Transmembrane protein 88 attenuates liver fibrosis by promoting apoptosis and reversion of activated hepatic stellate cells

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Abstract

Transmembrane protein 88(Tmem88) is a crucial inhibitor for Wnt/ β -catenin pathway in the development of myocardial cells. Due to the important role of β -catenin in the activation and proliferation of hepatic stellate cells(HSCs), it is necessary to investigate the function of Tmem88 in HSCs. In this study, we found that Tmem88 expression was decreased in the human liver fibrotic tissues, primary HSCs from fibrotic mice and activated HSC-T6 cells. Functionally, Tmem88 could inhibit HSCs activation and proliferation by blocking Wnt/ β -catenin pathway, and promoted the apoptosis of activated HSCs by initiating Bcl-2/Bax/Caspase3 pathway. Moreover, the level of DNA methyltransferase 3a(Dnmt3a) was upregulated in activated HSCs, and siRNA-mediated Dnmt3a silencing led to Tmem88 restoration. These results indicated that Tmem88 played an important role in HSCs activation, proliferation and apoptosis, and Tmem88 expression might be modulated by Dnmt3a.

Keywords: Liver fibrosis, Transmembrane protein 88(Tmem88), Hepatic stellate cells (HSCs), DNA methyltransferases(Dnmts), Wnt/ β -catenin.

1. Introduction

Liver fibrosis is the wound-healing respond to multiple factors that lead to liver injury(virus infection, alcohol abuse and high-fat diet)(Bataller and Brenner, 2005a; Moreira, 2007). Hepatic stellate cells (HSCs) are widely recognized as the main cell responsible for hepatic fibrogenesis(Reeves and Friedman, 2002; Sato et al., 2003). In

the usual, HSCs are quiescent and function as vitamin A storage(Friedman, 2008). However, quiescent HSCs will be converted into activated HSCs by transforming growth factor- β 1(TGF- β 1) and platelet-derived growth factor(Henderson and Iredale, 2007; Lee and Friedman, 2011). Activated HSCs will up-regulate the expression of alpha-smooth muscle actin(α -SMA), and remodel the liver architecture by synthesizing types I collagen(Coll α 1) and other extracellular matrix(ECM)(Brown et al., 2006; Rippe and Brenner, 2004). Therefore, uncovering the potential mechanisms of HSCs activation and exploring the possible strategies that inhibit HSCs activation will make a difference for the therapy of liver fibrosis.

Transmembrane protein 88(Tmem88) is a two-transmembrane protein with the inhibitory effect on Wnt/ β -catenin pathway(Lee et al., 2010). Emerging evidence suggests that Tmem88 has two isoforms: CRA-a(17kDa), which interacts with Wnt/ β -catenin pathway, and CRA-b (25kDa), which lacks the motif combined with Dvl proteins and therefore dose not regulate Wnt/ β -catenin pathway(Palpant et al., 2013). β -catenin is an important pro-fibrotic factor(Morrissey, 2003; Tan et al., 2014) and plays a crucial role in HSCs activation and proliferation(Ge et al., 2014; Jiang et al., 2006). Based on the observation mentioned above, we assumed that Tmem88(17kDa) could inhibit HSCs activation and proliferation by blocking Wnt/ β -catenin pathway. To verify this assumption, Tmem88 expression was examined in the human liver fibrotic tissues, primary HSCs from fibrotic mice and activated HSC-T6 cells, and the function of Tmem88 in activated HSCs was assessed by

modulating the level of Tmem88 expression. Furthermore, since the hypermethylated DNA of Tmem88 was found in primary HSCs from fibrotic mice, the relation between Tmem88 and DNA methylation was investigated.

2. Materials and methods

2.1. Specimen collection and mice treatment

Human liver fibrotic tissues were collected from several patients(n=10, male=6) undergoing partial liver resection in the First Hospital of Anhui Medical University. According to the Liver Cancer Study Group of Japan, the degree of fibrosis was classified as normal liver(Control) and mild to moderate fibrosis(Fibrosis). The causes of liver fibrosis included HBV infection(70%), alcohol abuse(20%) and HCV infection(10%). Informed consent was signed by all patients, and all experimental processes were in accordance with the Helsinki Declaration.

