Alcohol promotes renal fibrosis by activating Nox2/4-mediated DNA methylation of Smad7

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Abstract

Alcohol consumption causes renal injury and compromises kidney function. The underlying mechanism of the alcoholic kidney disease remains largely unknown. In this study, an alcoholic renal fibrosis animal model was firstly employed which mice received liquid diet containing alcohol for 4-12 weeks. The Masson’s Trichrome staining analysis showed that kidney fibrosis increased at week 8 and 12 in the animal model which was further confirmed by albumin assay, Western blot, immunostaining and real-time PCR of fibrotic indexes (collagen I and α-SMA). In vitro analysis also confirmed that alcohol significantly induced fibrotic response (collagen I and α-SMA) in HK2 tubular epithelial cells. Importantly, both in vivo and in vitro studies showed alcohol treatments decreased Smad7 and activated Smad3. We further determined how the alcohol affected the balance of Smad7 (inhibitory Smad) and Smad3 (regulatory Smad). Genome-wide methylation sequencing showed an increased DNA methylation of many genes and bisulfite sequencing analysis showed an increased DNA methylation of Smad7 after alcohol ingestion. We also found DNA methylation of Smad7 was mediated by DNMT1 in Ethyl alcohol (EtOH)-treated HK2 cells. Knockdown of Nox2 or Nox4 decreased DNMT1 and rebalanced Smad7/Smad3 axis, and thereby relieved EtOH-induced fibrotic response. The inhibition of reactive oxygen species by the intraperitoneal injection of apocynin attenuated renal fibrosis and restored renal function in the alcoholic mice. Collectively, we established novel in vivo and in vitro alcoholic kidney fibrosis models and found that alcohol induces renal fibrosis by activating oxidative stress-induced DNA methylation of Smad7. Suppression of Nox-mediated oxidative stress may be a potential therapy for long-term alcohol abuse-induced kidney fibrosis.

Key words: Alcohol; renal fibrosis; DNA methylation; NADPH Oxidases; Smad7
Introduction

Alcohol abuse is becoming a large social issue and its related diseases result in a heavy social-economic burden [1]. The long-term alcohol consumption is positively correlated with the risk of developing chronic kidney disease (CKD) [2,3], and associated with increased incidence of albuminuria, and is linked with a poor prognosis of CKD [4-9].

Accumulating evidence from basic research indicates alcohol abuse has detrimental effects on kidney. Although the liver is a primary organ most likely to be injured by alcohol, excessive alcohol intake destroys the structure and function of the kidneys, including tubular and glomerular dysfunction and electrolyte disorders [10]. Ethanol significantly increases reactive oxygen species (ROS) production, resulting in inflammatory response in kidney and renal cell apoptosis [11,12]. The chronic alcohol exposure also caused insulin resistance, β-cell dysfunction and hyperglycemia which potentially lead to glomerulosclerosis and interstitial fibrosis in kidney [13,14]. Hyperlipidemia induced by chronic alcohol consumption had the enhanced lipid oxidation, cytokine production and apoptosis in kidney [15,16]. Moreover, alcohol intoxication aggravated tubular abnormalities via oxidative stress and inflammation in the rhabdomyolysis-induced acute kidney injury (AKI) [17] and provoked renal fibrosis through myofibroblast infiltration and extracellular matrix (ECM) production in the ischemic reperfusion (IR)-induced AKI [18].

Given overwhelming data confirming the renal toxicity of alcohol abuse, there are significant gaps in the field of alcoholic renal disease. First, most studies have relied on animal models due to a dearth of in vitro models. Second, the mechanisms by which alcohol induces renal damage, especially at the late stage of renal fibrosis, are still obscure.

TGF-β1 plays a critical role in renal fibrosis by activating downstream regulatory Smads, including Smad2 and Smad3, via binding to TGF-β1 receptors. Activated
Smad2/3 form a complex with Smad4 and then translocate into the nucleus, bind to target genes and initiate transcription[19,20]. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase and degrades the TGF-β1 receptors, thereby negatively regulated TGF-β1/Smad signaling [21]. DNA methylation mediated by DNA methyltransferases, including DNMT1, DNMT3a and DNMT3b, may contribute to Smad7 reduction in liver fibrosis[22]. Additionally, previous studies showed that ROS could significantly induce DNA methyltransferases[23,24]. In the current study, we detected the suppression of Smad7 and activation of TGF-β/Smad3 in fibrotic kidney in response to alcohol, which was positively correlated with ROS production in kidneys, so we hypothesized that ROS-induced DNA methyltransferases may lead to DNA methylation and reduction of Smad7, thereby enhanced TGF-β/Smad3-mediated alcoholic renal injury.

Taken together, in the current study we aim to establish novel in vivo and in vitro alcohol-induced renal injury models and then studied the underlying mechanisms of alcoholic renal fibrotic response.

MATERIALS AND METHODS
Reagents and Materials
Antibodies Collagen I and total Smad3 were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA), phospho-Smad3, E-cadherin and PPAR-α was obtained from Cell Signaling (Cell Signaling Technology, Danvers, MA), α-SMA, SREBP-1 and β-actin were purchased from Bioss Biotechnology (Bioss Biotechnology, Bei Jing, China). Protein Assay Kit was obtained from Beyotime Institute of Biotechnology (Beyotime, Jiangsu, China). Lipofectamine 3000 was obtained from Science Biotechnology (Invitrogen, Beijing, China). BUN assay kit and creatinine (Cr) Assay kit (sarcosine oxidase) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Albumin Mouse ELISA Kit was obtained from Abcam (Abcam, Cambridge, MA). Reactive Oxygen Species Assay (DCF Assay) Kit and Dihydroethidium (DHE) were purchased from Beyotime Institute of Biotechnology
(Beyotime, Jiangsu, China). MASSON Staining kit was purchased from Zhuhai Besso Biotechnology Institute (Wuhan, China).

