

TRPM8 Deficiency Attenuates Liver Fibrosis Through S100A9-HNF4 α Signaling

Qiang Liu

Central South University

Xiaohua Lei

Central South University

Zhenyu Cao

Central South University

Ju Zhang

Central South University

Likun Yan

Central South University

Jie Fu

Central South University

Qing Tong

Central South University

Wei Qin

Central South University

Yaoli Shao

Central South University

Chun Liu

Central South University

Zhiqiang Liu

Central South University

Zicheng Wang

Central South University

Yuan Chu

Central South University

Ge Xu

Central South University

Siyuan Liu

Central South University

Xueyi Wen

Central South University

Hirofumi Yamamoto

Osaka University: Osaka Daigaku

Masaki Mori

Kyushu University: Kyushu Daigaku

Xin. M. Liang

Harvard Medical School

Xundi Xu (✉ xuxundi@csu.edu.cn)

Central South University

Research

Keywords: TRPM8, Liver fibrosis, ECM, Inflammation, S100A9, HNF4α

Posted Date: January 3rd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1205763/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Liver fibrosis represent a major global health care burden. Data emerging from recent advances have suggested TRPM8, a member of the transient receptor potential (TRP) family of ion channels, plays an essential role in various chronic inflammatory diseases. However, its role in liver fibrosis remains unknown. Herein, we assessed the potential effect of TRPM8 in liver fibrosis.

Methods

The effect of TRPM8 was evaluated using specimens obtained from classic murine models of liver fibrosis, namely wild-type (WT) and TRPM8^{-/-} (KO) fibrotic mice after carbon tetrachloride (CCl₄) or bile duct ligation (BDL) treatment. The role of TRPM8 was systematically evaluated using specimens obtained from the aforementioned animal models after various *in vivo* and *in vitro* experiments.

Results

Clinicopathological analysis has shown TRPM8 expression was upregulated in tissue samples from cirrhosis patients and fibrotic mice. TRPM8 deficiency not only attenuated inflammation and fibrosis progression in mice, but also helped to alleviate symptoms of cholangiopathies. Moreover, reduction in S100A9 and increase in HNF4α expressions were observed in liver of CCl₄ and BDL treated TRPM8 KO mice. Strong regulatory linkage between S100A9 and HNF4α was also noticed in L02 cells underwent siRNA-mediated S100A9 knockdown and S100A9 overexpressing plasmid transfection. Lastly, alleviative effect of a selective TRPM8 antagonist was confirmed *in vivo*.

Conclusion

These findings suggest TRPM8 deficiency may exert protective effects against inflammation, cholangiopathies and fibrosis through S100A9-HNF4α signaling mechanistically. M8-B might be a promising therapeutic candidate for liver fibrosis.

Introduction

Liver fibrosis, a major health care burden worldwide, represents the ubiquitous response of the liver to almost all chronic injuries predominantly arising from sustained viral infection, excessive alcohol consumption, metabolic aetiologies and autoimmune liver diseases [1, 2]. It is commonly characterized by the activation of hepatic stellate cells (HSCs) and excess accumulation of extracellular matrix (ECM) [3]. Despite global efforts in developing therapeutic interventions to treat this devastating disease, our

understanding of the complex mechanism of liver fibrosis remains limited and no acceptable treatment strategies currently exist. There is an urgent clinical need to better comprehend the underlying mechanisms and identify effective antifibrotic agents aimed at impeding or reversing liver fibrogenesis [4, 5].

Fibrogenesis is usually triggered by either chronic inflammation and oxidative stress, or repeated death of hepatocytes or biliary tract cells, promoting fibroblasts proliferate and ECM production at the injured site [6]. Previous studies have suggested that continuous inflammation from resident and infiltrating immune cells play an important role in liver fibrotic process [7]. In particular, liver macrophages, such as Kupffer cells and monocyte-derived infiltrating macrophages, have been proven to be major players during fibrogenesis by promoting the activation and survival of HSCs [8, 9]. Furthermore, during the recovery phase of chronic liver injury, the regression of liver fibrosis is often accompanied by a distinct restorative macrophage subpopulation [10]. Taken together, accumulating evidence has identified that macrophage-mediated inflammation is a vital factor that influences the progression of liver fibrosis.

Cholangiocytes, the epithelial cells lining the intrahepatic and extrahepatic bile ducts, are pivotal highly dynamic cells residing in a complex anatomic niche where they participate in hepatic canalicular bile modification, bile production and homeostasis [11, 12]. Cholangiocytes show increased proliferation in response to endogenous or exogenous signals/stimuli and are actively involved in inflammatory and reparative processes within the liver. Sustained stimulatory insults to these cells may induce the initiation and progression of cholangiopathies [13–15]. To date, the role of liver inflammation and fibrosis in cholangiopathies remains obscure.