Normal male C57BL/6 mice (about 20g) were purchased from the Animal Center of Anhui Medical University. The mice model of liver fibrosis was generated as previously described(Chen et al., 2016). Briefly, the CCL4 (20% in olive oil) was injected subcutaneously in mice back for 4 weeks(twice a week, 0.01 ml/g). XAV-939(Selleck Chemicals, USA) was injected intraperitoneally at a dose of 5mg/kg/day. All animal procedures complied with the guidelines which were approved by the University Animal Care and Use Committee.

2.2. Primary HSCs isolation

Mice were chosen randomly from the fibrosis group and control group(n=6/group).

Then, the mice were anesthetized with 10% chloral hydrate(0.15 ml/mouse) and fixed in experimental board. After the blood was flushed out, enzymes mixture, including protease E, collagenase IV and DNAase(Sigma, GER), was perfused to digest liver completely. Cells suspension was prepared via mincemeat, filtration and centrifuged in the cell-Nycodenz mixture(Sigma, GER) with density of 1.040-1.060 g/ml. To create discontinuous gradient, the cell-Nycodenz mixture was covered with Hank's fluid(Gibco, USA). Finally, HSCs were gathered by extracting the white cell layer from the gradient interface.

2.3. Cell culture

HSC-T6 cells(Fumeng Gene, China), an immortalized rat HSCs, were cultured in DMEM(Keygen Bio, China) supplemented with 5%(v/v) heat-inactivated FBS(Every Green, China). HSC-T6 cells were incubated at 37°C with 5% CO₂ and propagated every two days. HSC-T6 cells were activated by 10ng/ml TGF-β1 for 24h and treated by 10uM XAV-939 and 2uM 5-azadC(Sigma, USA) for 24h.

2.4. Total RNA isolation and quantitative real-time PCR

After splitting by TRIZOL reagents(Invitrogen, USA), total RNA was extracted from mice primary HSCs(n=3/group) and HSC-T6 cells. RNA quantification was determined by Nanodrop 2000(Thermo Scientific, USA). The mRNA levels of α-SMA, Tmem88, Collα1, β-actin and GAPDH were determined. The primer sequences(Sangon Biotech, China) were listed in **Table 1**. The mRNA level of other genes was normalized by β-actin/GAPDH. All samples were performed in triplicate

and repeated at least three times.

2.5. Transfection with Tmem88 plasmid

HSC-T6 cells were seeded in 6 wells plate for 12h and transfected with GV141-Tmem88 and GV141-Control(Shanghai Gene Corporation, China) in opti-MEM culture medium(Gibco, USA) by using LipofectamineTM2000 (Invitrogen, USA). The opti-MEM was replaced by DMEM after 6 h, and 10ng/ml TGF- β 1 (Peprotech, USA) was added for 24 h.

2.6. RNA interference analysis

The sequences of siRNA were as follows: Tmem88-siRNA (5'-CCAGAAUUUACAGGACUGATT-3', 5'-UCAGUCCUGUAAAUUCUGGTT-3'); DNA methyltransferase 1(Dnmt1)-siRNA (5'-CCCAGAGUAUGCACCAAUATT-3', 5'-UAUUGGUGCAUACUCUGGGTT-3'); DNA methyltransferase 3a(Dnmt3a)-siRNA (5'-GCGUCACACAGAAGCAUAUTT-3', 5'-AUAUGCUUCUGUGUGACGCTT-3'); DNA methyltransferase 3b(Dnmt3b)-siRNA (5'-AGAUGACAGGUGCCCAGAGUU-3', 5'-CUCUGGGCACCUGUCAUGUUU-3'); scrambled-siRNA (5'-UUCUCCGAACGUGUCACGUTT-3', 5'-ACGUGACACGUUCGGAGAATT-3'). All siRNA were purchased from the Shanghai GenePharma Corporation. HSC-T6 cells were seeded in 6 wells plate for 12h and transfected with siRNA in opti-MEM culture medium. The opti-MEM was replaced after 6 h by DMEM, and 10ng/ml TGF- β 1 was added for 24 h.

2.7. Cell proliferation assays

HSC-T6 cells were trypsinized and the number of cells was adjusted to 4×10^4 /ml. Then, 100ul cells suspension was added in each of 96 wells plate and the edge wells were filled with sterile PBS. After attachment, HSC-T6 cells were transfected with GV141-Tmem88 or GV141-Control for 6h in opti-MEM and incubated for 24h in DMEM or DMEM with 10ng/ml TGF- β 1. Then, 20ul MTT(5mg/ml, Sigma, USA) was added for 4h and supernatant was replaced by 150ul DMSO(Sigma, USA). The value of absorbance(A) was examined at the wavelength of 490 nm. Cell viability = the A value of transfection wells/the A value of control wells * 100%. All experiments were performed in triplicate and repeated at least three times.

