspase-3 were obtained from Cell Signaling (Danvers, MA, USA), whereas the antibodies against TonEBP, AR, and HSP70were obtained from Abcam (Cambridge, MA, USA). Adenoviruses expressing FXRα2 (AdFXRα2) and their control adenoviruses (Ad-VP16) were kindly provided by Dr. Peter Edwards at University of California, Los Angeles.

Real-Time PCR

Total RNA was extracted from mouse kidneys, primary mouse medullary collecting duct (MCD) cells, mouse inner medullary collecting duct cell line (mIMCD3) by using TRIZOL reagent (Biotek), which wasthen reversed to cDNA by using RevertAidTM First Strand cDNA Synthess Kit (Fermentas, USA) according to the manufacturer's instructions. RT-PCR was carried out by using cDNA as template in the PCR reaction with SYBR Green Mix (Bio-Rad, Hercules, CA). The reaction conditions included 94^{o}C for 5 min, followed by 35 cycles of 94 ^{o}C for 30 s, 59 ^{o}C for 30 s, 72 ^{o}C for 30 s, with a final extension at 72 ^{o}C for 5 min. β-Actin was used as an internal control. Quantitative values were obtained as threshold PCR cycle number

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(Ct). Each sample was measured in duplicate or triplicate in each experiment. The primer pairs used for amplifying interesting mouse genes were listed in Supplemental Table 1.

Western Blot Analysis

Proteins of mouse kidneys and MCD cells were extracted using an ice-cold lysis buffer containing phosphatase inhibitor and PMSF. The nuclear extracts were isolated by using the NE-PER Kit according to the manufacturer's instructions (78833, Pierce Biotechnology, Inc). The lysate was homogenized by using a sonic oscillator then centrifuged at 12,000 rpm for 10 minutes at 4^{o}C. Protein concentrations were determined by the bicinchoninic acid assay. Equal amounts of sample protein (30∼60 μg) mixed with 5×loading buffer were separated by 8%-12% (wt/vol) SDS/PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with 5% (wt/vol) skim milk at room temperature for 1 hour for blocking nonspecific binding sites and then incubated with selected primary antibodies at 4^{o}C overnight. The primary antibodies used in this study were mouse anti-FXR (1:500), rabbit anti-TonEBP (1:1,000), mouse anti–β-actin (1:1,000), rabbit anti-HSP70 (1:1,000), rabbit anti-AR (1:1,000), rabbit anti-procaspase3 (1:1,000),and rabbit anti-cleaved caspase-3 (1:1,000). After being washed for 5 minutes with TBS-T buffer for 5 times, the membrane was incubated at room temperature with 1:6,000 HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 2 h. The membrane was washed and detected by using chemiluminescence substrate (sc-2048, Santa Cruz

Biotechnology). The blot was visualized by exposure to X-ray film (Kodak XBT-1). Densitometric analysis was performed and protein expression level was quantified by Image J software.

Culture of Mouse Primary MCD Cells

Mouse primary MCD cells were cultured as previously described with minor modifications (1). Kidneys of male mice were rapidly removed and renal inner medullas including the papilla were cut in ice-cold saline into small pieces, then transferred to a hyperosmotic medium (600 mOsm) (Dulbecco's Modified Eagle Medium-Ham's F12 medium with 80mMNaCl and 120 mM urea). After minced, the medullas were digested in 10 ml of hyperosmotic medium containing 20 mg collagenase type CLS-II and 20 mg hyaluronidase for 40-60 min at 37^{o}C under continuous agitation. The cell suspension was centrifuged and the pellet was washed three times with pre-warmed, enzyme-free hyperosmotic medium. The cells were seeded in a hyperosmotic medium which contains 50 nM hydrocortisone, 5 pM 3, 3, 5- triiodo-L- thyronine, 1 nM sodium selenate, 5 mg/L transferrin and 20% FBS. In order to maintain AQP2 expression, the cells were grown on 24-mm transwells with 0.4 μm pores (3450, Corning). After reaching 70~80% confluence, the cells were transfered to the above-mentioned hyperosmotic medium without FBS for 12h before they were used for experiments. Mouse primary MCD cells were cultured in a hyperosmotic medium with ~600 mOsm all the time.