Transient receptor potential (TRP) cationic channels are known to be activated by various physical and chemical stimuli to promote signal transduction [16], among which TRPM8 is especially interesting because it can be activated by environmental stimuli [17, 18], play a critical role in numerous cancers [19] and inflammation-related diseases [20, 21]. However, its role in the pathogenesis of liver fibrosis have also remained unclear to date.

Here we report the functional significance of TRPM8 in liver fibrosis. The obtained data show an aberrantly upregulated TRPM8 expression in both fibrotic human clinical and mice specimens. Strong correlation between TRPM8 deficiency and alleviated liver fibrosis progression was also observed in CCl₄ and BDL treated mice. We also noticed TRPM8 ablation could attenuate various symptoms of cholangiopathy, including bile canalicular abnormalities, dysregulation of bile transporters and ductular reaction. Further, transcriptomic analysis of mRNAs using fibrotic mice liver specimens, coupled with both *in vitro* and *in vivo* assays, were conducted to understand the underlying molecular mechanism of TRPM8 in liver fibrosis. The results from this study suggest TRPM8 inhibition may lead to suppression in liver fibrogenesis through S100A9-HNF4 α signaling axis, which raised the possibility that TRPM8 might be served as a potential therapeutic target for anti-fibrosis therapy development.

Materials And Methods

Human serum and liver samples

Serum samples were obtained from 15 patients with clinically diagnosed cirrhosis and 10 healthy donors. Liver tissues were obtained from 10 patients with hepatic hemangioma (non-fibrotic samples) and 40 patients with cirrhosis who underwent liver transplantation at the Second Xiangya Hospital of Central South University. All study related experimental protocols were under the ethical guidelines of the 1975 Declaration of Helsinki Principles, and were approved by the Ethics Committee of the Second Xiangya Hospital of Central South University. Written informed consent was obtained from all human subjects. Healthy donors with no previously known liver abnormality and had not received any medication in the two weeks prior to sample collection participated in this study.

Reagents

CCl_4 (C112040) and olive oil (O108686) were purchased from Aladdin (Shanghai, China). The haematoxylin and eosin (H&E) staining kit (G1120), Sirius Red staining kit (G1471) and Masson's trichrome staining kit (G1340) were purchased from Solarbio (Beijing, China). TRIzol reagent was purchased from Invitrogen (Thermo Fisher SCIENTIFIC, MA, USA). A Universal Two-Step Test Kit (PV-9000) was purchased from ZsBio (Beijing, China). Following antibodies were used: anti-TRPM8 (GTX54866, GeneTex, California, USA), anti- α -SMA (BM0002, Boster, Beijing, China), anti-COL1A1 (BA0325, Boster, Beijing, China), anti-CK19 (GB11197, Servicebio, Wuhan, China), anti- β -actin (60008-1-AP, Proteintech, Rosemont, USA), anti-F4/80 (28463-1-AP, Proteintech, Rosemont, USA), anti-S100A9 (14226-1-AP, Proteintech, Rosemont, USA), and anti-HNF4 α (ab41898, Abcam, Cambridge, UK).

Animals and animal experiments

WT and TRPM8^{-/-} C57BL/6J mice were obtained from Jackson Laboratory (ME, USA). All mice received humane care throughout experiments according to the guidelines of Central South University and all animal experiments were approved by the University Committee on Use and Care of Animals.

6-week-old male WT and TRPM8^{-/-} mice were given intraperitoneal injections of CCl_4 to mimic toxic-induced fibrosis (1.0 ml/kg body weight, 1:4 dissolved in olive oil) or vehicle (olive oil) twice a week for 8 weeks (n = 5 per group). Two days after the last CCl_4 injection, mice were sacrificed and specimens were collected.

6-week-old male WT and TRPM8^{-/-} mice underwent ligation of the common bile duct (n = 5 per group) to mimic cholestatic liver fibrosis. 14 days after the BDL procedure, mice were sacrificed and specimens were collected.

For therapeutic studies, CCl_4 -injured C57BL/6J mice received either DMSO or 10 mg/kg M8-B hydrochloride intraperitoneal injection [22] every other day from the 7th to the 8th week (n = 5 per group), while BDL-injured mice received either DMSO or 10 mg/kg M8-B hydrochloride intraperitoneal injection every other day after 1 week of BDL treatment (n = 5 per group).

Histology analysis

Formalin-fixed, paraffin-embedded 4 µm-thick liver sections were stained with either hematoxylin and eosin (H&E) for cell morphology characterization, or Sirius Red and Masson's trichrome for quantitative assessment of liver fibrosis severity. Axio Scope A1 light microscopy (Carl Zeiss, Germany) was used to acquire digital images from random fields, and the most representative views of the sections are shown. Cell morphology and the severity of liver fibrosis were evaluated by experienced pathologists while blinded to the treatment groups.