2.8. Flow cytometer analysis

Cell cycle analysis: HSC-T6 cells were trypsinized and collected in 15ml centrifuge tubes. The cells suspension was centrifuged(1000g, 3-5min), mixed sufficiently and fixed in cold ethanol(70%, 1ml) at 4 °C for 12-24h. After being centrifuged and resuspended, the HSC-T6 cells were added with 0.5 ml mixture(RNase and PI) and incubated for 30min at 37 °C in dark place. Flow cytometer(BD Biosciences) was used to detect the red fluorescence and scattered light at 488nm wavelength. The analysis of DNA content and scattering light was processed by using the ModFit software(Verity Software House, USA). All experiments were repeated for three times.

Cell apoptosis analysis: HSC-T6 cells were collected from suspension by

centrifugation, and binding buffer(400ul) was added to resuspend the cells(about 1×10^6 /ml). Then, 5ul Annexin V-FITC(5min, 2-8 °C, dark) and PI were added(5min, 2-8 °C, dark), successively. When the preparation was completed, cells suspension were detected by flow cytometer(BD Biosciences) within 1h and data fitting was made by FlowJo software(TreeStar, USA). All samples were assayed in triplicates.

2.9. Western blot analysis

Human liver tissues, primary mice HSCs(n=3/group) and HSC-T6 cells were lysed to extract the total protein by using RIPA lysis buffer(Beyotime, China). After being quantified by Nanodrop 2000 (Thermo Scientific, USA), the protein samples were adjusted to roughly equal concentration. The supernatant was mixed with proteins loading buffer, and heated at 100°C for 10min. After electrophoresis, blotting, blocking, primary antibodies and secondary antibodies incubation, the membranes were subjected to image development. The categories and concentrations of primary antibodies: anti-Bcl-2 and anti-Bax(Rabbit, 1:200, Boster, China); anti-Tmem88 (Rabbit, 1:300, Santa Cruz, USA); anti- β -actin(Rabbit, 1:3000, Bloworld, USA); anti- α -SMA and anti-Coll α 1(Rabbit, 1:300, Bioss, China), anti- β -catenin(Mouse, 1:300, Santa Cruz, USA); anti-C-myc, anti-CyclinD1 and anti-Caspase3(Rabbit, 1:500, Cell signaling, USA); anti-Dnmt1 and anti-Dnmt3a(Rabbit, 1:1000, Abcam, UK); anti-Dnmt3b(Rabbit, 1:500, ImmunoWay, USA).

2.10. Immunohistochemistry

Liver tissues were formalin-fixed and paraffin-embedded, and wax were sliced and

toasted for 1h at 60 °C. Sections were xylene-dewaxed and hydrated in gradient ethanol, antigen retrieval by immersing in 0.01M citrate buffer and heated to boiling by microwave(98 °C-100 °C) for 15min. After antigen retrieval, the sections were blocked by 5% BSA (37 °C, 20min) and conjugated with primary antibody against Tmem88, α -SMA and β -catenin(4 °C, 12-24h) and secondary antibody (37°C ,60min). The expression of Tmem88, α -SMA and β -catenin was visualized by strept avidin-biotin complex(SABC, 37 °C, 30min) and 3,3'-diaminobenzidine (DAB, microscopic detection about 5min) staining. Then, the sections were hematoxylin-redyed and dehydrated in gradient ethanol(30, 50, 70, 80, 90, 95, 100% for 3min, respectively) and xylene for 20min. Finally, the sections were mounted by gums and subjected to microscopic examination.

2.11. Double immunofluorescence staining

Fresh liver tissues were immersed in freezing medium and stored at -80 °C. Then, the liver tissues were sliced and washed by PBS for 3 times(5min each time). 10% BSA (30min, room temperature) was dropped in the sections to block some unspecific binding sites. The mixed solution of primary antibodies(rabbit polyclonal anti-Tmem88 and goat monoclonal anti- α -SMA were diluted in 1:200) was added in the sections at 4°C overnight. Then, a mixture of fluorescently-labeled secondary antibodies(anti-goat cmcy-3 and anti-rabbit FITC were diluted in 1:200) was added (2h, 37°C, dark). The nuclear staining was achieved by dropping DAPI(5min, 37°C, dark). After washed by PBS and mounted by gum, the sections were visualized by

fluorescence microscope(Leica, Germany).