Cell Viability Assay

Cell viability was determined by the MTT assay as previously described (2). Mouse inner medullary collecting duct cell line (mIMCD3) was grown to 70-80% confluence in 24-well plates and subjected to hypertonic stress for 6 hours in the presence or absence of GW4064 (2.5 μM) and CDCA (50 μM), or pretreatment with an FXRα2 expressing adenovirus (25 MOI) for 36 hours. The MTT solution (5 mg/ml) was then added to the medium to reach the final concentration at 0.5 mg/ml. The cells were cultured for another 4 hours. At the end of the incubation, the medium was carefully removed and the MTT formazan crystals were dissolved in 500μl DMSO by incubating at room temperature in darkness for 15 minutes. At last, the absorbance was measured at 570 nm by using a microplate reader.

Flow Cytometry of Apoptosis

Flow cytometry was used to determine the apoptotic cells as previously described (3). In brief, Cultured mIMCD3 were either subjected to hyperosmotic challenge for 6 hours in the presence or absence of GW4064(2.5 μM) and CDCA (50μM) or infected with Ad-FXRα_2(25 MOI) or Ad-VP16 (25 MOI) for 36 hours before 6-hour hyperosmotic challenge. After hypertonic stress, both floating and adherent cells were collected and washed with ice-cold PBS for three times. After centrifuging, the cell pellets were resuspended in the Annexin V Binding Buffer at a concentration of 0.25-1.0$\times 10^7$ cells/ml. 100 μl of cell suspension were then transferred to a 5 ml test tube and 5 μl of FITC-conjugated Annexin V were added and incubated for 15

minutes at room temperature in the darkness. 5 μl of propidium iodide was then added and incubated at room temperature for another 5 minutes in the dark. At last, 400 μl of Annexin V Binding Buffer were added to each tube and the cells were analyzed by FACScan analysis with Cellquest software (Becton Dickinson).

Immunohistochemistry

Mouse kidneys were fixed with 4% (wt/vol) PFA in PBS, dehydrated, embedded in paraffin. Then the blocks were sectioned for 3μm. Paraffin-embedded renal tissue were dewaxed, hydrated and quenched in 3% H_2O_2 for 8 minutes to remove endogenous hydroxyl peroxidase activity. After three times 5-minute wash, nonspecific binding sites were blocked by incubation with 5% BSA in PBS for 30min at 37^{o}C. Then sections were incubated with primary antibodies at 4^{o}C overnight. The sections were then incubated with a horseradish peroxidase-conjugated secondary antibody (Zhong-shan Golden Bridge, Beijing) at 37^{o}C for 30 min. Peroxidase in these sections were visualized with diaminobenzidine (DAB) and counterstained with hematoxylin.

TUNEL Assay

Apoptosis of renal medullary collecting duct cells was determined using in Situ Cell Death Detection Kit (POD, Roche) as previously reported (3). Briefly, paraffin-embedded renal tissues were dewaxed and hydrated and quenched in 3% H_2O_2 for 10 minutes to remove endogenous hydroxyl peroxidase activity. The sections

were pretreated with proteinase K and incubated with TdT labeling reaction mixture at 37^{o}C for 1 hour. After terminating the reaction by three 5-minute washes with PBS, the sections were incubated in the Converter-POD at 37^{o}C for 30 minutes, and then stained with diaminobenzidine (DAB) to detect apoptotic cells. In order to identify renal collecting ducts, renal sections were incubated with an AQP2 antibody (Millipore) at 37^{o}C for 1 hour. After three 5-minute washing with PBS, the sections were incubated with an alkaline phosphatase-conjugated secondary antibody by the use of the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium kit (2).At last, the sections were counterstained with hematoxylin.