Transmission electron microscopy

Liver tissue obtained from CCl₄-treated mice was thin-sectioned first followed by 2.5% glutaraldehyde fixation in phosphate-buffered saline by perfusion via the inferior vena cava. Transmission electron microscopy was performed as described.

Immunohistochemistry (IHC)

The expression of TRPM8, α-SMA, COL1A1, F4/80, CK19, S100A9 and HNF4α in the liver was evaluated using immunohistochemical staining. Briefly, the tissue sections were deparaffinized, hydrated and incubated in 3% hydrogen peroxide, to block endogenous peroxidase. Antigen retrieval was performed by heating in 10 mM sodium citrate buffer (pH 6.0) for 20 min, and then incubated with primary antibodies against TRPM8 (1:200 dilution), α-SMA (1:500 dilution), COL1A1 (1:500 dilution), F4/80 (1:1000 dilution), CK19 (1:1000 dilution), S100A9 (1:400 dilution) or HNF4α (1:500 dilution) antibodies at 4 °C overnight. A universal two-step test kit was used to treat the samples after they had been incubated with appropriate primary antibodies. Finally, sections were counterstained with haematoxylin for 10 min, dehydrated and sealed with neutral balsam before a light microscopy equipped (Olympus, Hamburg, Germany) with a digital camera was used to photograph the regions of interest. Three images at randomly selected locations were acquired for each tissue section.

Serum biochemistry

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using automatic biochemical analysers (Abbott, Chicago, USA). Human serum S100A9 was measured using a S100A9 ELISA kit (CSB-E11834h, CUSABIO, Wuhan, China) following manufacturer's recommended procedure.

Cell experiments

L02 cells were purchased from Cell Bank of Xiangya Medical School, Central South University. L02 cells were cultured for 24 h before exposed to TRPM8 siRNA, S100A9 siRNA (RiboBio, Guangzhou, China) or S100A9 plasmid (GeneChem, Shanghai, China). Lipofectamine™ 3000 transfection reagent was used to treat L02 cells following the manufacturer's instructions. Transfection medium was replaced with complete culture medium after 6 h. Cells were collected for further investigations after 48 h.

Primary mouse hepatocytes were prepared from male WT and TRPM8^{-/-} mice following the well-established collagenase perfusion method [23]. After purifying the primary mouse hepatocytes, cells were collected for further investigations.

qRT-PCR and RNA-seq analysis

Total RNA was extracted from frozen liver tissue using TRIzol reagent following manufacturer's instructions. Relative mRNA expression levels were calculated using the relative quantification method ($2^{-\Delta\Delta CT}$). All used PCR primer sequences can be found in supplementary Table S1. A melting curve of each amplicon was determined to verify its specificity. Genes were normalized to β -actin as an internal control. Liver RNA samples were submitted for RNA-seq analysis at Annoroad Gene Technology Co., Ltd.

Western blot analysis

Proteins in lysates were resolved by SDS-PAGE electrophoresis and transferred to PVDF membrane (Millipore Corporation, MA, USA). The blotted membrane was blocked and immunoblotted with TRPM8 (1:500 dilutions), α -SMA (1:500 dilutions), COL1A1 (1:500 dilutions), S100A9 (1:500 dilutions), HNF4 α (1:1000 dilutions) primary antibodies at 4 °C overnight. β -Actin (1:5000 dilution) was used as a control. After washing with TBST, the membranes were incubated with secondary antibodies (1:5000 dilution) for 1 h at room temperature, followed by detection with enhanced chemiluminescence system. ImageJ was used to quantify the grey values for each target protein bands.

Statistical analysis

Data were expressed as mean \pm SD and analyzed using GraphPad Prism 7. Statistical differences between two groups were analyzed by the unpaired Student t test with a two tailed distribution. Differences between multiple groups of data were analyzed by one-way ANOVA with Bonferroni correction. *P*-value of less than 0.05 was considered to be statistically significant.

Results

TRPM8 expression is elevated in liver fibrosis

Immunohistochemical staining indicated TRPM8 expression in human cirrhotic liver tissue was significantly upregulated compared to healthy donors (Fig. 1A). The cohort of 40 patients included hepatitis B-related cirrhosis, alcoholic cirrhosis and autoimmune hepatitis-related cirrhosis. Immunohistochemical analysis also revealed an enhanced hepatic TRPM8 expression in cirrhotic tissues compared to normal human liver tissues, regardless of etiology (Fig. 1B). Western blot analysis further illustrated an elevated TRPM8 expression in both CCl₄- and BDL-induced liver fibrotic mice (Fig. 1C, D). Taken together, these data suggest that TRPM8 may be involved in hepatic fibrogenesis.