2.12. Biochemistry analysis

Serum was extracted from mice blood by centrifugation(3,000 g, 10 minutes) and stored at -80°C. Reitman-Frankel method was used to detect the activities of ALT and AST.

2.13. Statistical analysis

All data were expressed as mean $\pm$ SD. Student's t-test was used for two samples and one-way ANOVA was used for multiple samples. The difference had a statistical significance, $P < 0.05$.

3. Results

3.1. Tmem88 expression was decreased in the human liver fibrotic tissues, primary HSCs from fibrotic mice and TGF- β 1-activated HSC-T6 cells.

First of all, human liver fibrotic tissues were collected to investigate the level of Tmem88 expression. Western Blot showed that the expression of α -SMA and Coll α 1 was up-regulated in the fibrosis group, and Tmem88 expression was down-regulated in the fibrosis group(**Fig.1A**). Secondly, mice model of liver fibrosis was established to examine Tmem88 expression. Up-regulated activities of ALT and AST(**Fig.1B**), prominent hepatic steatosis, necrosis, fibrosis and increased α -SMA(**Fig.1C**) suggested that mice model of liver fibrosis had been established successfully. Immunohistochemistry staining showed that Tmem88 expression was decreased in

fibrotic mice(**Fig.1C**). Double immunofluorescence staining showed that Tmem88-positive area and α -SMA-positive area were highly overlapped, which indicated that Tmem88 might be mainly expressed in HSCs(**Fig.1D**). Therefore, the protein and mRNA levels of Tmem88 in mice primary HSCs were examined. Western Blot showed that the level of Tmem88 was down-regulated in the primary HSCs from fibrotic mice(**Fig.1E**). Finally, Tmem88 expression was examined in TGF- β 1-activated HSC-T6 cells by Western Blot and quantitative real-time PCR. We found that the levels of α -SMA and Coll α 1 were elevated and the expression of Tmem88 was reduced in TGF- β 1-treated HSC-T6 cells(**Fig.1F**). Taken together, Tmem88 expression was decreased in the human liver fibrotic tissues, primary HSCs from fibrotic mice and TGF- β 1-activated HSC-T6 cells.

3.2 Pharmacological blockade of Wnt/ β -catenin signaling improved liver fibrosis and attenuated the activation of HSC-T6 cells.

β -catenin inhibitor(XAV-939) was used to confirm the role of β -catenin in hepatic fibrogenesis and HSCs activation. As showed in **Fig.2A**, the activities of ALT and AST were down-regulated in CCL4+XAV-939 group. Moreover, XAV-939 could significantly improve the steatosis and necrosis, and attenuate α -SMA expression and collagen deposition in fibrotic mice(**Fig.2B**). Western Blot showed that XAV-939 could inhibit the expressions of β -catenin and fibrotic markers(α -SMA and Coll α 1) in fibrotic mice(**Fig.2C**). *In vitro*, XAV-939 could inhibit the expressions of β -catenin and activation markers(α -SMA and Coll α 1) in TGF- β 1-activated HSC-T6

cells(**Fig.2D**). These results suggested that β -catenin played an important role in hepatic fibrogenesis and HSCs activation, and blockade of Wnt/ β -catenin signaling improve liver fibrosis and attenuate HSCs activation.

3.3 Tmem88 over-expression inhibited HSC-T6 cells activation and proliferation by blocking Wnt/ β -catenin signaling, and promoted the apoptosis of activated HSC-T6 cells.

To explore the function of Tmem88 in HSCs activation, HSC-T6 cells were transfected with Tmem88 plasmid(GV141-Tmem88). Compared with TGF- β 1+GV141-Control, the expression of Tmem88 was significantly increased in TGF- β 1+GV141-Tmem88 group(**Fig.3A**). Moreover, Tmem88 over-expression significantly inhibited the expressions of Coll α 1 and α -SMA in the levels of mRNA and protein(**Fig.3B**). These results suggested that GV141-Tmem88 could up-regulate effectively Tmem88 expression and inhibited the expressions of α -SMA and Coll α 1 in activated HSCs.