Immunofluorescence staining

Primary cultured inner medullary collecting duct (IMCD) cells from wild-type or FXR$^{-/-}$ mice that were grown to proper confluence were fixed with 4% (wt/vol) paraformaldehyde (PFA) at room temperature for 15minutes on a rocking platform. After three 5-minute washes with PBS, the cells were permeabilized in 0.1% Triton X-100 in PBS for 10 minutes. After blocked by 5% BSA in PBS for 10 minutes, the cells were incubated with primary antibodies at 4^{o}C overnight. After washed, the cells were incubated with appropriate dyLight 488 (green) or dyLight 594 (red)-conjugated secondary antibodies at 37^{o}C for 30 minutes. Nuclei were stained with 4', 6'-diamidino-2-phenylindole (DAPI). Images were obtained through a confocal microscope.

Luciferase Assay.

mIMCD3 cells grown to 50-60% confluence were transiently transfected with a FXRE-luciferease reporter plasmid by using Lipofectamine 3000 (Invitrogen) according to the standard protocols by the manufacturer.β-galactosidase reporter plasmid was used as a control for transfection efficiency. After 24-hour transfection, the cells were treated with hyperosmotic (600mOsm) for 6 h. After being washed twice with cold PBS, the cells were lysed with 1×luciferase lysis buffer (Luciferase Assay Kit, Promega). Luciferase activity was determined by using luminometer (Turner BioSystems). Luciferase levels of each sample was normalized by β-galactosidase activity. All experiments were repeated at least three times.

Statistical Analyses

All data were shown as the mean±S.E.M. The data were analyzed by using the Prism software package (GraphPad Software). An unpaired two-sided student's t test was used to analyze significant differences. Value of $p < 0.05$ was considered to be statistically significant.

Supplemental Figure Legends

Fig. S1. FXR was constitutively expressed in the principal cells of the inner medullary collecting ducts. Mouse medullary collecting ducts were prepared and primary medullary collecting duct (MCD)cells were cultured. Three preparations of MCD cells were used for detecting FXR expression. (*A*) RT-PCR analysis of FXRα mRNA expression. (*B*) Immunoblot assay of FXR protein expression. (*C*) Immunofluorescence study showing nuclear localization of FXR protein (red). MCD cells were transfected with an AQP2-FGFP expression vector (green). The nucleus was visualized by staining with DAPI (blue).

Fig. S2. Hypertonicity induced FXR expression in primary medullary collecting duct cells. Primary MCD cells were cultured in a hypertonic medium with 600 mOsm and then exposed to increased hypertonicity for 6h. (*A*) FXR mRNA expression was significantly up-regulated by hyperosmotic stress in a dose-dependent manner. MCDs were treated with hypertonicity for indicated doses. $^{***}P<0.001$ *vs.* basic condition (600 mOsm) (n=4). (*B*) Hypertonicity increased FXR protein expression. $^{*}P<0.05$ *vs.* basic condition (n=3). (*C*) Hypertonicity (900 mOsm) induced FXR protein expression in a time-dependent manner. MCDs were exposed to hypertonicity for indicated times. FXR protein expression was examined by Western blot and quantitated by using densitometry. The results are representative of at least three independent experiments. $^{**}p< 0.01$ *vs.* basic condition (600 mOsm) (n=3).

Fig. S3. Collecting duct-specific deletion of FXR gene accelerated medullary collecting duct cell apoptosis in mice with 24-hour water restriction. Wild-type (FXRf/f) and collecting duct-specific FXR knockout (CD-FXR-/-) mice were generated by crossing floxed FXR mice with an aquaporin 2 (AQP2) promoter-driven Cre mouse (AQP2 Cre). Apoptotic cells were identified by the TUNEL assay. (*A*)Apoptotic cells in renal medullas of FXRf/f and CD-FXR-/- mice with water deprivation (WD) for 24 hours. Apoptotic cells were shown in dark brown (arrows). (*B*)Quantitative analysis of apoptotic cells in renal medullas of mice. $^{***}p<0.01$ *vs.*FXRf/f control mice, $^{\#\#}p<0.01$ *vs.* FXRf/f with WD(n=4).