TRPM8 deficiency attenuates liver fibrosis in mice

As illustrated in Fig. 2A, H&E, Sirius Red and Masson's trichrome staining of the liver tissue all showed CCl₄-induced TRPM8^{-/-} mice had significantly attenuated liver injury and fibrosis compared with WT mice. This finding was also supported by the significantly reduced α-SMA and COL1A1 expression in TRPM8 deficient mice. Moreover, serum ALT and AST levels were significantly reduced in TRPM8^{-/-} mice (Fig. 2B). Western blot analysis further showed suppressions of α-SMA and COL1A1 in CCl₄-induced TRPM8 KO group than WT (Fig. 2C). Additionally, CCl₄-treated TRPM8^{-/-} mice had a greater reduction in hepatic mRNA expression of several profibrotic genes, such as α-SMA, COL1A1, TGF-β1, and TIMP-2 (Fig. 2D).

Similar histopathological staining of BDL-injured mice liver tissues also provided direct evidence that knocking out TRPM8 significantly alleviated fibrogenesis. The results of IHC (Fig. 3A) and western blot (Fig. 3C) showed a greatly reduced α-SMA and COL1A1 expression in TRPM8^{-/-} mice compared with the WT. In addition, remarkable reductions in serum ALT and AST levels were noticed in BDL-injured TRPM8^{-/-} mice (Fig. 3B), so were all the mRNA expression of fibrogenic genes (Fig. 3D). These results indicate that TRPM8 deficiency observably improves liver fibrosis.

TRPM8 deficiency ameliorates bile canalicular abnormalities, dysregulation of bile transporters, ductular reaction and liver inflammation in fibrotic liver

Electron microscopy revealed not only irregularly dilated and tortuous bile canaliculi, but also lack of microvilli in CCl₄-treated WT (Fig. 4A). Dysregulation of hepatic bile acid transporters, such as significantly lowered bile salt export pump (BSEP) mRNA and elevated MDR3 mRNA expression were observed in both CCl₄- and BDL-injured WT mice as illustrated in Fig. 4B and 4C, respectively. Furthermore, the levels of CK19 expression in liver tissues of both CCl₄- and BDL-injured WT mice were significantly elevated than that of the injured TRPM8^{-/-} mice (Fig. 4D, F). Moreover, as ductular reaction is often associated with inflammatory cell infiltration in portal region³⁷, murine macrophage-specific F4/80 marker was used to study the inflammatory response of the liver. Expectedly, markedly more F4/80 positive cells were found in liver samples of WT fibrotic mice than that of the TRPM8^{-/-} groups (Fig. 4D, F). Consistently, significant reduction in transcription levels of inflammatory genes and chemokines (such as IL-6, IL-1β, TNFα, and MCP-1) were detected in liver tissues of both CCl₄- and BDL-injured KO mice (Fig. 4E, G).

TRPM8 deficiency alleviates liver fibrosis by regulating S100A9-HNF4α signaling

After filtering the genes with a greater than 2-fold change and a *P*-value of less than 0.05, transcriptomic analysis of mRNAs in CCl₄-injured WT and TRPM8^{-/-} mouse liver revealed 118 upregulated and 39 downregulated genes. Among them, genes are closely related to inflammation and fibrosis, such as S100A9, Col6a3 and CTGF, were noticed to show a significant reduction in TRPM8^{-/-} mice (Fig. 5A). Importantly, as the serum concentration of S100A9 are often elevated in patients with liver cirrhosis than healthy donors (Fig. 5B), we then have investigated the relationship between S100A9, an inflammation-

related factor, and HNF4 α , a well-known master regulator of liver-specific gene expression in fibrogenesis. Results of IHC and western blot assays suggested TRPM8 ablation would lead to the downregulation of S100A9 and upregulation of HNF4 α in CCl $_4$ -injured mice (Fig. 5C, D). Similar trend was also observed in liver specimens obtained from BDL-induced fibrotic mice (Fig. 5E, F). Furthermore, identical modulatory effects of TRPM8 on S100A9 and HNF4 α expression were confirmed in primary hepatocytes isolated from both WT and TRPM8 $^{-/-}$ mice (Fig. 5G), as well as L02 cells (Fig. 5H). The aforementioned negative regulatory relationship between S100A9 and HNF4 α expression was subsequently demonstrated in L02 cells underwent either siRNA-mediated S100A9 knockdown (Fig. 5I) or S100A9 overexpressing plasmid transfection (Fig. 5J). Taken together, these results imply TRPM8 deficiency may attenuate liver fibrosis by downregulating the expression of the pro-inflammatory factor S100A9 while promoting the expression HNF4 α .

Inhibition of TRPM8 ameliorates murine liver fibrosis

TRPM8 deficiency seemed to significantly attenuate the progression of liver fibrosis in fibrotic mice, we then investigated the potential therapeutic effect of a TRPM8 specific inhibitor (M8-B hydrochloride) on liver fibrosis. H&E, Sirius red and Masson's trichrome staining assays verified M8-B treatment was able to delay the development of fibrosis induced by CCl $_4$. Consistently, IHC staining also showed M8-B significantly reduced α -SMA and COL1A1 expression in the liver of fibrotic mice (Fig. 6A). Biochemical tests of the serum samples revealed M8-B markedly reduced both ALT and AST levels, indicating an improved liver functional state (Fig. 6B). Similarly, M8-B treatment led to a noticeable suppression in α -SMA and COL1A1 expression by western blot assay (Fig. 6C); and the mRNA levels of profibrogenic markers, α -SMA, COL1A1, TGF- β 1 and TIMP-2 by qRT-PCR detection (Fig. 6D). Identical treatment was given to BDL-induced liver fibrotic mice, yielded data were likewise comparable (Fig. S1). In summary, these results strongly suggest inhibiting TRPM8 may be a promising therapeutic approach for treating liver fibrosis.

Discussion

The present study shows that TRPM8 is upregulated in both human liver fibrosis tissues and specimens collected from two murine fibrosis models, namely the CCl $_4$ -induced and BDL-treated. Notably, obtained data suggests genetic inactivation of TRPM8 is able to intervene and effectively attenuate the progression of liver fibrosis in mice. Furthermore, the treatment of M8-B, a specific inhibitor of TRPM8, demonstrated the antifibrotic potential in CCl $_4$ and BDL-induced murine models. These findings suggested that TRPM8 could be a novel therapeutic target for liver fibrosis.

Extensive studies have elucidated the key role of inflammation in pathogenesis of liver fibrosis [7]. Activation of proinflammatory cytokines and chemokines, including IL-6, IL-1 β , TNF α and MCP-1, can activate HSCs, promote ECM secretion and deposition, and then exacerbate liver fibrosis [7]. In this study, we found that TRPM8 inhibition could reduce F4/80 positive cell population and inhibit the transcript

levels of IL-6, IL-1 β , TNF α and MCP-1 in murine models of liver fibrosis. These findings suggest TRPM8 ablation may alleviate liver fibrosis through inhibiting inflammatory response.

In addition to its role in fibrosis, inflammation has also been central in discussions related to cholangiopathies, partly owing to the current prevailing view that although the activating injury event is often unknown in many cases, features that characterize activated cholangiocytes include not only increased proliferation, but more importantly elevated level of pro-fibrotic and pro-inflammatory secretions [24, 25]. Results of the TRPM8 inhibition experiment have briefly shown targeting TRPM8 was effective in not only restoring irregularly dilated and tortuous bile canaliculi and dysregulated hepatic bile acid transporters such as BSEP and MDR3, but also attenuating ductular reaction, mainly in term of reduced number of activated cholangiocytes. In general, despite the lack of more in-depth investigation, these data lay the basic groundwork for hypothesizing hepatic fibrosis alleviation might be achievable through an alternative route, that is TRPM8 blockage may lead to less pro-inflammatory cytokines secretion that suppresses the activation of cholangiocytes, and thus improving liver fibrosis.

Furthermore, RNA sequencing with morphological examination were conducted to reveal molecular mechanism of TRPM8 in the pathogenesis of liver fibrosis. Molecules related to inflammation and cholangiopathies were screened and verified using both tissue samples and *in vitro* assays. Dysregulation of S100A9 and HNF4 α were identified in genetic intervened murine fibrotic models. *In vitro* assays also elucidated targeting TRPM8 could give rise to S100A9 - HNF4 α signaling.

S100A9, a member of S100 protein family, is released from either inflammatory cells or damaged cells, and binds to toll-like receptor 4 or the receptor for advanced glycation end products, thereby promoting inflammatory response [26, 27]. A growing body of evidence suggests strong S100A9 association with various inflammatory and fibrotic diseases [28–30]. Our results have also shown S100A9 expression was significantly elevated in serum samples from cirrhosis patients compared to healthy donors, while significant reduction in liver samples from CCl $_4$ or BDL-treated TRPM8 $^{-/-}$ mice. Therefore, it is hypothesized that TRPM8 mediates the inflammatory response by regulating the expression of S100A9.

HNF4 α , the central transcriptional regulator of hepatocyte gene expression, differentiation and function maintenance, has been shown to take a central regulatory role in not only the development of biliary cancer [31, 32], but also the formation of microvilli, maintenance of normal liver function, and alleviation of liver fibrosis [33, 34]. In accordance with these findings, our data show that inhibition of TRPM8 upregulated HNF4 α in genetic models of TRPM8 treated by CCl $_4$ and BDL, suggesting its involvement in the process of liver fibrosis. At the same time, *in vitro* analysis also confirmed that S100A9 negatively regulate expression of HNF4 α in L02 cells. These data suggested that TRPM8 deficiency upregulates the expression of HNF4 α via downregulate of S100A9 expression. Certainly, additional study will be needed to illustrate the detailed mechanism.

In conclusion, our results suggest that TRPM8 inhibition may alleviate liver fibrosis contributed by inflammation and cholangiopathies through S100A9-HNF4 α signaling. TRPM8 may be a potential

therapeutic target for liver fibrosis.

Declarations

Acknowledgments

Not applicable.

Funding

This study was supported by National Natural Science Foundation of China (81670111) and Hunan Provincial Key Research and Development Program (2019SK2242).

Authors' contributions

X.D.X. and X.M.L. conceptualized the study, designed experiments, and contributed data interpretation. Q.L. performed most of the experiments, data acquisition and analyses. X.H.L., Z.Y.C., J.Z. and L.K.Y. contributed to the construction of animal models, provided essential clinical patient specimens. J.F., Q.T., W.Q. and Y.L.S. contributed to mouse liver tissue immunohistochemistry experiments and performed critical assessment of histology. C.L., Z.Q.L., Z.C.W., Y.C. and G.X. contributed to human specimen related administrative, technical or material support. X.M.L., S.Y.L., X.Y.W. performed statistical analyses. Q.L., X.M.L., X.D.X. wrote the manuscript. H.Y. and M.M. provided critical reviews of the manuscript. All authors read and approved the final manuscript.

Data Availability

RNA-seq data have been deposited to Sequence Read Archive (SRA) in The National Center for Biotechnology Information (NCBI) with the accession number PRJNA666513. All the other relevant data of the study, including clinical data, are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

Human samples were processed following standard operating procedures approved by the Ethical Committees of Central South University (Approval No. 2020048). Animal care and procedures were approved by the Animal Care Committee of Central South University (Approval No. 2020308).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

References

1. Friedman SL. Mechanisms of hepatic fibrogenesis. *GASTROENTEROLOGY* 2008;134:1655.
2. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. *Annu Rev Pathol* 2011;6:425.
3. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol* 2017;14:397.
4. Fallowfield JA, Iredale JP. Targeted treatments for cirrhosis. *Expert Opin Ther Targets* 2004;8:423.
5. Bonis PA, Friedman SL, Kaplan MM. Is liver fibrosis reversible? *N Engl J Med* 2001;344:452.
6. Lotersztajn S, Julien B, Teixeira-Clerc F, Grenard P, Mallat A. Hepatic fibrosis: molecular mechanisms and drug targets. *Annu Rev Pharmacol Toxicol* 2005;45:605.
7. Koyama Y, Brenner DA. Liver inflammation and fibrosis. *J CLIN INVEST* 2017;127:55.
8. Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. *HEPATOLOGY* 2015;61:1066.
9. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *NAT REV IMMUNOL* 2017;17:306.
10. Baeck C, Wei X, Bartneck M, Fech V, Heymann F, Gassler N, Hittatiya K, Eulberg D, Luedde T, Trautwein C, Tacke F. Pharmacological inhibition of the chemokine C-C motif chemokine ligand 2 (monocyte chemoattractant protein 1) accelerates liver fibrosis regression by suppressing Ly-6C(+) macrophage infiltration in mice. *HEPATOLOGY* 2014;59:1060.
11. O'Hara SP, Tabibian JH, Splinter PL, LaRusso NF. The dynamic biliary epithelia: molecules, pathways, and disease. *J HEPATOL* 2013;58:575.
12. Tabibian JH, Masyuk AI, Masyuk TV, O'Hara SP, LaRusso NF. Physiology of cholangiocytes. *COMPR PHYSIOL* 2013;3:541.
13. Lazaridis KN, Strazzabosco M, Larusso NF. The cholangiopathies: disorders of biliary epithelia. *GASTROENTEROLOGY* 2004;127:1565.
14. Park SM. The crucial role of cholangiocytes in cholangiopathies. *GUT LIVER* 2012;6:295.
15. Banales JM, Huebert RC, Karlsen T, Strazzabosco M, LaRusso NF, Gores GJ. Cholangiocyte pathobiology. *Nat Rev Gastroenterol Hepatol* 2019;16:269.
16. Clapham DE, Runnels LW, Strubing C. The TRP ion channel family. *NAT REV NEUROSCI* 2001;2:387.
17. Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D. The menthol receptor TRPM8 is the principal detector of environmental cold. *NATURE* 2007;448:204.
18. Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A. A TRP channel that senses cold stimuli and menthol. *CELL* 2002;108:705.
19. Yee NS. Roles of TRPM8 Ion Channels in Cancer: Proliferation, Survival, and Invasion. *Cancers (Basel)* 2015;7:2134.
20. Naumov DE, Perelman JM, Kolosov VP, Potapova TA, Maksimov VN, Zhou X. Transient receptor potential melastatin 8 gene polymorphism is associated with cold-induced airway

- hyperresponsiveness in bronchial asthma. *RESPIROLOGY* 2015;20:1192.
21. Straub RH. TRPV1, TRPA1, and TRPM8 channels in inflammation, energy redirection, and water retention: role in chronic inflammatory diseases with an evolutionary perspective. *J Mol Med (Berl)* 2014;92:925.
 22. Almeida MC, Hew-Butler T, Soriano RN, Rao S, Wang W, Wang J, Tamayo N, Oliveira DL, Nucci TB, Aryal P, Garami A, Bautista D, Gavva NR, Romanovsky AA. Pharmacological blockade of the cold receptor TRPM8 attenuates autonomic and behavioral cold defenses and decreases deep body temperature. *J NEUROSCI* 2012;32:2086.
 23. Seglen PO. Preparation of isolated rat liver cells. *Methods Cell Biol* 1976;13:29.
 24. Lazaridis KN, Strazzabosco M, Larusso NF. The cholangiopathies: disorders of biliary epithelia. *GASTROENTEROLOGY* 2004;127:1565.
 25. Banales JM, Huebert RC, Karlsen T, Strazzabosco M, LaRusso NF, Gores GJ. Cholangiocyte pathobiology. *Nat Rev Gastroenterol Hepatol* 2019;16:269.
 26. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. *BIOCHEM PHARMACOL* 2006;72:1622.
 27. Vogl T, Eisenblatter M, Voller T, Zenker S, Hermann S, van Lent P, Faust A, Geyer C, Petersen B, Roebrock K, Schafers M, Bremer C, Roth J. Alarmin S100A8/S100A9 as a biomarker for molecular imaging of local inflammatory activity. *NAT COMMUN* 2014;5:4593.
 28. Hara A, Sakamoto N, Ishimatsu Y, Kakugawa T, Nakashima S, Hara S, Adachi M, Fujita H, Mukae H, Kohno S. S100A9 in BALF is a candidate biomarker of idiopathic pulmonary fibrosis. *Respir Med* 2012;106:571.
 29. Zhong A, Xu W, Zhao J, Xie P, Jia S, Sun J, Galiano RD, Mustoe TA, Hong SJ. S100A8 and S100A9 Are Induced by Decreased Hydration in the Epidermis and Promote Fibroblast Activation and Fibrosis in the Dermis. *AM J PATHOL* 2016;186:109.
 30. Tammaro A, Florquin S, Brok M, Claessen N, Butter LM, Teske G, de Boer OJ, Vogl T, Leemans JC, Dessing MC. S100A8/A9 promotes parenchymal damage and renal fibrosis in obstructive nephropathy. *CLIN EXP IMMUNOL* 2018;193:361.
 31. Watt AJ, Garrison WD, Duncan SA. HNF4: a central regulator of hepatocyte differentiation and function. *HEPATOLOGY* 2003;37:1249.
 32. Saha SK, Parachoniak CA, Ghanta KS, Fitamant J, Ross KN, Najem MS, Gurumurthy S, Akbay EA, Sia D, Cornella H, Miltiadous O, Walesky C, Deshpande V, Zhu AX, Hezel AF, Yen KE, Straley KS, Travins J, Popovici-Muller J, Gliser C, Ferrone CR, Apte U, Llovet JM, Wong KK, Ramaswamy S, Bardeesy N. Mutant IDH inhibits HNF-4alpha to block hepatocyte differentiation and promote biliary cancer. *NATURE* 2014;513:110.
 33. Yue HY, Yin C, Hou JL, Zeng X, Chen YX, Zhong W, Hu PF, Deng X, Tan YX, Zhang JP, Ning BF, Shi J, Zhang X, Wang HY, Lin Y, Xie WF. Hepatocyte nuclear factor 4alpha attenuates hepatic fibrosis in rats. *GUT* 2010;59:236.

34. Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. NAT GENET 2003;34:292.

Figures

Figure 1

TRPM8 is highly expressed in the livers of cirrhotic patients and fibrotic mice. (A) Representative images of immunohistochemical staining of TRPM8 in healthy control and cirrhotic human liver tissues, and its statistical summary. Scale bars, 100 μ m. (B) Cirrhotic patients were divided into three different categories according to their etiologies. chronic hepatitis B (CHB), alcohol-related liver disease (ALD), autoimmune hepatitis (AIH). (C, D) Representative western blot analysis of hepatic TRPM8 expression in CCl₄ and BDL-induced liver fibrosis model (n = 3 per group). Results are expressed as the mean \pm SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Figure 2

Liver fibrosis is attenuated in TRPM8^{-/-} mice after CCl₄ treatment.

(A) Representative histology of H&E, Sirius Red, Masson's trichrome and IHC staining for α -SMA and COL1A1 in the liver of WT and TRPM8^{-/-} mice induced by CCl₄ (n = 5 per group). Quantification of positive staining areas were measured by Image J software. Scale bars, 100 μ m. (B) Liver function was assessed by measuring the serum levels of ALT and AST in mice (n = 5 per group). (C) Immunoblotting analyses of α -SMA and COL1A1 expression in the mouse liver (n = 3 per group). (D) Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR (n = 5 per group). Results are expressed as the mean \pm SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Figure 3

TRPM8 deficiency alleviates BDL-induced liver fibrosis in mice.

(A) Representative images of H&E, Sirius Red, Masson's trichrome and IHC staining for α -SMA and COL1A1 in the liver of WT and TRPM8^{-/-} mice operated with BDL (n = 5 per group). Quantification of positive staining areas were measured by Image J software. Scale bars, 100 μ m. (B) Liver function was assessed by measuring the serum levels of ALT and AST in mice (n = 5 per group). (C) Immunoblotting

analyses of α -SMA and COL1A1 expression in the mouse liver (n = 3 per group). (D) Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR (n = 5 per group). Results are expressed as the mean \pm SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure 4

TRPM8 deficiency attenuates bile canalicular abnormalities, dysregulation of bile transporters, ductular reaction and liver inflammation in fibrotic liver.

(A) Electron microscopy images showing bile canaliculi (arrows) in liver sections obtained from CCl₄-treated WT and TRPM8^{-/-} mice. (B, C) Hepatic mRNAs of bile acid transport genes were measured by qRT-PCR in liver specimens obtained from CCl₄- or BDL-treated WT and TRPM8^{-/-} mice (n = 5 per group). (D, F) Histology of CK19 and F4/80 IHC staining in liver sections obtained from CCl₄- or BDL-treated WT and TRPM8^{-/-} mice. Scale bars, 100 μ m. (E, G) Hepatic mRNAs of IL-6, IL-1 β , TNF α and MCP-1 were measured by qRT-PCR in liver specimens obtained from CCl₄- or BDL-treated WT and TRPM8^{-/-} mice (n = 5 per group). Results are expressed as mean \pm SD. * P < 0.05, ** P < 0.01.

Figure 5

TRPM8 regulates the expression of S100A9 and HNF4 α .

(A) Volcano plot and Heatmap presentation of significantly up- and downregulated genes in liver specimens obtained from CCl₄-treated TRPM8^{-/-} mice compared to WT (n = 3 per group). (B) Serum S100A9 expressions in liver cirrhosis patients and healthy donors. (C, E) Histology of S100A9 and HNF4 α IHC staining in liver sections obtained from CCl₄- or BDL-treated WT and TRPM8^{-/-} mice (n = 5 per group). Scale bars, 50 μ m. (D, F) Expressions of S100A9 and HNF4 α were detected by IHC using liver specimens obtained from CCl₄- or BDL-treated WT and TRPM8^{-/-} mice (n = 3 per group). (G) Immunoblotting assays of TRPM8, S100A9 and HNF4 α in primary hepatocytes from WT and TRPM8^{-/-} mice. (H) Immunoblotting assays of TRPM8, S100A9 and HNF4 α in L02 cells transfected with TRPM8 siRNA after 48 hours. (I, J) Immunoblotting assays of S100A9 and HNF4 α in L02 cells transfected with S100A9 siRNA or plasmid after 48 hours. Results are expressed as mean \pm SD. ** P < 0.01.

Figure 6

TRPM8 inhibitor mitigates liver fibrosis in CCl₄-treated mice.

(A) H&E, Sirius Red, Masson's trichrome and IHC staining for α -SMA and COL1A1 in liver sections of CCl₄-treated mice (n = 5 per group). Image J was used to quantify positive stained areas. Scale bars, 100 μ m. (B) Serum levels of ALT and AST were measured in mice (n = 5 per group). (C) Expressions of α -SMA and COL1A1 were detected by immunohistochemistry (n = 3 per group). (D) Hepatic mRNAs of fibrogenic genes were measured by qRT-PCR assays in mice treated with M8-B or DMSO after CCl₄ induction (n = 5 per group). Results are expressed as mean \pm SD. * P < 0.05, ** P < 0.01.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.tif](#)
- [FigureS1.tif](#)
- [SupplementaryTableS1.docx](#)