Animal model of alcoholic kidney injury

Mice were purchased from the Jiangsu Provincial Experimental Animal Center. All animal experiment protocols were approved by the Anhui Medical University Animal Experimental Ethics Committee and conducted in Anhui Medical University. The male C57BL/6 mice (aged 6–8 weeks) were acclimated with the Lieber-DeCarli liquid diet control ad libitum for 1 week. Afterward, mice received either Lieber-DeCarli diet ethanol (6% vol/vol) ethanol or Lieber-DeCarli diet control (isocaloric maltose dextrin) for 4, 8 and 12 weeks. Mice were randomly assigned into five groups (n = 6-8): Group 1, mice received control diet for 4 weeks; Groups 2-4, mice received ethanol diet for 4, 8 and 12 weeks respectively [25]; Group 5, mice received ethanol diet and intraperitoneal injection of apocynin (0.1mg/kg) twice per week (Santa Cruz, CA, USA) for 8 weeks. Mice were sacrificed by exsanguination under anesthesia with inhaled 5% isoflurane in room air. Kidney, liver, heart and lung tissues, blood, 24-hour urine samples were collected for further analysis.

Cell culture

Human kidney tubular epithelial cell line (HK2), mouse tubular epithelial cell line (mTEC) and TβRII dominant negative tubular epithelial cell line were provided by Prof. Hui Yao Lan (The Chinese University of Hong Kong) and seeded in 6-well plates and cultured in HyClone™ DMEM/F12 medium with 5% FBS at 37°C, 5% carbon dioxide incubator. After the overnight starving in DMEM/F12 medium supplemented without FBS, HK2 cells received ethanol (100mM) treatment for 1 to 4 days respectively in the same culture condition. The optimal dose of ethanol treatment was determined by our pilot experiment. The apocynin (100μM) was pretreated for 12 h before being exposed to ethanol. The in vitro experiments were repeated 3-4 times.

MTT Assay
HK2 cells were seeded in 96-well plates and treated by Ethyl alcohol (EtOH) at doses of 6.25 to 400 mM for 24 hours or times of 0.75 to 96 hours at 100 mM. Cell viability was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay followed by the manufacture’s instructions.

**Knockdown of Nox2, Nox4 and DNMT1, DNMT3a, DNMT3b in tubular epithelial cells**

Nox4 was knocked down by the transfection with shRNA plasmid (GeneChem Co. Ltd., Shanghai, China) with LipofectamineTM 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions as previously described [26,27]. Nox 2, DNMT1, DNMT3a and DNMT3b were knocked down by siRNA transfection (GenePharm, Shanghai, China) with the same protocols.

**RNA extraction and real-time PCR**

Total RNA was extracted by RNeasy Isolation Kit (Qiagen, Valencia, CA, USA) and converted into cDNA by the Reverse transcription kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed by using the Bio-Rad iQ SYBR Green supermix with Opticon2 (Bio-Rad, Hercules, CA) as previously described [28]. The sequences of primers are as follows:

Human α-SMA, forward 5’-ATCAAGGAGAAACTGTGTTATGTAG-3’, reverse 5’-GATGAAGGATGGCTGGAACAGGGTC-3’;

Human Col-1, forward 5’-TCTAGACATGTTCAGCTTTGTGGAC-3’, reverse 5’-TCTGTACGCAGGTGATTGGTG-3’;

Human β-actin, forward 5’-CGCCGCCAGCTCACCATG-3’, reverse 5’-CACGATGGAGGGGAAGACGG-3’;

Mouse β-actin, forward 5’-CATTGCTGACAGGATGCAGAA-3’, reverse 5’-ATGGTGCTAGGAGCCAGAGC-3’;

Mouse Col-1, forward 5’-TGTAAACTCCCTCCACCCCA-3’, reverse 5’-TCGTCTGTTTCCAGGGTTGG-3’;

Mouse α-SMA, forward 5’-CGGGCTTTGCTGGTGATG-3’.
reverse 5’-CCCTCGATGGATGGGAAA-3’;
Mouse DNMT1, forward 5’-AGGCGGAAATCAAAGGAGGA-3’,
reverse 5’-GTTTTGCGACTGGATCTCTG-3’;
Mouse DNMT3a, forward 5’-GGTTCGGAGATGGCAAGTTC-3’,
reverse 5’-TGGAGGACTTTAGTAGATGGC-3’;
Mouse DNMT3b, forward 5’-TGCAAGAGCAGAGATGGGAG-3’,
reverse 50-ACTTGCCATCACCACACAC-3’.
Mouse ALDH1A1, forward 5’-ACCAAAGGGACCACCTTCCA-3’
reverse 5’-ACATTGCTGGTTTGGCTCAG-3’;
Mouse ALDH3A1, forward 5’-CCTGCGCAAGAATGAATGGA-3’;
reverse 5’-TCTGACGAGTCTTTGCCACA-3’;
Mouse ALDH2, forward 5’-TTCTCTTCGCGCCCATATCT-3’,
reverse 5’-AGAAAGCCTAGCCAGAGTGG-3’;
The ratio for the mRNA of interest was normalized to β-actin and presented as the mean ± S.D.