Flow cytometric analysis showed that the percentage of G0/G1 phase was decreased in TGF- β 1 group and increased in TGF- β 1+GV141-Tmem88 group(**Fig.3C**). MTT assay suggested that the viability of HSC-T6 cells was obviously increased in TGF- β 1 group and significantly decreased in TGF- β 1+GV141-Tmem88 group(**Fig.3D**). These results indicated that Tmem88 over-expression could inhibit TGF- β 1-induced HSC-T6 cells proliferation. Moreover, the effect of Tmem88 over-expression on Wnt/ β -catenin signaling was examined

because of the interaction between Wnt/ β -catenin pathway and Tmem88. As showed in **Fig.3E**, the expressions of β -catenin, C-myc and CyclinD1 was significantly increased in TGF- β 1 group, and decreased in TGF- β 1+GV141-Tmem88 group. Given these results, Tmem88 over-expression could inhibit HSC-T6 cells proliferation and Wnt/ β -catenin activation induced by TGF- β 1.

Flow cytometric analysis showed that Tmem88 over-expression increased the percentage of early apoptosis and advanced apoptosis in activated HSC-T6 cells (**Fig.3F**). Moreover, Tmem88 over-expression obviously up-regulated the ratios of Bax/Bcl-2 and Cleaved-caspase3/pro-caspase3(**Fig.3G**). Therefore, Tmem88 over-expression could increase the apoptosis of activated HSC-T6 cells.

3.5 Tmem88 silencing enhanced HSC-T6 cells activation and Wnt/ β -catenin activation, and inhibited the apoptosis of activated HSC-T6 cells.

Tmem88-siRNA was used to further confirm the function of Tmem88 in activated HSC-T6 cells. As presented in **Fig.4A&Fig.4B**, Tmem88-siRNA significantly up-regulated the mRNA and protein levels of Coll α 1 and α -SMA compared with Scrambled-siRNA. Moreover, the expressions of β -catenin, CyclinD1 and C-myc were significantly increased(**Fig.4C**), and the ratios of Bax/Bcl-2 and Cleaved-caspase3/pro-caspase3 were decreased in Tmem88-siRNA group(**Fig.4D**). These results suggested that Tmem88 silencing enhanced HSC-T6 cells activation and β -catenin activation, and inhibited the apoptosis of activated HSC-T6 cells.

3.6 Tmem88 expression was associated with DNA hypermethylation and restored

by Dnmt3a-siRNA in activated HSC-T6 cells.

The pilot experiments showed Tmem88 DNA was hypermethylated in primary HSCs from fibrotic mice(data not yet published). To determine the relation between Tmem88 expression and DNA methylation, the DNA methylation inhibitor 5-aza-2'-deoxycytidine(5-azadC, 2uM) was used. Western Blot showed that Tmem88 expression was increased in TGF- β 1+5-azadC group(**Fig.5A**). Then, the protein levels of Dnmt1, Dnmt3a and Dnmt3b of mice primary HSCs and HSC-T6 cells were examined. The result showed the expressions of Dnmt1, Dnmt3a and Dnmt3b was elevated in primary HSCs from fibrotic mice(**Fig.5B**) and activated HSC-T6 cells(**Fig.5C**). Therefore, HSC-T6 cells were transfected with Dnmt1-siRNA, Dnmt3a-siRNA, Dnmt3b-siRNA and Scrambled-siRNA to determine which Dnmt was responsible for the alteration of Tmem88 expression. As showed in **Fig.5D**, Tmem88 expression was restored in TGF- β 1+Dnmt3a-siRNA group. Taken together, Tmem88 expression was associated with DNA hypermethylation and restored by Dnmt3a-siRNA in activated HSC-T6 cells.

4. Discussion

Liver fibrosis is a common pathological process of varied chronic liver diseases (Bataller and Brenner, 2005b; Connolly et al., 2009). The most important characteristic of liver fibrosis is ECM deposition(Lee and Friedman, 2011) and activated HSCs are the most main cells which synthesized ECM(Friedman, 2008). Therefore, the majority of anti-fibrotic therapies was designed to inhibit HSCs

proliferation and activation or promote HSCs apoptosis. Our study demonstrated that Tmem88 could ameliorate TGF- β 1-induced HSCs activation and proliferation supported by the level of activation markers(Col1 α 1 and α -SMA), cell viability assay and cell cycle analysis. Furthermore, Tmem88 could initiate Bcl-2/Bax/Caspase3 pathway, followed by the increased apoptosis rate in activated HSCs. These results indicated that Tmem88 attenuated liver fibrosis by promoting apoptosis and reversion of activated HSCs.