Fig. S4.Ad-FXRα2infection facilitated TonEBP nuclear translocation in FXR$^{-/-}$ primary MCD cells. Primary MCD cells were cultured from FXR$^{-/-}$ mice and infected withAd-FXRa2.FXR and TonEBP protein expression was assessed by immunofluorescence. Note that Ad-FXRα2 infection rescued FXR expression in the nucleus (green)and promoted nuclear translocation of TonEBP protein (red). The nucleus was stained in blue by DAPI.

Supplemental Table 1. Primer pairs used for amplifying mouse genes.

β- actin forward	5’ AGCCATGTACGTAGCCATCC 3’
β- actin_reverse	5’ GCTGTGGTGGTGAAGCTGTA 3’
FXRα forward	5’ TGGGTACCAGGGAGAGACTG 3’
FXRα reverse	5’ CGGAAGAAACCTTTGCAGCC 3’
TonEBPforward	5’ ACCTCTTCCAGCCCTACCAT 3’
TonEBP_reverse	5’ CTTCGGGGTTGATGGATGCT 3’
AR_forward	5’ GACACTTGGACGGCTATGGA 3’

AR_reverse	5’ ATGCCTTTGCTGTGGCAGTA 3’
HSP70 forward	5’ CCGACAAGGAGGAGTTCGTG 3’
HSP70 reverse	5’ ACAGTAATCGGTGCCCAAGC 3’

References

1. Wang F*, et al.* (2014) Prostaglandin E-prostanoid4 receptor mediates angiotensin II-induced (pro)renin receptor expression in the rat renal medulla. *Hypertension* 64(2):369-377.
2. Zheng S*, et al.* (2014) Metformin induces renal medullary interstitial cell apoptosis in type 2 diabetic mice. *Journal of diabetes* 6(2):132-146.
3. Han Q*, et al.* (2011) AMPK potentiates hypertonicity-induced apoptosis by suppressing NFkappaB/COX-2 in medullary interstitial cells. *Journal of the American Society of Nephrology : JASN* 22(10):1897-1911.