Western blot analysis
The pulverized kidney tissue and HK2 cells were lysed by RIPA-Buffer (Beyotime, Jiangsu, China). The protocol of western blot was used previously [28,29]. The primary antibodies, collagen I, α-SMA, β-actin, phospho-Smad3 and total Smad3 were incubated overnight at 4°C and treated with IRDye 800-conjugated secondary antibody (1:10000, Rockland immunochemicals, Gilbertsville, PA, USA) at room temperature for 1.5h, finally detected by Li-Cor/Odyssey infrared image system (LI-COR Biosciences, Lincoln, NE, USA) and quantified by the Image J software (NIH, Bethesda, MD, USA)[30].

DCF Assay
Alcohol induced cellular oxidative stress were measured by the DCF (2’,7’-dichlorofluorescein) Assay Kit (Beyotime, Jiangsu, China). DCF, the oxidized products of ROS, was measured by fluorescence microscopy with excitation of 488
nm and emission of 525 nm in HK2 cells treated by ethanol (100mM) for 1 to 4 days [26].

DHE Staining

DHE (Dihydroethidium) was used as a fluorescent probe for the detection of ROS generation specific for superoxide and hydrogen peroxide (Beyotime, Jiangsu, China). HK2 cells were treated by ethanol (100mM) for 1 to 4 days, stained by DHE, and measured under fluorescence microscopy using an excitation wavelength between 480-520 nm and an emission wavelength between 570-600 nm. [26].

Determination of MDA and GSH

The levels of MDA and GSH in cell or in mouse tissues were detected by a commercial kit (Jiancheng Co., Nanjing, China) according to the manufacturer’s instructions [26].

Masson’s Trichrome staining, Immunohistochemistry analysis and Haematoxylin and eosin (HE) staining

Kidney, and liver tissues were fixed with 4% paraformaldehyde and then dehydrated, paraffin-embedded, and sliced (3-5 μm). The tissue sections were stained with the Masson’s Trichrome staining reagent according to the manufacturer’s instructions (Zhuhai Besso Biotechnology Institute, Wuhan, China), and then observed with a microscope (Olympus, Japan) at 200 × magnification and analyzed by the Image Analysis System. (AxioVision 4, Carl Zeiss, Jena, Germany)[28].

Immunohistochemistry was performed in paraffin sections using a microwave-based antigen retrieval technique. Sections were incubated with rabbit anti-Collagen I and rabbit anti-α-SMA overnight at 4 °C. And then drop the secondary antibody and DBA (3,30-diaminobenzidine tetrahydrochloride) for color development. The results were analyzed by Image Analysis System (AxioVision 4, Carl Zeiss, Jena, Germany)[28,31].
Kidney, heart, lung and liver tissues were stained with the HE staining reagent performed in paraffin sections in Wuhan servicebio technology CO., LTD for histological analysis.

**Oil red O staining**

was performed in frozen sections using Oil red O stain (Solarbio, Beijing, China). Sections were fixed with 4% paraformaldehyde for 10 minutes and washed with PBS for three times. After embathed with 60% isopropanol for 20 second, sections were stained with Oil red O for 15 minutes. Then washed with 60% isopropanol. After washed with PBS for three times, the formed fat drops were observed by a microscope.

**Microalbuminuria**

Twenty-four-hour urine samples were collected one day before the sacrifice of mice. Microalbumin and urinary creatinine level were measured with Albumin Mouse ELISA Kit (Abcam, Cambridge, MA) and Creatinine Assay Kit (Nanjing, China), according to instructions supplied. Results are expressed as the urine microalbumin to creatinine ratio (μg/mg).

**Smad7 gene methylation level detection**

DNA was extracted from the kidney tissue using the genome extraction kit (Generay Biotechnology, Shanghai, China) and the isolated DNAs were treated with sodium bisulfite using the Methylamp DNA modification kit (QIAGEN, Germany) according to the manufacturer’s protocols. PCR primers were as follows: F: GGAA GTG TAGGGA ATTTATTTTAT, R: TATCTACACRCCTCCCTTTAAA. The resulting PCR products were purified using a MinElute Gel Extraction Kit (Generay, Shanghai, China) and cloned into pTG19-T vector (Generay). Individual clones were grown and plasmids were purified using a Plasmid DNA Purification Kit.
(Generay). For each condition, at least 10 clones of each sample were selected and sequenced by the Shanghai Generay Biotech Co., Ltd.

**Determination of blood pressure and blood alcohol concentration**

Blood pressure was measured using a tail-cuff method. Briefly, mice were gently restrained in a holder and warmed to 37 °C by a heating platform under the holder. Animals were acclimated in the environment for 5–10 min after occlusion cuffs and pulse transducers (Softron, BP-2010 Series) were placed on the tail. The values of at least 10 readings for each mouse were used for blood pressure. The levels of blood alcohol were determined by headspace GC according to the manufacturer’s instructions.

**Statistical analyses**

Data was analyzed by two sample t-test or one-way analysis of variance (ANOVA) followed by Tukey post hoc tests using GraphPad Prism 5 software. A p-value less than or equal to 0.05 (two tailed) was considered as the statistical significance.

**RESULTS**

**Establishment of novel alcohol-induced renal fibrosis mouse model.**

Protocol for model establishment was listed in Figure 1A. We found although mice in Lieber-DeCarli ethanol liquid diet-feed group lose weight significantly in the first 4 weeks, their body weight then increased to comparable level to control group. (Figure 1B). As the level of serum ethanol increased significantly compared with control group (supplementary Figure 1A), serum urea nitrogen and microalbumin analysis indicated alcohol treatment declined the renal function compared the control diet (Figures 1C and 1D). We also found that ethanol treatment had no impact on blood pressure (Supplementary Figure 1B). HE staining showed the EtOH-induced damage in mouse kidney and liver (Figures 1E), and HE staining failed to detect EtOH-induced damage in mouse heart and lung (Supplementary Figure 2). Oil red O staining detected the lipid deposition in the kidneys of alcohol-treat mice (Figures 1F).
Moreover, we detected the key factors in regulating lipid metabolism. Western blot and quantitative data showed that SREBP-1 increased and PPAR-α reduced in a time-dependent manner in alcohol-injured kidneys (Figure 1G).