Wnt/ β -catenin pathway is important for liver physiology and pathology including hepatic development, bile duct homeostasis(Monga, 2014), liver fibrosis(Guo et al., 2012), cirrhosis(Shackel et al., 2001) and hepatocellular carcinoma(Vilchez et al., 2016). Activation of Wnt/ β -catenin leads to nuclear translocation of β -catenin and transcription of target genes(C-myc, Cyclin D1)(Habas and Dawid, 2005; Logan and Nusse, 2004). Available evidences suggest that β -catenin is up-regulated in activated HSCs and participates in the HSCs activation and proliferation(Cheng et al., 2008; Ge et al., 2014). Consistent with these findings, we found that β -catenin expression is increased in fibrotic mice and activated HSC-T6 cells, and pharmacological blockade of Wnt/ β -catenin signaling could improve liver fibrosis and attenuate HSCs activation. Tmem88 is a two-transmembrane-type protein with inhibitory effect on the Wnt/ β -catenin signaling(Lee et al., 2010; Palpant et al., 2013). In our study, it was demonstrated that β -catenin and its downstream proteins(C-myc and Cyclin D1) were increased in activated HSCs, and Tmem88 could inhibit Wnt/ β -catenin activation and

transcription of target genes(C-myc, Cyclin D1). Therefore, Tmem88 might reverse HSCs activation and proliferation by inhibiting Wnt/ β -catenin signaling.

DNA hypermethylation is orchestrated in the process of HSC activation (Page et al., 2016). DNA is methylated by DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) which add the methyl group to the cytosine(Cedar and Bergman, 2012; Iacobazzi et al., 2013). Dnmt3a and Dnmt3b are “de novo” methyltransferases that bind and methylate un-methylated DNA, and Dnmt1 is a maintenance methyltransferase that has a higher catalytic activity for semi-methylating DNA(Anestopoulos et al., 2015). Three DNA methyltransferases(Dnmt1, Dnmt3a, Dnmt3b) synergistically maintain the hypermethylated status of DNA. Usually, gene expression is inhibited due to DNA hypermethylation(Nomoto et al., 2007). In current study, we demonstrated that Tmem88 expression was decreased in the primary HSCs from fibrotic mice and TGF- β 1-activated HSC-T6 cells. Moreover, another group in our laboratory found that Tmem88 DNA was hypermethylated in primary HSCs from fibrotic mice(data not yet published). Therefore, we hypothesized that the decreased expression of Tmem88 in activated HSCs was led by DNA hypermethylation. Our study found that Tmem88 expression was significantly increased by DNA methylation inhibitor 5-azadC, reflecting the potential relation between Tmem88 expression and DNA hypermethylation. Furthermore, the expressions of Dnmt1, Dnmt3a and Dnmt3b were increased in HSC-T6 cells. Therefore, Dnmt1-siRNA, Dnmt3a-siRNA and Dnmt3b-siRNA were used to investigate the role of DNA methyltransferases in

activated HSC-T6 cells. The result showed that siRNA-mediated Dnmt3a silencing resulted in Tmem88 restoration in TGF- β 1-activated HSC-T6 cells rather than Dnmt1-siRNA or Dnmt3b-siRNA. These results suggested that Tmem88 expression was associated with DNA hypermethylation and Dnmt3a could inhibit Tmem88 expression by methylating Tmem88 DNA in activated HSCs.

Collectively, our findings suggested that Tmem88 played a pivotal role in HSCs activation, proliferation and apoptosis and might have the protective effect on liver fibrosis(**Fig.6**). The potential mechanism was to inhibit the activation of Wnt/ β -catenin signaling. Moreover, Tmem88 expression might be regulated by Dnmt3a in activated HSCs. To the best of our knowledge, this is the first report about the pathological role of Tmem88 in liver fibrosis. This study offers us some new insights that Tmem88 can serve as a potential pharmacological target against liver fibrosis.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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Table 1 Primers used in RT-qPCR.

Figure1 Tmem88 expression was decreased in the human liver fibrotic tissues, primary HSCs from fibrotic mice and TGF- β 1-activated HSC-T6 cells.