Figure S1

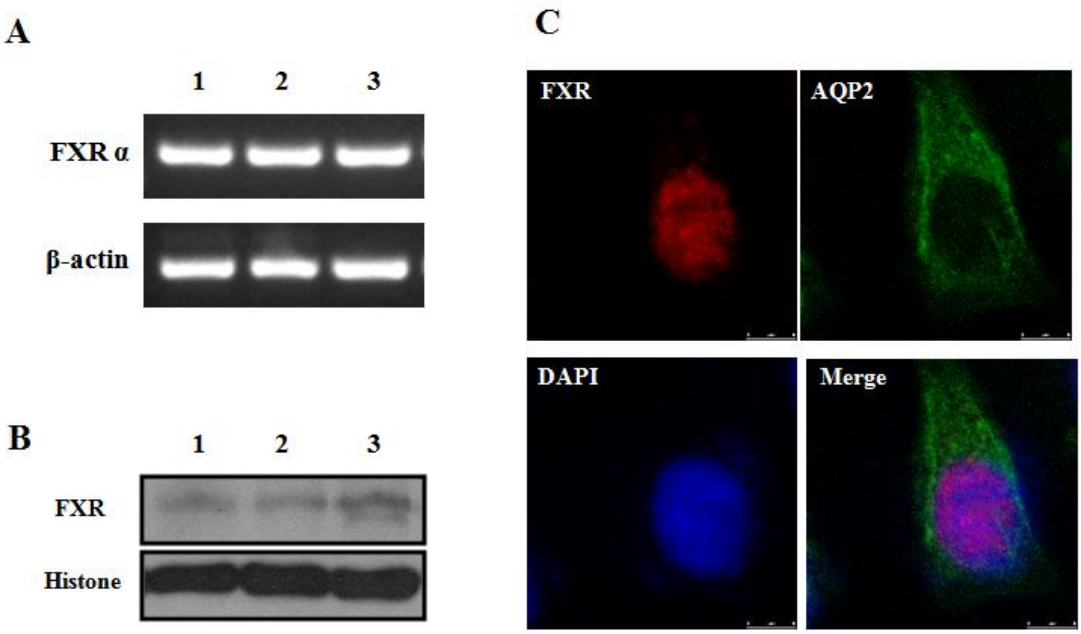
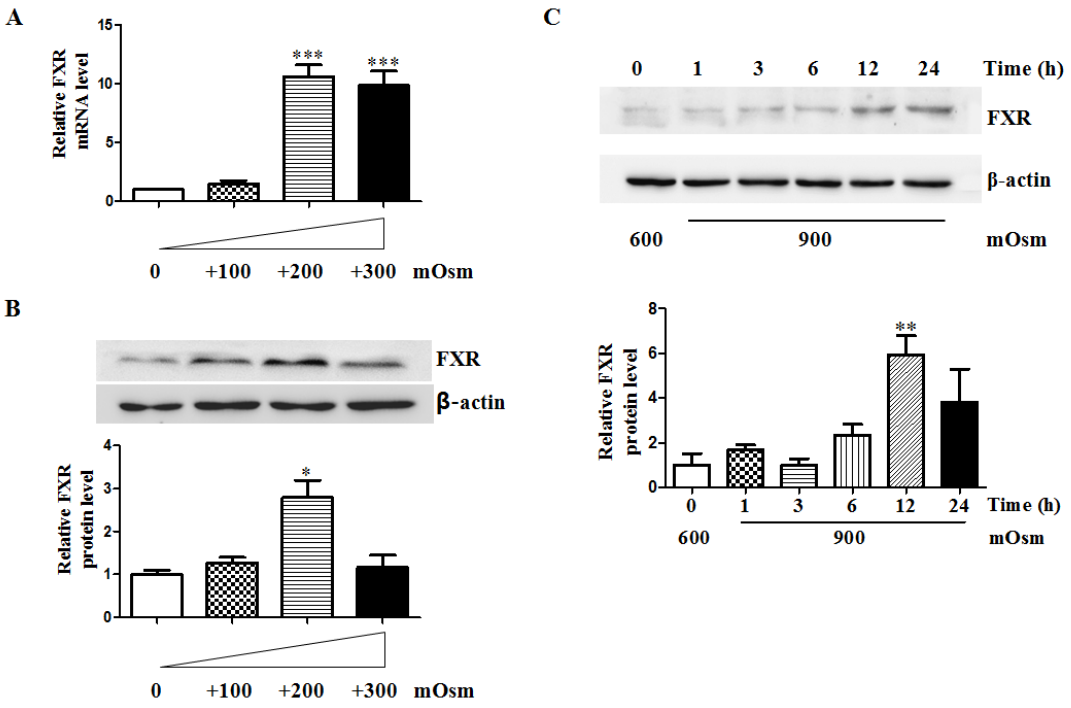


Figure S2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Relative FXR mRNA level
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10
5
0
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0
+100
+200
+300
mOsm
B
FXR
β-actin
Relative FXR protein level
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3
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+100
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12
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Time (h)
FXR
β-actin
600
900
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Relative FXR protein level
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Figure S3

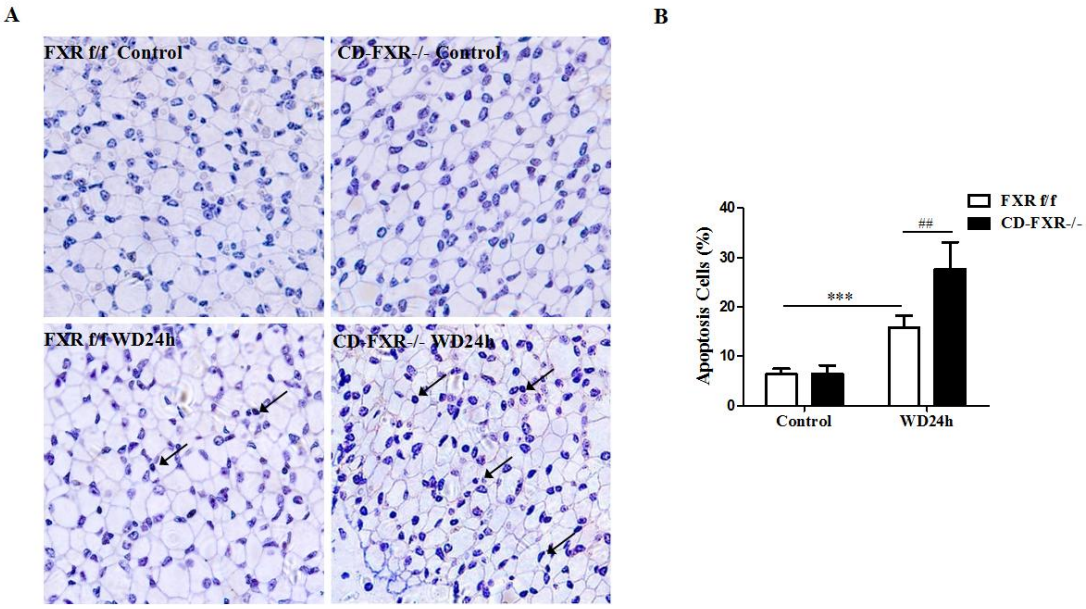
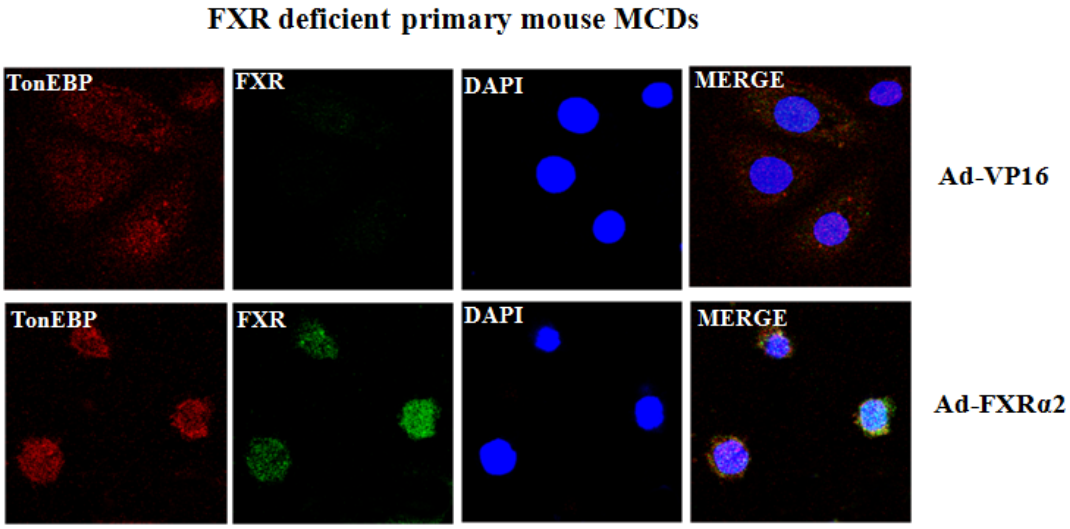

A
FXR f/f Control
CD-FXR-/- Control
FXR f/f WD24h
CD-FXR-/- WD24h
B
FXR f/f
CD-FXR-/-
Apoptosis Cells (%)
40
30
20
10
0
***
##
Control
WD24h

Figure S4


FXR deficient primary mouse MCDs
TonEBP
FXR
DAPI
MERGE
Ad-VP16
TonEBP
FXR
DAPI
MERGE
Ad-FXRα2