**Alcohol induced a fibrotic response in mice and human HK2 cell lines**

Masson’s Trichrome staining and quantitative data showed total collagens increased at week 4 and peaked at week 8 in kidney, but not in liver (Figures 2A). The immunohistochemistry analysis showed alcohol diet significantly increased Collagen Type I (Col.I) in kidneys (Figures 2B). Western blot analysis and real-time PCR results showed alcohol diet had significant inductions of fibrotic indexes, Col.I and α-SMA (Figures 2C). We also established an alcohol-induced cellular fibrosis model by treating human kidney tubular epithelial cells (HK2) with an optimized dose of ethanol (100mM). MTT assay showed that ethanol reduced cell viability in a dosage- and time-dependent manner (Figures 3A). Western blot analysis showed that ethanol decreased E-cadherin and increased Col.I and α-SMA in a time-dependent manner (Figure 3B). The mRNA levels of Col.I and α-SMA were increased and peaked at 12-hours in cells treated with ethanol (100mM) (Figure 3C).

**Alcohol induced aberrant methylation in kidney**

The whole-genome methylation sequencing revealed significant changes of protein methylation in alcoholic kidneys (Figures 4A). We then examined the protein levels of the three major DNA methyltransferases, DNMT1, DNMT3a and DNMT3b in ethanol treated HK2 cells and in mice using Western blot analysis. Results showed that all three methyltransferases were significantly elevated in alcohol-stimulated tubular epithelial cells and alcoholic mice (Figures 4B and 4C, respectively).

**Alcohol reduced Smad7 and activated TGF-β1/Smad3 signaling-driven fibrosis in kidney**

We previously reported that interrupting TGF-β1/Smad3 and Smad7 signaling leads to renal fibrosis in various renal disease models[28,29,32,33]. We further analyzed gene
methylation with bisulfite sequencing and found Smad7 methylation significantly upregulated in the alcohol group (Figure 5A). Western blot data showed ethanol significantly reduced Smad7 and increased phosphorylated Smad3 in alcoholic kidney (Figure 5B). In addition, we tested the effects of other organic solvents, like dimethyl sulfoxide and isopropanol, on Smad7 expression to confirm the specificity of ethanol treatment, the results showed that ethanol, but not dimethyl sulfoxide or isopropanol, reduced the Smad7 level significantly (Supplementary Figure 3). Real-time PCR also indicated that TGF-β1 mRNA was significantly induced by the alcohol consumption (Figure 5C). Furthermore, EtOH-induced fibrotic response in TβRII wild type (WT) cells was significantly suppressed in EtOH-treated TβRII dominant negative (DN) cells (Figure 5D). Consistent findings from in vitro study also indicated the imbalance of Smad3 and Smad7 signaling in ethanol-treated HK2 cells (Figure 5E).

DNMT1 promoted the loss of Smad7 in alcohol-treated mTECs
To determine which DNA methyltransferases played the critical role in alternating Smad7 methylation, we successfully knocked down the methyltransferases of DNMT1, DNMT3a, and DNMT3b in mTECs (Figure 6A, B). Among the EtOH-treated mTECs with knockdown of DNMT1, DNMT3a, and DNMT3b respectively, inhibition of DNMT1 significantly restored Smad7 levels compared with alcohol-treated empty vector cells (Figure 6C).

Alcohol induced reactive oxygen species production in vivo and in vitro
We next investigated the underlying mechanisms by which alcohol induced DNMT1 and resulted in the imbalance of Smad3/Smad7. We found that MDA levels were significantly increased in kidneys, while GSH, an antioxidant index, decreased in vivo (Figures 7A and 7B), suggesting the significant increase of ROS levels by alcohol. In addition, DCF fluorescence data showed that alcohol increased ROS in HK2 cells, which was further confirmed by DHE staining (Figures 7D, E). Nox2 and Nox4 are two important Nox family members that mediate ROS production[34]. We found that
Nox2 and Nox4 levels increased after alcohol ingestion in mice (Figure 7C). These results were consistent with the findings that Nox2 and Nox4 were highly induced in EtOH-treated HK2 cells in a time-dependent manner (Figure 7F).

**Alcohol promoted renal fibrosis through Nox2/4-dependent mechanisms**

To determine the function of Nox2 and Nox4 in EtOH-treated tubular epithelial cells, we silenced Nox2 and Nox4 respectively (Figure 8A and 9A). Western blot and real-time PCR results show knockdowns of either Nox2 or Nox4 suppressed EtOH-induced fibrosis (Collagen I and α-SMA) in cells (Figures 8B&C and 9B&C). Moreover, Western blot results showed that silencing Nox2 or Nox4 alleviated DNMT1 protein level and restored the balance of Smad7/Smad3 ratio (Figure 8D and 9D). These results suggested that both Nox2 and Nox4 affected DNA methylation and caused imbalance of Smad7/Smad3 in EtOH-induced fibrosis.

**Apocynin prevented alcohol-induced renal fibrosis by suppressing DNA methyltransferase and rebalancing Smad3/Smad7 signaling**

Next, we determined if apocynin, a NADPH oxidase inhibitor, prevented alcohol-induce renal fibrosis. We found that alcohol induced Nox2 and Nox4 were significantly reduced in mice receiving apocynin twice per week for 8 weeks (Figure 10A). Consistently, apocynin decreased levels of ROS and improved renal function in mice as demonstrated by GSH, MDA and BUN assays analysis (Figures 10B, 10C and 10D). Treatment of apocynin attenuated total collagen deposition (Figure 11A) as well as other fibrosis indexes (Collagen I and α-SMA) in kidneys. Importantly, apocynin reduced DNMT1 level and thereby restored the balance of Smad7 and Smad3 signaling in alcoholic kidneys (Figure 11B, 11C and 11D). The findings were further confirmed by *in vitro* study that apocynin attenuated EtOH-induced fibrotic response in tubular epithelial cells (Figures 11Eand 11F). Additionally, treatment of apocynin didn’t alter the level of aldehyde dehydrogenases (ALDHs) (Supplementary Figure 4).
DISCUSSION

In the present study, we established and validated novel models of alcohol-induced renal fibrosis both in vivo and in vitro. We provided experimental evidence showing alcohol impaired renal function and increased ECM deposition mice as well as promoted fibrotic response in tubular epithelial cells. The models established by us may be used to elucidate mechanisms of alcoholic kidney injury and serve as a useful platform to screen drugs for the injury. We also demonstrated that alcohol promoted renal fibrosis by activating Nox2/4-mediated DNA methylation of Smad7, which enhanced Smad3-mediated renal fibrosis. Importantly, inhibition of ROS by apocynin protected against alcoholic renal fibrosis.

We measured the detrimental effects of chronic, heavy alcohol intake on kidneys in a novel murine model modified from the NIAAA model[25]. Results show alcohol aggravated renal injury and fibrotic response in a time-dependent manner; deposition of collagen and accumulation of α-SMA+ myofibroblasts were key characteristics. These findings were consistent with a series of well-designed studies by Dr. McIntyre’s group, which showed that chronic ethanol ingestion induced oxidative kidney injury and fibrosis [35-37]. Of note, the renal fibrotic response may be led by the abnormal repair from the ethanol-induced acute kidney injury, which need to be further determined[38]. Additionally, it is important to note that limited in vitro data has been collected due to lack of in vitro models for evaluating alcohol-induced kidney injury; this is clearly an obstacle for understanding the mechanism more deeply. Given this, we established a new model by treating tubular epithelial cells with ethanol at various time points. We found that ethanol stimulation decreased the E-cadherin while increasing protein level of collagen and α-SMA in a time-dependent manner.

We also found that DNMT1-mediated DNA methylation of Smad7 is the central mechanism for alcohol-induced renal fibrosis. We, and others, identified that activation of TGF-β1/Smad3 signaling and loss of Smad7 enhanced renal fibrosis in
various kidney disease models [39,28,29,32,19,33,40]. In the present study, genome-wide methylation sequencing and bisulfite sequencing showed that alcohol induced DNA methylation of Smad7 led to downregulation of Smad7 and overactivation of TGF-β/Smad3 signaling. By knocking down three major DNA methyltransferases (DNMT1, 3a and 3b), we showed that DNA methylation of Smad7 was mediated by DNMT1. Previous results showed that DNMT1 is induced in TGF-β-treated mouse kidney fibroblasts and regulates the expression of genes involved in fibroblast activation [41]. In addition, silencing the DNMT1 gene decreased Smad7 expression in response to TGF-β1 in liver fibrosis [22]. All these lines of evidence indicate DNMT1-mediated imbalance of Smad7/Smad3 signaling mediates alcohol-induced renal fibrosis.

Interestingly, we found that Nox-mediated ROS production plays a role in the induction of DNA methyltransferases in alcohol-stimulated kidneys. The increased expression of DNA methyltransferases caused by oxidative stress is gaining attention [42-45]. Kidneys are rich in alcohol-oxidizing enzyme [46], which may be associated with a complex interaction between alcoholic kidney injury and EtOH-induced oxidative stress [47]. Oxidative stress is a key trigger in kidney injury caused by alcohol, directly or indirectly. NADPH, specifically Nox2 and Nox4, are important enzymes in mediating oxidative stress-induced renal injury [48,36,47,34]. Indeed, Nox4 contributes to several types of renal diseases, including diabetic nephropathy, hypertensive nephropathy and obstructive nephropathy [34]. In the current study, we found alcohol induced Nox2 and Nox4 production both in vivo and in vitro. And, blocking these enzymes reduced ECM production in response to alcohol, indicating these enzymes are involved in alcohol-induced renal fibrosis. It is worth noting that the alcohol-induced fibrotic response peaked at week 8 and reduced at week 12, consistent with the finding that acute ethanol administration causes a dose-dependent injury of the antioxidant system [47]. It is possible that prolonged alcohol stimulation may activate antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, thereby protecting against renal fibrosis in a negative feedback loop
Finally, we evaluated the therapeutic effect of ROS cleavage on alcoholic renal fibrosis. Apocynin, inhibition NADPH oxidases activity and ROS generation and scavenging, was intraperitoneally injected to inhibit ROS production in alcohol-fed mice [48,27,53,54]. Apocynin restored renal function and suppressed alcohol-induced renal fibrotic response, which was further confirmed in EtOH-stimulated HK2 cells. These results indicate that targeting Nox-mediated ROS production may be a viable therapy for treating alcohol-induced renal injury.

Of note, in our model, we found that ethanol treatment caused hepatic injury and steatosis, but no significant fibrosis in liver. However, we found evidence showing that ethanol diet induced fibrotic response in kidney, we presumed that liver may be an organ with better metabolic and regenerative capacities compared with kidney.

In conclusion, based on our novel in vivo and in vitro alcoholic renal injury models, we found that Nox2/Nox4-mediated ROS production induced DNA methylation of Smad7 via DNMT1-dependant mechanism (Figure 12). Suppression of Smad7 and activation of Smad3 appears to promote alcohol-induced renal fibrosis. We further showed that inhibition of Nox-mediated oxidative stress by apocynin protected against alcoholic renal injury. Taken together, these data underscore a potential therapy to treat long-term alcohol abuse-induced kidney fibrosis by targeting ROS production.