Figure2 Pharmacological blockade of Wnt/ β -catenin signaling improved liver fibrosis and attenuated the activation of HSC-T6 cells.

Figure2 Tmem88 over-expression inhibited HSC-T6 cells activation and proliferation by blocking Wnt/ β -catenin signaling, and promoted the apoptosis of activated HSC-T6 cells.

Figure4 Tmem88 silencing enhanced HSC-T6 cells activation and β -catenin activation, and inhibited the apoptosis of activated HSC-T6 cells.

Figure5 Tmem88 expression was associated with DNA hypermethylation and restored by Dnmt3a-siRNA in activated HSC-T6 cells.

Figure 6 Overview of Tmem88 in liver fibrosis.

Figure 1 Tmem88 expression was decreased in the human liver fibrotic tissues, primary HSCs from fibrotic mice and TGF-β1-activated HSC-T6 cells. A. the protein level of Tmem88 in human liver fibrotic tissues, $p < 0.05$, $p < 0.01$ vs control. B. the activities of ALT and AST in the serum from control and fibrotic mice, $n=6/\text{group}$, $p < 0.01$ vs control. C. pathology observation of mice liver sections stained with hematoxylin and eosin (H&E) and Masson ($\times 200$), and the levels of α -SMA and Tmem88 were analyzed by immunohistochemistry ($\times 200$). D. double immunofluorescence staining of α -SMA (green) and Tmem88 (red), representative views from control and fibrotic mice were presented ($\times 200$). E. the protein and mRNA expressions of Tmem88 in primary HSCs from control and fibrotic mice, $n=3/\text{group}$, $p < 0.01$ vs control. F. the protein and mRNA expressions of Tmem88 in TGF-β1-activated HSC-T6 cells, the HSC-T6 cells were activated by 10ng/ml TGF-β1 for 24h, $p < 0.01$ vs control.

Figure 2 Pharmacological blockade of Wnt/ β -catenin signaling improved liver fibrosis and attenuated the activation of HSC-T6 cells. A. the activities of ALT and AST in the serum from control mice, fibrotic mice and XAV-939(5mg/kg/day)-treated fibrotic mice, $n=6/\text{group}$, $^{**}p < 0.01$ vs control, $^{##}p < 0.01$ vs CCL4. B. pathology observation of mice liver sections stained with hematoxylin and eosin (H&E) and Masson($\times 200$), and the levels of α -SMA and Tmem88 were analyzed by immunohistochemistry($\times 200$). C. the protein levels of β -catenin, α -SMA and Cola1 in the liver tissues from control mice, fibrotic mice and XAV-939(5mg/kg/day)-treated fibrotic mice. $^{**}p < 0.01$ vs Control, $^{##}p < 0.01$ vs CCL4. D. the protein levels of β -catenin, α -SMA and Cola1 in 10uM XAV-939-treated activated HSC-T6 cells, the HSC-T6 cells were treated by 10ng/ml TGF- β 1 and 10uM XAV-939 for 24h, $^{**}p < 0.01$ vs Control; $^{##}p < 0.01$ vs TGF- β 1.

Figure 3 Tmem88 over-expression inhibited HSC-T6 cells activation and proliferation by blocking Wnt/ β -catenin signaling, and promoted the apoptosis of activated HSC-T6 cells. The HSC-T6 cells were transfected by GV141-Tmem88 and GV141-Control for 6h, and treated by 10ng/ml TGF- β 1 for 24h. A. the protein and mRNA levels of Tmem88 in the GV141-Tmem88 transfected HSC-T6 cells, $^{**}p < 0.01$ vs TGF- β 1+ GV141-Control. B. the protein and mRNA levels of Tmem88, Colla1 and α -SMA in the GV141-Tmem88 transfected HSC-T6 cells, $^*p < 0.05$, $^{**}p < 0.01$ vs Control; $^{\#}p < 0.05$, $^{##}p < 0.01$ vs TGF- β 1+ GV141-Control. C. the cell cycle of GV141-Tmem88 transfected HSC-T6 cells. D. the cell viability of GV141-Tmem88 transfected HSC-T6 cells, $^{**}p < 0.01$, TGF- β 1 vs Control, GV141-Tmem88 vs GV141-Control, TGF- β 1+GV141-Tmem88 vs TGF- β 1+GV141-Control. E. the protein levels of β -catenin, CyclinD1 and C-myc in the GV141-Tmem88 transfected HSC-T6 cells, $^*p < 0.05$, $^{**}p < 0.01$ vs Control; $^{\#}p < 0.05$, $^{##}p < 0.01$ vs TGF- β 1+ GV141-Control. F. FACS analysis was used to examined apoptosis rate alteration in HSC-T6 cells from TGF- β 1, TGF- β 1+GV141-Tmem88 and TGF- β 1+GV141-Control group. G. the expression of apoptosis-relative proteins(Bcl-2, Bax, Pro-caspase3, Cleaved-caspase3) in the GV141-Tmem88 transfected HSC-T6 cells, $^*p < 0.05$, $^{**}p < 0.01$ vs TGF- β 1+GV141-Control.