Clinical perspectives
(i) Alcohol consumption causes renal injury but the underlying mechanism of the alcoholic kidney disease remains largely unknown.

(ii) Established novel in vivo and in vitro alcoholic kidney fibrosis models and found that alcohol induces renal fibrosis by activating oxidative stress-induced DNA
methylation of Smad7.

(iii) Suppression of Nox-mediated oxidative stress may be a potential therapy for long-term alcohol abuse-induced kidney fibrosis.

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Author Contributions
X.M. Meng conceived, designed the study, H.Y. Chen, J. Li and H. Han interpreted data and wrote the main manuscript text. L. Gao, Q. Chen, J.G. Wen, J. Jin and W.J. Ni, Y. Huang and T.T. Ma contributed to data analysis and discussion. Q. Yang, J.N. Wang, L. Jiang, W.F. Wu, B. Wei, Q.Y. Ma, X.Q. Liu, did experiments and data analysis. All authors read and approved the manuscript.

List of Abbreviations
AKI acute kidney injury
ALDH acetaldehyde dehydrogenase
APO apocynin
α-SMA α-smooth muscle actin
CKD chronic kidney disease
Col.I collagen I
DNMT1 DNA methyltransferase 1
ECM extracellular matrix
EtOH ethyl alcohol
HK2  Human kidney tubular epithelial cell line
NOX  NADPH Oxidase
ROS  reactive oxygen species
TEC  tubular epithelial cell
TGF-β transforming growth factor-β

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**Figure Legends**

**Figure 1. Establishment and validation of alcohol-induced renal fibrosis model in vivo.** (A) Protocol for Animal model establishment; (B) Body weight of mice; (C, D) Renal function tests. Results of BUN and urinary albumin/creatinine ratio indicate that alcohol impaired renal function; (E) HE staining in kidney and liver; (F) Oil red O staining in kidney. (G) Western blot analysis and quantitative data of SREBP-1 and PPAR-α. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control. W: week; EtOH, Ethyl alcohol; BUN, blood urea nitrogen; NC, normal control. Data represent the mean ± SEM for 6-8 mice.

**Figure 2. Alcohol induced fibrotic response in mice.** (A) Masson’s Trichrome staining of kidney and liver; (B) Immunohistochemistry of Col.I; (C, D) Western blot analysis and quantitative data of Col.I and α-SMA, Western blotting analysis show alcohol induced renal fibrosis, which was further confirmed by Real-time PCR for mRNA. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control. NC, normal control. Data represent the mean ± SEM for 6-8 mice.

**Figure 3. Alcohol induced fibrotic response in human HK2 cell lines.** (A) Cell viability of EtOH-treated HK2 cells by MTT Assay. (B) Western blot analysis and
Results demonstrate that EtOH increased the mRNA level of Col.I, and α-SMA in HK2 cells. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control. h: hour; d: day; EtOH, Ethyl alcohol; NC, normal control. Data represent the mean±SEM for 3-4 independent experiments.

**Figure 4. Methylation analysis in alcohol induced fibrosis models.** (A) Hierarchical cluster analysis of differentially methylated region (DMR) by the whole-genome methylation sequencing of alcoholic kidneys and control kidneys. Red color indicates relatively up-regulated genes, and green color indicates down regulated genes. Only genes passing the significance change (P < 0.05 and fold-change >2) are shown; (B, C) Western blot analysis and quantitative data of three kinds of methylated enzymes. Results demonstrate that alcohol increased levels of DNMT1, DNMT3a and DNMT3b *in vivo* and *in vitro*. *p < 0.05, ***p < 0.001 compared to the control. W: week; EtOH, Ethyl alcohol; NC, normal control. Data represent the mean±SEM for 3-4 independent experiments or 6-8 mice.

**Figure 5. Alcohol caused the imbalance of Smad3 and Smad7 signaling.** (A) Bisulfite sequencing for the methylation status of indicated genes. White circles represented unmethylated CpGs, and black circles represented methylated CpGs. Methylated shown in yellow, unmethylated shown in blue, and not present shown in grey; (B) Western blot analysis and quantitative data of p-Smad3 and Smad7 in mice; (C) Real-time PCR of TGF-β1 in mice. (D) Western blot analysis and quantitative data of Col.I and α-SMA in TβRII WT/DN cells; (E) Western blot analysis and quantitative data of p-Smad3 and Smad7 in HK2 cells. Results demonstrated that alcohol decreased the protein level of Smad7 and increased the level of phosphorylated Smad3 in HK2 cells. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control. ##p<0.01, ###p<0.001 compare to EtOH-treated WT cells. W: week; EtOH, Ethyl alcohol; KD, knockdown; NC, normal control; WT, wild type. DN, dominant negative; Data represent the mean ± SEM for 6-8 mice or 3-4 independent
experiments.

Figure 6. DNMT1 promoted the loss of Smad7 in alcohol treated mTECs. (A, B) Western blot analysis and Real-time PCR show DNMT1, DNMT3a and DNMT3b were knocked down in mTEC cells; (C) Western blot analysis and quantitative data of Smad7. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control. #p<0.05 compared to EtOH-treated EV. EV, empty vector; EtOH, Ethyl alcohol; KD, knockdown. Data represent the mean ± SEM for 6-8 mice or 3-4 independent experiments.

Figure 7. Alcohol enhanced reactive oxygen species (ROS) production in vivo and in vitro. (A, B) Levels of glutathione (GSH) and MDA in mice. Results demonstrate that alcohol induced oxidative stress in mice; (C) Western blot analysis and quantitative data of Nox2 and Nox4; (D, E) Intracellular levels of ROS by DCF Assay and DHE staining; (F) Western blot analysis and quantitative data of Nox2 and Nox4. Results of ROS assay demonstrate that alcohol induced cell oxidative stress in HK2 cells. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control. d: day; W: week; Data represent the mean ± SEM for 6-8 mice or 3-4 independent experiments.

Figure 8. Alcohol promoted renal fibrosis via Nox2-dependent mechanisms. (A) Verification of Nox2 knockdown in HK2 cells; Results show that Nox2 was downregulated by transfection of Nox2 siRNA; (B) Western blot analysis and quantitative data of Col.I and α-SMA in Nox2 silenced HK2 cells; (C) Real-time PCR of Col.I and α-SMA in Nox2 silenced HK2 cells. Results demonstrate that silence Nox2 significantly reduced the mRNA level of Col.I and α-SMA; (D) Western blot analysis and quantitative data of the DNMT1, Smad7, and phosphorylation of Smad3. **p < 0.01 compared to the control. # p < 0.05, ## p < 0.01 compared to Nox2 EV group. EV, empty vector; EtOH, Ethyl alcohol; KD, knockdown. Data represent the mean ± SEM for 3-4 independent experiments.
Figure 9. Alcohol promoted renal fibrosis via Nox4-dependent Mechanisms. (A) Verification of Nox4 knockdown in HK2 cells; Results show that Nox4 was downregulated by transfection of Nox4 shRNA; (B) Western blot analysis and quantitative data of Col.I and α-SMA in Nox4 silenced HK2 cells; (C) Real-time PCR of Col.I and α-SMA in Nox4 silenced HK2 cells. Results demonstrate that silence Nox4 significantly reduced the mRNA level of Col.I and α-SMA; (D) Western blot analysis and quantitative data of the DNMT1, Smad7 and phosphorylation of Smad3. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control. ¦p < 0.05, ¦¦p < 0.01, ¦¦¦p < 0.001 compared to NOX4 EV group. EV, empty vector; EtOH, Ethyl alcohol; KD, knockdown. Data represent the mean ± SEM for 3-4 independent experiments.

Figure 10. Intraperitoneal Injection of apocynin prevented alcohol-induced ROS. (A) Western blot analysis and quantitative data of Nox2 and Nox4; (B, C) Levels of glutathione (GSH) and MDA; (D) Results of BUN show that apocynin prevented the decline of kidney function caused by alcohol. **p < 0.01, ***p < 0.001 compared to the control. ¦¦p < 0.01, ¦¦¦p < 0.001 compared to EtOH without apocynin. NC, normal control; EtOH, Ethyl alcohol; APO, Apocynin. Data represent the mean ± SEM for 6-8 mice.

Figure 11. Apocynin inhibited alcohol-induced fibrosis by reducing the ROS-dependent activation of DNMT1 while restoring the balance of Smad7 and Smad3 both in vivo and in vitro. (A) Masson’s Trichrome staining and score of severity; (B) Immunohistochemistry of Col.I; (C) Western blot analysis and quantitative data of Col.I, α-SMA, DNMT1, Smad7 and phosphorylation of Smad3; (D) Real-time PCR of Col.I and α-SMA. (E) Western blot analysis and quantitative data of Col.I and α-SMA. Results demonstrate that APO inhibited the fibrosis in HK2 cells treated by alcohol. (F) Real-time PCR in HK2 cells. Results demonstrate that APO inhibited the mRNA level of Col.I and α-SMA. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control. ¦p < 0.05, ¦¦p < 0.01, ¦¦¦p < 0.001 compared to EtOH without apocynin. NC, normal control; EtOH, Ethyl alcohol; APO, Apocynin. Data
represent the mean ± SEM for 6-8 mice or 3-4 independent experiments.

Figure 12. Mechanisms of alcohol-induced kidney fibrosis and potential targets for treatment. Chronic alcohol consumption induced Nox2/4-mediated ROS production, and thereby enhanced DNA methylation of Smad7 via DNMT1-dependant mechanism, this finally triggered Smad3 activation and renal fibrosis.

Supplementary Figure 1. Blood alcohol concentration and systolic blood pressure in alcohol-feeding mice. (A) Blood alcohol concentration in mice. Result showed that blood ethanol concentration was increased in model group compared with control; (B) Blood pressure; Result showed that blood pressure was not significant altered when treated with alcohol and apocynin. *p < 0.05 compared to the control. NC, normal control; EtOH, Ethyl alcohol; APO, Apocynin. Data represent the mean ± SEM for 6-8 mice.

Supplementary Figure 2. Treatment of alcohol has little effect on heart and lung. (A) HE staining in heart samples; (B) HE staining in lung samples. NC, normal control. Data represent the mean ± SEM for 6-8 mice.

Supplementary Figure 3. Other organic solvents like dimethyl sulfoxide and isopropanol had little effect on Smad7 level. (A) Western blot analysis and quantitative data showed that ethanol, instead of dimethyl sulfoxide and isopropanol, reduced the protein level of Smad7. *p < 0.05 compared to the control. NC, normal control; EtOH, Ethyl alcohol; DMSO, dimethylsulfoxide. Data represent the mean ± SEM for 6-8 mice.

Supplementary Figure 4. Level of alcohol dehydrogenases in the kidney. (A) Real-time PCR results showed that mRNA level of ALDH1A1, ALDH2 and ALDH3A1 were induced in model group and treatment of apocynin fail to alter their
levels. *p < 0.05 compared to the control. NC, normal control; EtOH, Ethyl alcohol; APO, Apocynin; ALDH, acetaldehyde dehydrogenase. Data represent the mean ± SEM for 6-8 mice.
A. Animal Model

EtOH-fed groups: 6% EtOH liquid diet (change diets at 3:00–5:00 pm)

Liquid diet acclimatization
1 week control liquid diet ad libitum
1 week control liquid diet ad libitum

Pair-fed group: control liquid diet (change diets at 3:00–5:00 pm)

B. Body weight

| Week | NC | 4W | 8W | 12W |
|------|----|----|----|-----|
| 1    |    |    |    |     |
| 2    |    |    |    |     |
| 3    |    |    |    |     |
| 4    |    |    |    |     |
| 5    |    |    |    |     |
| 6    |    |    |    |     |
| 7    |    |    |    |     |
| 8    |    |    |    |     |
| 9    |    |    |    |     |
| 10   |    |    |    |     |
| 11   |    |    |    |     |
| 12   |    |    |    |     |

(E) HE staining

NC | Model (4 weeks) | Model (8 weeks) | Model (12 weeks)

Kidney

Liver

F. Oil red O staining (kidney)

NC | Model (4 weeks) | Model (8 weeks) | Model (12 weeks)

G. Western blot (kidney)

SREBP-1 (54 kDa) | PPAR-α (51 kDa) | β-actin (42 kDa)

| Model | 4W | 8W | 12W |
|-------|----|----|-----|
| NC    |    |    |     |
|       |    |    |     |
|       |    |    |     |
|       |    |    |     |

Figure 1
A. Masson’s Trichrome staining

B. IHC of Col.1

C. Western blot (mice)

D. Real-time PCR (mice)

Figure 2
**Figure 3**

**A. MTT Assay (HK2)**

![Graph showing cell viability for various concentrations of EtOH](image)

**B. Western blot (HK2)**

- **EtOH** / (100 mmol) - NC, 1d, 2d, 3d, 4d
- **E-cadherin** (90 kDa)
- **Col.I** (130 kDa)
- **α-SMA** (42 kDa)
- **β-actin** (42 kDa)

![Western blot images](image)

**C. Real-time PCR (HK2)**

- **α-SMA** protein
- **Col.I** mRNA
- **α-SMA** mRNA

![Graphs showing mRNA levels for various etoh concentrations](image)
Figure 4
A. Bisulfite sequencing

| CpG Position | Unmethylated | Methylated | Not present |
|--------------|--------------|------------|-------------|
| 1            | 1.0          | 0          | 0           |
| 2            | 3.0          | 0          | 0           |
| 3            | 6.0          | 0          | 0           |
| 4            | 5.0          | 0          | 0           |
| 5            | 4.0          | 0          | 0           |
| 6            | 7.0          | 0          | 0           |
| 7            | 7.0          | 0          | 0           |
| 8            | 1.0          | 0          | 0           |

Legend: • unmethylated | □ methylated | □ not present

B. Western blot (mice)

C. Real-time PCR (mice)

D. Western blot

E. Western blot (HK2)

Figure 5
**Figure 6**

**A. Western blot (mTEC)**

- DNMT1 (178 kDa)
- DNMT3a (100 kDa)
- DNMT3b (94 kDa)
- β-actin (42 kDa)

**B. Real-time PCR (mTEC)**

- DNMT1 mRNA
- DNMT3a mRNA
- DNMT3b mRNA

**C. Western blot (mTEC)**

- Smad7 (46 kDa)
- β-actin (42 kDa)

- Smad7 protein

*EV vs. KD*

**Legend**

- *p < 0.05
- **p < 0.01
- ***p < 0.001
- #p < 0.05 compared to control
- **p < 0.01 compared to control
- *p < 0.05 compared to control
- **p < 0.01 compared to control
- ***p < 0.001 compared to control
A. GSH (mice)

B. MDA (mice)

C. Western blot (mice)

D. DCF Assay of ROS

E. DHE staining

F. Western blot (HK2)

Figure 7
Figure 9

A. Western blot (HK2)

Nox4-EV  Nox4-KD

Nox4 (67 kDa)

β-actin (42 kDa)

B. Western blot (HK2)

Nox4-EV  Nox4-KD

Col I (130 kDa)

α-SMA (42 kDa)

β-actin (42 kDa)

C. Real-time PCR (HK2)

Col I mRNA

α-SMA mRNA

D. Western blot (HK2)

DNMT1 protein

Smad7 protein

p-Smad3 protein

Figure 9
A. Western blot

Nox2 (65 kDa)
Nox4 (67 kDa)
β-actin (42 kDa)

Model
NC 8W 8W+APO

Nox2 protein

Ratio (Nox2/β-actin)

NC EtOH EtOH+APO

Nox4 protein

Ratio (Nox4/β-actin)

NC EtOH EtOH+APO

B. GSH

GSH level (μmol/L)

NC EtOH EtOH+APO

C. MDA

MDA level (μmol/mL)

NC EtOH EtOH+APO

D. BUN level

BUN level (μmol/L)

NC EtOH EtOH+APO

Figure. 10
Chronic Alcohol Consumption

Nox2

Nox4

ROS

DNMTs

Smad7

Smad3

Apocynin

Renal Fibrosis

Figure 12
A. Western blot (HK2)

|       | NC    | EtOH  | DMSO  | Isopropanol |
|-------|-------|-------|-------|-------------|
| Smad7 |       |       |       |             |
| 46 kDa|       |       |       |             |
| β-actin|       |       |       |             |
| 42 kDa|       |       |       |             |

Supplementary Figure 3

Ratio (Smad7/β-actin)

- NC
- EtOH
- DMSO
- Isopropanol

* Indicate significant difference.