A.

B.

C.

D.

Figure 4 Tmem88 silencing enhanced HSC-T6 cells activation and β -catenin activation, and inhibited the apoptosis of activated HSC-T6 cells. The HSC-T6 cells were transfected by Tmem88-siRNA and Scrambled-siRNA for 6h, and treated by 10ng/ml TGF- β 1 for 24h. A. the mRNA level of Tmem88, Col1a1 and α -SMA were assessed in the Tmem88-siRNA transfected HSC-T6 cells, $^{**}p<0.01$ vs Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs TGF- β 1+Scrambled-siRNA. B. the protein levels of Tmem88, Col1a1 and α -SMA were assessed in the Tmem88-siRNA transfected HSC-T6 cells, $^{**}p<0.01$ vs Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs TGF- β 1+Scrambled-siRNA. C. the protein levels of β -catenin, CyclinD1 and C-myc were assessed in the Tmem88-siRNA transfected HSC-T6 cells, $^{*}p < 0.05$, $^{**}p<0.01$ vs Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs TGF- β 1+Scrambled-siRNA. D. the protein levels of Bcl-2, Bax, Pro-caspase3 and Cleaved-caspase3 were assessed in the Tmem88-siRNA transfected HSC-T6 cells, $^{*}p < 0.05$, $^{**}p<0.01$ vs TGF- β 1+Scrambled-siRNA.

Figure 5 Tmem88 expression was associated with DNA hypermethylation and restored by Dnmt3a-siRNA in activated HSC-T6 cells. A. the protein levels of β-catenin, α-SMA and Colα1 in 2uM 5-azadC-treated activated HSC-T6 cells, the HSC-T6 cells were treated by 10ng/ml TGF-β1 and 2uM 5-azadC for 24h, * $p < 0.01$ vs Control; ## $p < 0.01$ vs TGF-β1. B. the protein levels of Dnmt1, Dnmt3a and Dnmt3b in the primary HSCs from control and fibrotic mice, n=3/group, * $p < 0.05$, ** $p < 0.01$

vs Control. C. the protein level of Dnmt1, Dnmt3a and Dnmt3b in activated HSC-T6 cells, the HSC-T6 cells were treated by 10ng/ml TGF- β 1 for 24h, $p<0.05$, $p<0.01$ vs Control. D. the protein level of Tmem88 in the Dnmts-siRNA transfected HSC-T6 cells, the HSC-T6 cells were transfected by Dnmt1-siRNA, Dnmt3a-siRNA, Dnmt3b-siRNA and Scrambled-siRNA for 6h, and treated by 10ng/ml TGF- β 1 for 24h, $p<0.05$, $p<0.01$ vs Control; $p<0.05$, $p<0.01$ vs TGF- β 1+Scrambled-siRNA.

Figure 6 Overview of Tmem88 in liver fibrosis.

Table 1 Primers used in RT-qPCR.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
Mouse		
Colla1	TGTAAACTCCCTCCACCCCA	TCGTCTGTTTCCAGGGTTGG
α-SMA	CGGGCTTTGCTGGTGATG	CCCTCGATGGATGGGAAA
Tmem88	GCCCTGTTGGTCACTGGATT	GAACCTGGTGAGACTGGGATT
GAPDH	GGACCTCATGGCCTACATGG	TAGGGCC TCTCTTGCTCAGT
Rat		
Colla1	GATCCTGCCGATGTCGCTAT	TGTAGGCTACGCTGTTCTTGCA
α-SMA	CGAAGCGCAGAGCAAGAGA	CATGTCGTCCCAGTTGGTGAT
Tmem88	ACTCCCAGTTTCTGCGTTCT	AATCCAATGACCAACAGGGT
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC