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Original Research

Myocyte Enhancer Factor 2A Contributes to the TGF-β1-Mediated Cholangiocyte Epithelial to Mesenchymal Transition and Senescence in Cholestatic Liver Fibrosis

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Abstract

Background: Cholangiocytes are primary targets in chronic cholestatic liver diseases. Myocyte enhancer factor 2A (MEF2A) is a transcription factor with a crucial role in some fibrogenic diseases. However, whether it contributes to cholestatic liver fibrosis is still obscure. **Methods**: A bile duct–ligated (BDL) mouse model was established to detect MEF2A expression during cholestatic liver fibrosis. In addition, human intrahepatic biliary epithelial cells (HIBECs) were transfected with lentivirus-expressing shMEF2A (LV-shMEF2A) to regulate the expression of MEF2A *in vitro*. Biomarkers of epithelial to mesenchymal transition (EMT), senescence, and fibrogenesis were evaluated using various assays: Quantitative real-time polymerase chain reaction (qRT-PCR), western blotting, senescence-associated β-galactosidase (SA-β-gal), and immunofluorescence. Furthermore, MEF2A expression and cytoplasm translocation induced by transforming growth factor β1 (TGF-β1) in HIBECs were determined by qRT-PCR, western blotting, and immunofluorescence. The expression of TGF-β1-induced MEF2A, EMT, senescence, and fibrosis markers inhibited by p38 MAPK signaling were evaluated by western blotting. Finally, the peripheral blood from primary biliary cholangitis (PBC) patients and healthy controls (HCs) was collected to analyze expression of MEF2A using Enzyme-linked immunosorbent assay (ELISA). **Results**: We found that MEF2A expression increased in liver tissues of BDL mice, and positively related to the extent of fibrosis. Silencing MEF2A in HIBECs restrained TGF-β1-induced EMT, senescence, and fibrotic reaction. Moreover, TGF-β1 enhanced the expression of MEF2A and induced its cytoplasm translocation in a concentration- and time-dependent manner, partially through interacting with p38 MAPK. The expression of MEF2A was also higher in the serum of PBC patients than in HCs, and positively correlated with fibrosis degree. **Conclusions**: Our study demonstrates that MEF2A is a central mediator linking TGF-β1-induced EMT and senescence in HIBECs. We propose it as a novel biomarker of fibrogenesis in cholestatic liver fibrosis. We also suggest inhibiting MEF2A as a potential strategy in treating cholestatic liver fibrosis.

Keywords: MEF2A; EMT; senescence; fibrosis; cholangiocytes

1. Introduction

Cholestatic liver diseases, such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), are characterized by an impairment of bile secretion or excretion, usually lead to liver fibrosis and cirrhosis, and even liver failure [1,2]. The clinical management dilemma of these diseases are linked with poor understanding of their pathogenesis, late diagnosis and lack of effective cure except for liver transplant [2]. Liver fibrosis plays a crucial role in the disease's progression and is closely related to clinical outcomes in cholestatic liver diseases [3]. Therefore, developing new strategies for ameliorating or reversing fibrogenesis is paramount to managing these diseases.

Cholangiocytes are the principal cells responsible for cholestasis and liver fibrosis. They can activate hepatic stellate cells (HSCs) and fibroblasts, causing overproduction of extracellular matrix and fibrotic progression [4]. Cholangiocytes may also act as fibrogenic cells in the liver by undergoing epithelial to mesenchymal transition (EMT) and enhanced senescence [5,6]. Liver sections from patients with PBC contain cholangiocytes with enhanced EMT markers, suggesting that preventing EMT may restrain or reverse liver fibrosis [7]. Cholangiocyte senescence and its senescence-associated secretory phenotype are hallmarks of PBC, contributing to the paracrine activation of HSCs and increased liver fibrosis [8]. Evidence shows transforming growth factor β1 (TGF-β1) is the most potent cytokine that perpetuates the fibrogenic response of the liver. It may do so by positively regulating EMT and senescence of cholangiocytes [9,10].

Myocyte enhancer factor 2A (MEF2A) is a transcription factor from the MEF2 family within the MADS-box superfamily [11]. It participates in numerous cellular pro-

cesses, including neuronal differentiation, muscular development, and cellular growth control [12]. The important roles of MEF2A in human cancers, cardiovascular diseases, and neurodegenerative diseases have been demonstrated by a great deal of evidence [13–17]. Further, it may also have a role in liver fibrosis. For example, MEF2A is involved in the activation of cultured rat HSCs [18]. Furthermore, high levels of MEF2A are present in human cirrhotic liver tissues and freshly isolated human HSCs [19]. By consulting the HUMAN PROTEIN ATLAS database (https://www.proteinatlas.org/), we found that MEF2A was not only expressed in HSCs, but also in cholangiocytes/bile duct epithelial cells. However, whether and how cholangiocyte-derived MEF2A participates in cholestatic liver fibrosis are still unclear.

In the present study, we quantified the levels of MEF2A in liver sections from bile duct–ligated (BDL) mice and peripheral blood from patients with PBC to explore its involvement in cholestatic liver fibrosis. We also assessed the function of MEF2A in TGF-β1-induced EMT, senescence, and fibrosis in human intrahepatic biliary epithelial cells (HIBECs). Our findings identify MEF2A as a novel inducer of cholestatic liver fibrosis.

2. Materials and Methods

2.1 Experimental Animals

Eight- to ten-week-old specific pathogen-free C57BL/6 male mice were maintained under specific pathogen-free conditions, and offered free access to sterile water and food. Their weight ranged between 20 and 25 g. Animal experiments were approved by the Institutional Animal Care and Use Committee of Jining Medical University.

2.2 Bile Duct Ligation (BDL) Mouse Model

After a week of adaptive feeding, the mice were randomly assigned to a sham (n = 10) or BDL group (n = 20). BDL was performed as previously described [20]. Briefly, the mice were anesthetized with 2% pentobarbital sodium (40 mg/kg) (Sigma-Aldrich, St. Louis, USA). We first isolated the common bile duct and the left and right hepatic ducts, and then ligated the left and right hepatic ducts and the hepatic portal and duodenal portions of the common bile duct, respectively. At last, the abdomen was closed. The sham group was used as a control and underwent laparotomy without ligation. After 7 or 28 days, the mice were killed and their liver tissues were harvested. It was further manipulated for qRT-PCR, western blotting, histological, and immunohistochemical analyses.

2.3 Histology and Fluorescent Immunohistochemistry of Mouse Liver

Paraformaldehyde-fixed liver tissue samples were cut into 4-μm-thick paraffin-embedded sections and stained with hematoxylin-eosin. They were viewed under a light microscope to assess the fibrosis stage according to the METAVIR scoring system [21]. Before immunostaining, antigen retrieval was performed with 10 mM sodium citrate buffer in microwave. Sequentially, permeabilization was performed with TritonX-100 (Solarbio, Beijing, China). Slices were incubated with 5% Bovine Serum Albumin (BSA) in phosphate buffer saline (PBS) for 30 min at room temperature and then incubated at 37 °C for 1 h with a primary anti-MEF2A antibody (Abcam, Cambridge, UK, 1:500). After rinsing 3 times with PBS, slices were incubated with fluorescently conjugated secondary antibodies at room temperature for 15 min. The sections were washed 5 times in PBS and 3 times in 5% BSA, followed by adding 15 μL mounting media containing DAPI stain and add a coverslip. Scanning of slices was performed using a laser scanning confocal microscope (Zeiss LSM800, Oberkochen, Germany).

2.4 Lentiviral Vectors for MEF2A Silencing

Lentiviral vectors expressing an shRNA targeted against the murine MEF2A transcript (LV-shMEF2A) or negative control (LV-NC) were constructed by GenePharma (China). The shMEF2A targeting sequence was 5′-GCAGCCAGCTCAACGTTAACA-3′, and that of the negative control was 5′-TTCTCCGAACGTGTCACGT-3′.

2.5 Cell Culture and Lentiviral Transfection

Human intrahepatic biliary epithelial cells (HIBECs) were purchased from the BeNa Culture Collection (China). They were cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL) at 37 °C and 5% CO_2. The cells (1×10^5 per well) were transfected with LV-shMEF2A or LV-NC following the manufacturer's protocol (multiplicity of infection = 30). In brief, LV-shMEF2A or LV-NC was mixed with 10 mg/mL polybrene and added to the cell suspension. The cell culture medium was replaced with fresh RPMI 1640 12 to 16 h after transfection, and the transfected cells were cultured continuously. Green fluorescent protein was detected by fluorescence microscopy to assess the transfection efficiency 72 h after transfection.

2.6 qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA). Complementary DNA (cDNA) was reverse transcribed with an All-In-One 5× RT reagent kit (abm, Zhenjiang, Jiangsu, China) following the manufacturer's protocol, and was stored at –20 °C until use. The quantitative PCR assay was performed using an UltraSYBR Mixture (CWBIO, Taizhou, Jiangsu, China) with the following conditions: 1 min at 95 °C, 40 cycles at 95 °C, 15 s; and 40 cycles at 60 °C, 30 s. The primers were synthesized by ShengGong Biotech (Shanghai, China), and their sequences are listed in Table 1. The relative expression of GAPDH was used as an endogenous reference to quantify the relative mRNA expression of target genes. The tran-

Table 1. The primers using in qRT-PCR analysis.

Gene	Species	DNA sequence (sense 5′–3′)	DNA sequence (anti-sense 5′-3′)
MEF2A	Human	TGCGACAGCCCAGACCCTG	GAGGTGGCAGACCAGGTGCG
MEF2A	Mouse	CAGGTGGTGGCAGTCTTGG	TGCTTATCCTTTGGGCATTCAA
α-SMA	Human	GGCTCT GGGCTCTGTAAGG	CTCTTGCTCTGG GCTTCATC
E-cadherin	Human	TTCTGCTGCTCTTGCTGTTT	TGGCTCAAGTCAAAGTCCTG
N-cadherin	Human	GGTGGAGGAGAAGAAGACCAG	GGCATCAGG CTCCACAGT
vimentin	Human	GCCCTTAAAGGAACCAATGA	AGCTTCAACGGCAAAGTTCT
GAPDH	Human	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

script level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7 Western Blotting

Cells were lysed using a RIPA lysis buffer containing 10% phosphatase and proteinase inhibitor (Beyotime, Shanghai, China). An equal amount of protein was loaded and separated on 10% sodium dodecyl sulphate-polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). They were blocked with 5% non-fat milk for 2 h at room temperature and incubated overnight at 4 °C with primary antibodies: anti-MEF2A (Abcam, 1:1000), anti-E-cadherin (Abcam, 1:1000), anti-N-cadherin (Abcam, 1:1000), anti-vimentin (Abcam, 1:1000), anti-α-SMA (Abcam, 1:1000), anti-P21 (Abcam, 1:1000), anti-p38 (Danvers, MA, USA, 1:1000), anti-JNK (CST, 1:1000), anti-ERK (CST, 1:1000), or anti-GAPDH (Boster Bio, Shanghai, China, 1:1000). The HRP-conjugated secondary antibodies were added to the membranes and incubated for 2 h at room temperature. The membranes were visualized in a drak room using an ECL reagent (Beyotime) following the manufacturer's instructions.

2.8 Senescence-Associated β-Galactosidase (SA-β-Gal) Assay

The SA-β-Gal activity was assessed using a senescence β-galactosidase staining kit (Beyotime) by the manufacturer's instructions. Briefly, the cells were washed with PBS and fixed with SA-β-Gal staining fixative for 15 min at room temperature. After rewashing twice with PBS, the SA-β-Gal staining solution was added, and the cells were incubated overnight at 37 °C in the dark. The cells were observed under a light microscope, and the senescent cells were identified by blue color.

2.9 Cell Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. They were permeabilized with 0.01% Triton X-100 (Solarbio, Beijing, China) for 5 min and blocked in 10% fetal bovine serum for 1 h at room temperature. For the staining, the cells were incubated with a primary rabbit anti-α-SMA (Abcam, 1:500) overnight at 4 °C, and then incubated with an Alexa Fluor 488- or 590-conjugated secondary antibody (Santa Cruz Biotechnology, California, USA, 1:200) for 1 h. Nuclei were visualized with 4′-6-diamidino-2-phenylindole (Beyotime, 5 mg/mL), and images were captured using a confocal microscope (Zeiss LSM800, Oberkochen, Germany, Germany).

Table 2. Baseline clinical characteristics of analyzed PBC patients.

Parameters	Patients (n = 15)
Age (years)	56.5 ± 9.3
Gender	
Male	3
Female	12
Duration of PBC (years)	5.9 ± 5.2
Liver stiffness measurement (LSM, kPa)	12.0 ± 5.7

2.10 Patients

Serum MEF2A levels of 15 patients with PBC and 15 healthy controls (HCs) was analyzed in this study. All participants were inpatients from the Department of Gastroenterology, Affiliated Hospital of Jining Medical University, China. All fulfilled the requirements for PBC diagnosis specified in the European Association for the Study of the Liver clinical practice guidelines [22]. The participants gave their written informed consent to partake in the study. The Institutional Review Board for Clinical Research of the Affiliated Hospital of Jining Medical University approved the study. Clinical characteristics of included patients are shown in Table 2.

2.11 ELISA

Serum MEF2A levels were tested using a human MEF2A ELISA kit (FineTest, China). Peripheral blood was collected in EDTA anti-coagulated tubes and centrifuged at 1000 × g and 4 °C for 20 min. The supernatant was aspirated, and the assay was performed following the manufacturer's instructions. Briefly, the capture antibody was incubated in 96-well plates at 4 °C overnight. After blocking with assay diluents, the standards and samples were added to the designated wells and incubated at 37 °C for 2 h. The plates were incubated with the detection antibody for 1 h and Horseradish Peroxidase (HRP) for 30 min. They were washed with 0.05% Tween-PBS before adding the 3,3',5,5'-tetramethylbenzidine sulfate (TMB) substrate to develop color. Absorbance was measured using a mi-

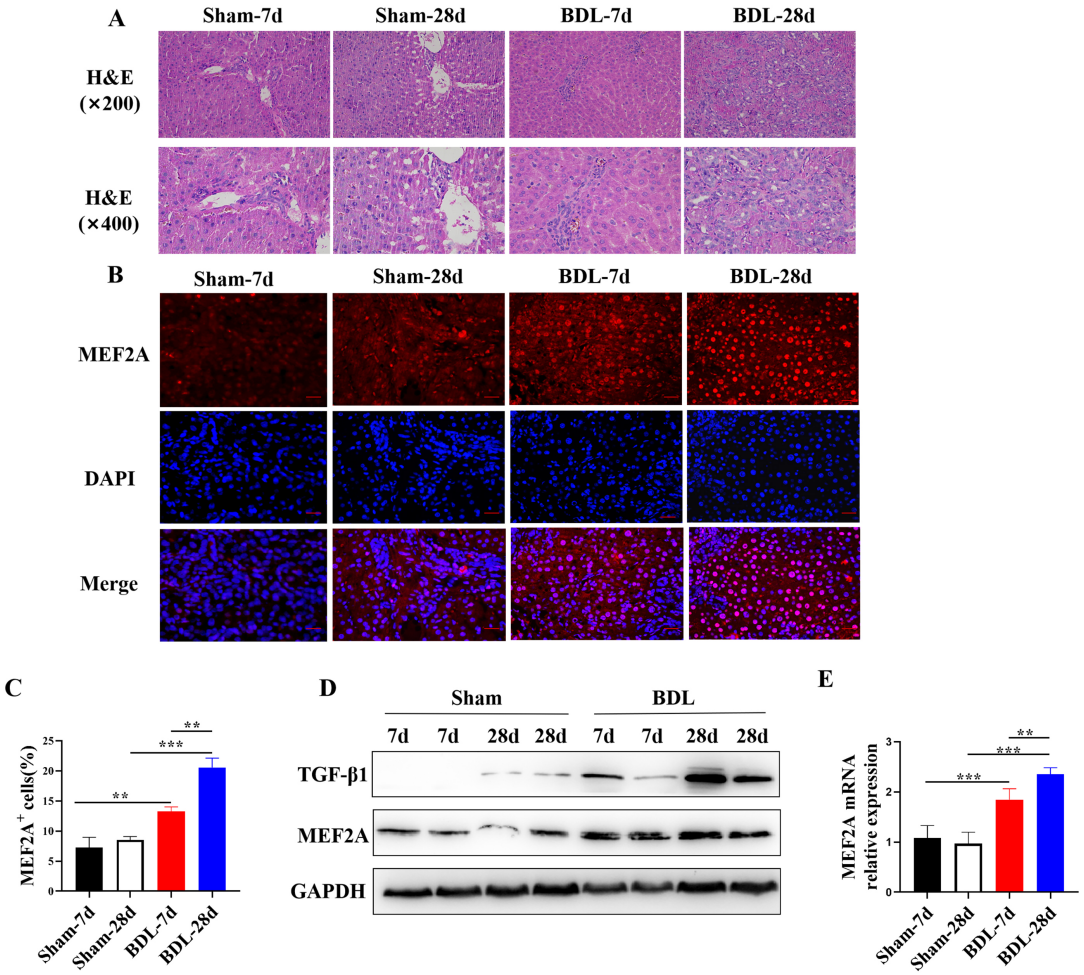

Fig. 1. MEF2A is overexpressed in livers of BDL mice and positively correlates with degree of fibrosis. Bile duct-ligated model was established using C57BL/6 mice to induce liver fibrosis. Sham mice were used as control. Mice were sacrificed 7 or 28 days after BDL surgery, and liver tissues were harvested. (A) Histological examination of liver sections with H&E staining. Scale bar represents 50 μm. (B) Expression of MEF2A in liver tissues of BDL and sham mice detected with immunohistochemical staining using second antibodies labeled with fluorochromes. Original magnification $\times$ 200. Scale bar represents 50 μm. (C) Percentages of MEF2A$^+$ cells in mice livers shown in (B). $^{**}p < 0.01$, $^{***}p < 0.001$. (D) MEF2A and TGF-β1 protein expression in liver tissues of BDL and sham mice identified by western blotting. Reference protein was GAPDH. (E) Relative MEF2A mRNA expression in liver tissues of BDL and sham mice examined by qRT-PCR. Gene expression was normalized to GAPDH in each group. $^{**}p < 0.01$, $^{***}p < 0.001$.

croplate spectrophotometer (BioTek, Vermont, USA).

2.12 Statistical Analysis

All experiments were performed in triplicates, and data were expressed as the mean $\pm$ standard deviation (SD). Differences between two groups were analyzed with the *t* test. A one-way ANOVA was recruited to compare differences among multiple groups. Statistical Package for Social Sciences (v 20.0) (Chiago, IL, USA) was used for all data analyses. Significant differences were considered when $p < 0.05$.

3. Results

3.1 MEF2A is Considerably Overexpressed in Livers of BDL Mice

Bile duct-ligated mice are a widely used model to study cholestatic liver fibrosis, including PBC. Therefore, we examined the expression of MEF2A in the livers of BDL mice. They were sacrificed 7 or 28 days after BDL surgery, and their liver tissues were harvested. Histological examination of liver sections showed that BDL surgery induced liver fibrosis in mice (Fig. 1A), confirming the successful establishment of the BDL model. Fluorescent immunohistochemistry analysis localized MEF2A expression in the liver and revealed that the percentage of MEF2A$^+$ cells in BDL-mice was markedly higher than the control (Fig. 1B,C). We also examined protein levels of MEF2A

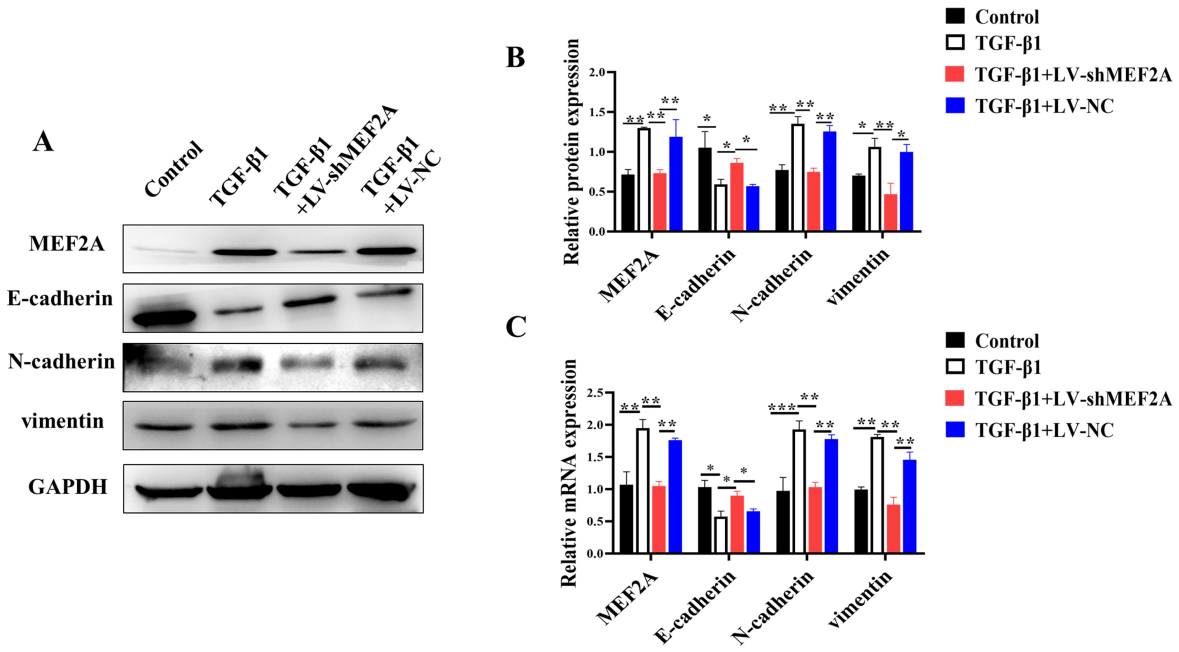

Fig. 2. Silencing MEF2A inhibits TGF-β1-induced EMT in HIBECs. HIBECs were cultured and treated with TGF-β1 (15 ng/mL) or in combination with LV-shMEF2A (lentiviral vector expressing shRNA against MEF2A) or LV-NC (empty vector). (A) Expression of MEF2A protein and EMT markers in HIBECs assessed by western blotting. Reference protein was GAPDH. (B) Quantification of MEF2A and EMT markers in cells depicted in (A). *$p < 0.05$, **$p < 0.01$. (C) Relative expression of MEF2A and EMT markers evaluated by qRT-PCR. Gene expression was normalized to GAPDH in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

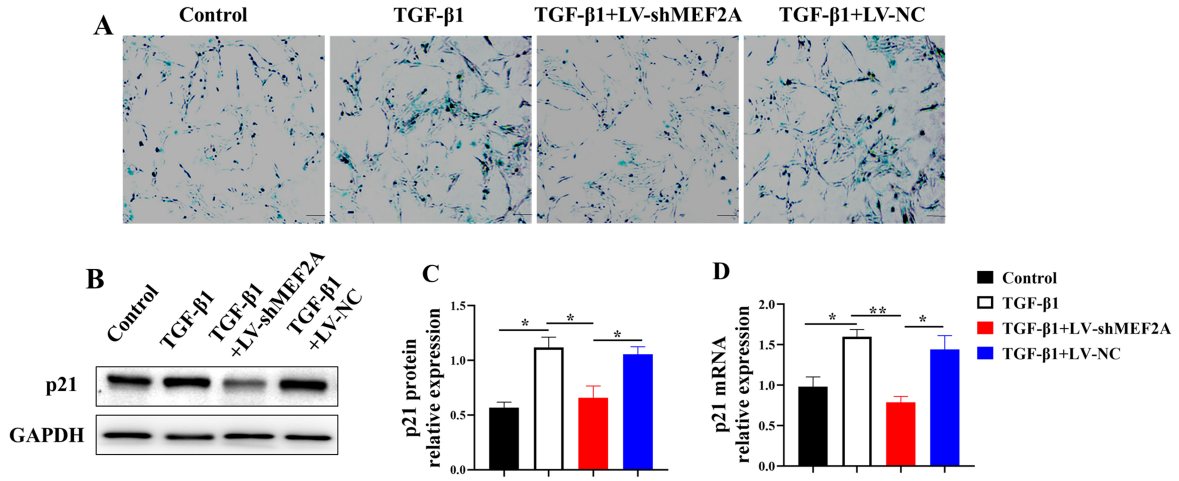

Fig. 3. Silencing MEF2A decreases TGF-β1-induced cellular senescence in HIBECs. HIBECs were cultured and treated with TGF-β1 (15 ng/mL) or in combination with LV-shMEF2A (lentiviral vector expressing shRNA against MEF2A) or LV-NC (empty vector). Cells were harvested 96 h posttreatment to determine senescence. (A) SA-β-galactosidase staining of the senescent cells (blue). Original magnification $\times$ 100. Scale bar = 50 μm. (B) Expression of p21, a senescence marker, in HIBECs detected by western blotting. Reference protein was GAPDH. *$p < 0.05$, **$p < 0.01$. (C) Quantification of p21 levels in cells shown in (B). (D) Relative expression of p21 mRNA evaluated by qRT-PCR. Gene expression was normalized to GAPDH in each group. *$p < 0.05$, **$p < 0.01$.

and TGF-β1 in the liver by western blotting. In BDL-mice liver tissues, which were substantially increased compared with those of the control (Fig. 1D). Moreover, MEF2A mRNA levels were consistent with protein levels (Fig. 1E).

3.2 Silencing MEF2A Inhibits TGF-β1-Induced EMT in HIBECs

Evidence suggests that TGF-β1 induces EMT in cultured cholangiocytes [23]. Hence, we assessed whether silencing MEF2A affects TGF-β1-induced EMT. We treated HIBECs with TGF-β1 or in combination with a lentiviral

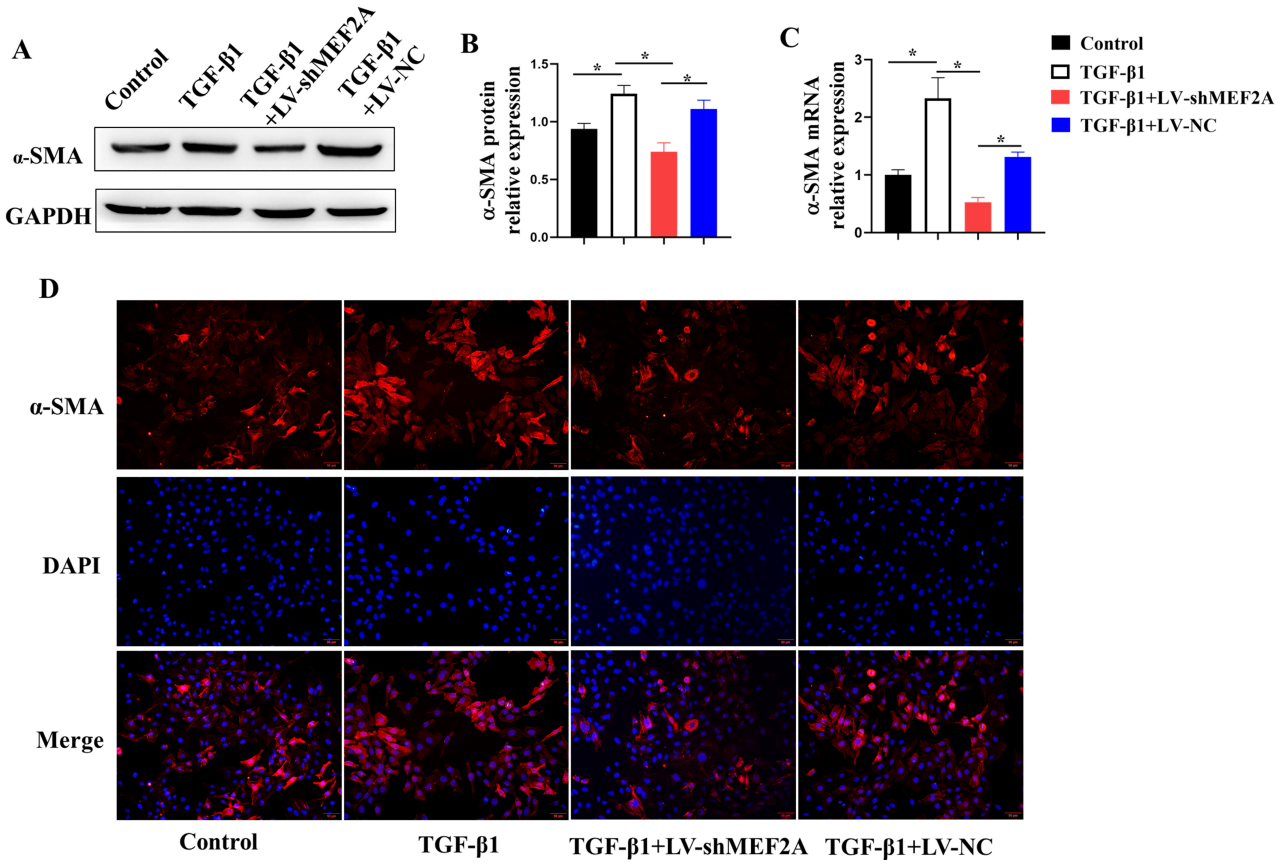

Fig. 4. Silencing MEF2A alleviates fibrogenesis in HIBECs. HIBECs were cultured and treated with TGF-β1 (15 ng/mL) or in combination with LV-shMEF2A (lentiviral vector expressing shRNA against MEF2A) or LV-NC (empty vector). (A) Expression of α-SMA protein in HIBECs identified by western blotting. (B) Quantification of α-SMA protein levels in cells shown in (A). (C) Relative expression of α-SMA mRNA in HIBECs evaluated with qRT-PCR. *$p < 0.05$, **$p < 0.01$ vs. negative control. (D) Immunofluorescence assay demonstrating α-SMA expression in HIBECs. Original magnification $\times$ 200. Scale bar = 50 μm. Red, α-SMA$^+$ cells; blue, DAPI-counterstained nuclei.

vector expressing shRNA against MEF2A (LV-shMEF2A) or the empty vector (LV-NC). Western blotting and qRT-PCR assays verified the silencing of MEF2A with approximately 50% knock-down efficiency compared with control and LV-NC groups (Fig. 2). In addition, TGF-β1 significantly decreased the expression of E-cadherin but increased N-cadherin and vimentin, which were significantly altered in the presence of LV-shMEF2A (Fig. 2). These results indicate that MEF2A acts as a positive modulator of EMT in HIBECs.

3.3 Silencing MEF2A Decreases TGF-β1-Induced Cellular Senescence in HIBECs

Cholangiocyte senescence contributes to the pathogenesis of PBC and liver fibrosis [24]. HIBECs were transfected with TGF-β1 in combination with LV-shMEF2A or LV-NC to investigate the effect of MEF2A on their senescence. About 96 h post-transfection, cells were stained with SA-β-gal assay, and those positive for SA-β-gal activity were determined. TGF-β1 substantially increased the number of senescent HIBECs than that in the control group. When MEF2A was silenced, the proportion of the positive cells in the LV-shMEF2A-transfected group was significantly lower than that in LV-NC-transfected group (Fig. 3A). Thus, silencing MEF2A decreases the number of senescent HIBECs. Moreover, qRT-PCR and western blotting revealed that transcript and protein levels of p21, a marker of senescence [25], significantly decreased after the MEF2A knockdown (Fig. 3B,C). These data suggest that MEF2A silencing reduces HIBEC senescence.

3.4 Silencing MEF2A Alleviates Fibrogenesis in HIBECs

TGF-β1 is considered the most potent cytokine that maintains the fibrogenic response in the liver. We treated HIBECs with TGF-β1 or in combination with LV-shMEF2A or LV-NC to explore whether MEF2A affects the TGF-β1-promoted fibrogenic response in these cells. Expression levels of profibrogenic marker α-SMA were examined by qRT-PCR, western blotting, and immunofluorescence assays. The TGF-β1 treatment significantly increased protein and mRNA levels of α-SMA in HIBECs compared with the control (Fig. 4A–C). Conversely,

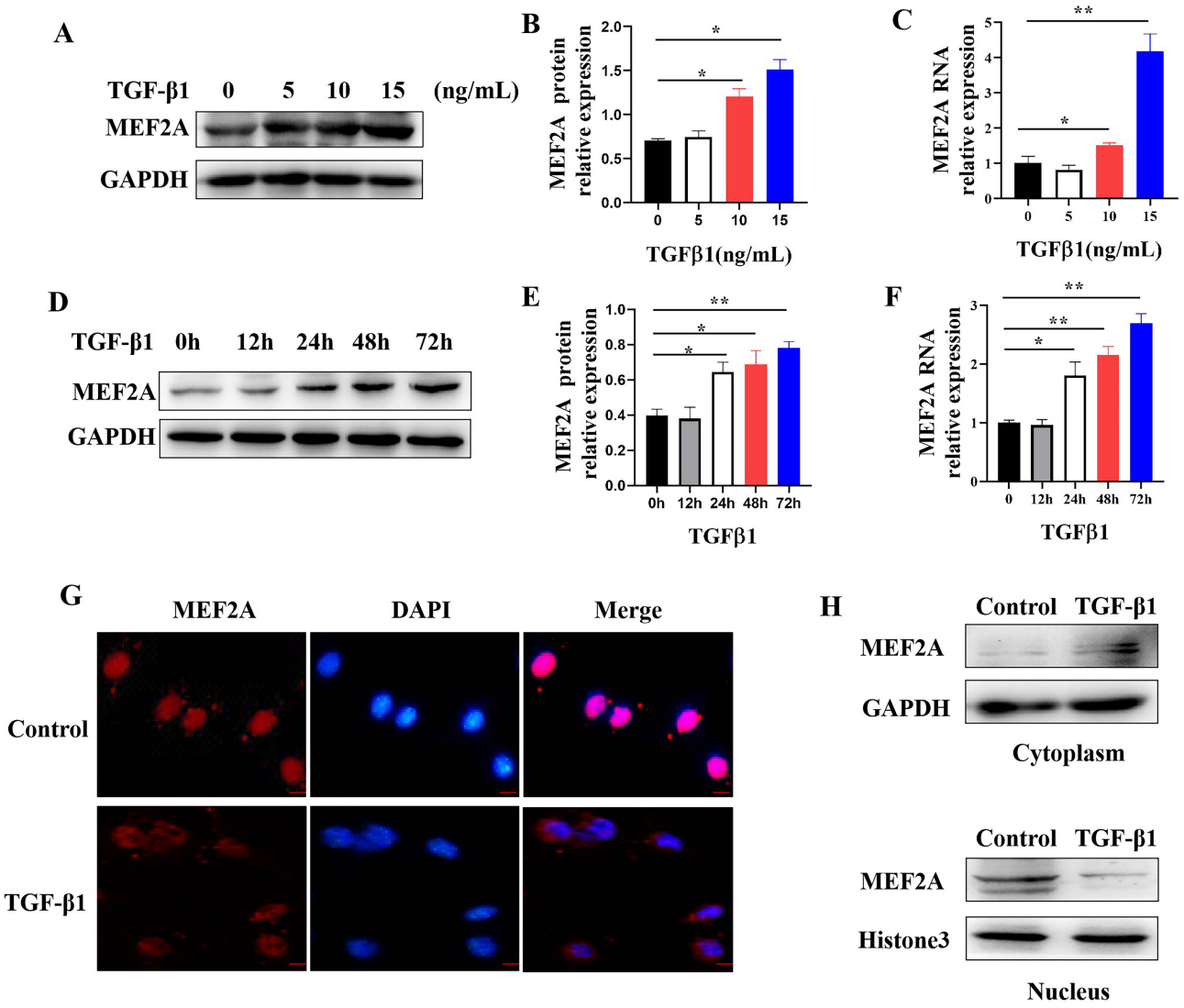

Fig. 5. Regulating MEF2A expression and cytoplasm translocation by TGF-β1 in HIBECs. (A–C) HIBECs were treated for 48 h with TGF-β1 at various concentrations: 0, 5, 10, and 15 ng/mL. Expression levels of MEF2A protein and mRNA were examined across different TGF-β1 concentrations by western blotting and qRT-PCR. *$p < 0.05$, **$p < 0.01$ vs. control. (D–F) HIBECs were treated with TGF-β1 (15 ng/mL) at various time points: 0, 12, 24, 48, and 72 h. MEF2A protein and mRNA expression levels were analyzed at each time point by western blotting and qRT-PCR. *$p < 0.05$, **$p < 0.01$ vs. control. (G) Immunofluorescence analysis of MEF2A localization in HIBECs. Red, MEF2A; blue, DAPI-counterstained nuclei (scale bar = 50 μm). (H) Western blotting assay demonstrating MEF2A levels in cytoplasm and nuclei of HIBECs.

MEF2A knockdown significantly decreased the levels of α-SMA induced by TGF-β1 (Fig. 4A–C). Immunofluorescence staining of HIBECs also demonstrated elevated α-SMA protein under the TGF-β1 treatment and its reduction upon MEF2A knockdown (Fig. 4D). These results imply that MEF2A promotes fibrogenesis in HIBECs.

3.5 Regulating MEF2A Expression and Cytoplasm Translocation via p38 MAPK Signaling Mediates TGF-β1-Induced EMT, Senescence, and Fibrosis in HIBECs

TGF-β1 is a central regulator of EMT, senescence, and liver fibrosis. We stimulated HIBECs with TGF-β1 to determine whether it could induce MEF2A expression and cytoplasm translocation. We determined its expression and subcellular localization with qRT-PCR, western blotting and immunofluorescence assays. The TGF-β1 treatment increased the MEF2A expression in HIBECs in a concentration- and time-dependent manner (Fig. 5A–F). Furthermore, TGF-β1 induced its translocation from the nucleus to the cytoplasm (Fig. 5G,H).

The MAPK pathway may play a role in the pathogenesis of tissue fibrosis, acting downstream of TGF-β1 [26], and p38 directly activates MEF2A, promoting its nuclear activation [27]. Hence, we tested the roles of MAPK signalings (p38, JNK, and ERK) in TGF-β1-induced MEF2A

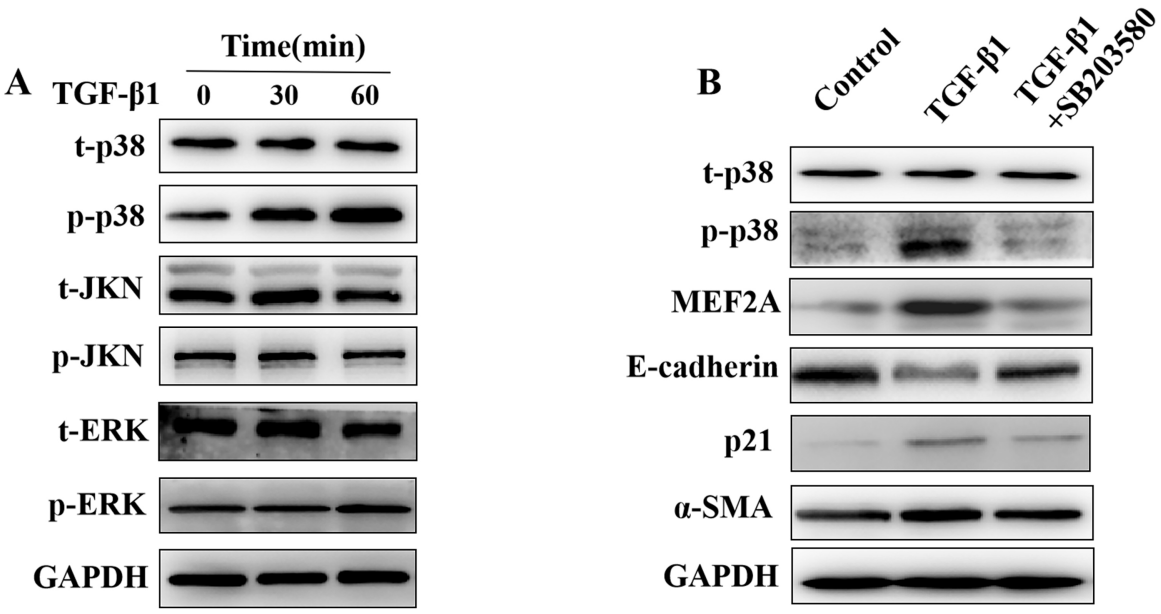

Fig. 6. Regulation of the expression of MEF2A, E-cadherin, p21, and α-SMA by TGF-β1-p38 MAPK in HIBECs. (A) HIBECs were treated with TGF-β1 for different lengths of time (0, 30 min, and 60 min). Levels of phosphorylated p38 (p-p38), total p38 (t-p38), p-JNK, t-JNK, p-ERK and t-ERK were determined by western blotting. (B) Western blotting assay showing p-p38, p38, MEF2A, E-cadherin, p21, and α-SMA levels in HIBECs after TGF-β1 treatment with or without SB 203580 inhibitor.

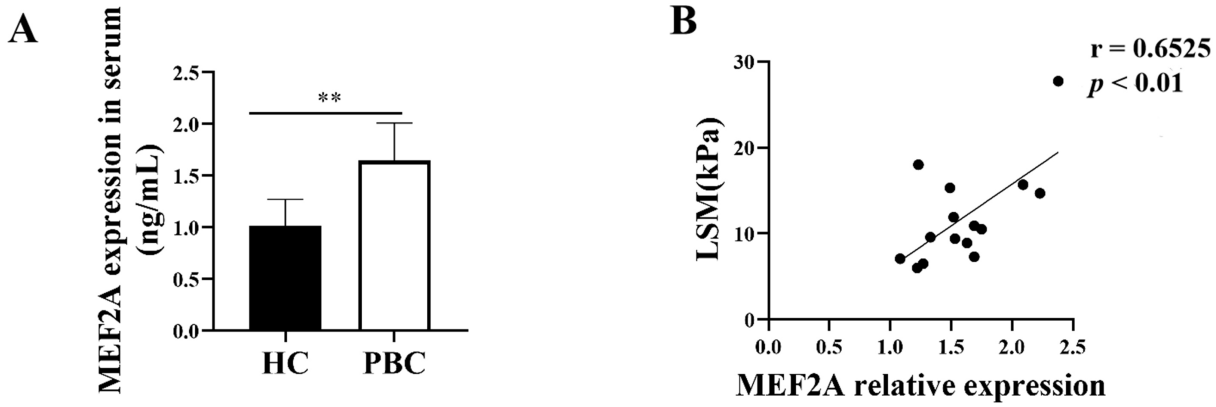

Fig. 7. MEF2A expression increases in patients with PBC and correlates with the extent of liver fibrosis. (A) Peripheral blood samples were collected from patients with PBC (n = 15) and healthy controls (HCs) (n = 15). Expression of serum MEF2A was detected by ELISA. **$p < 0.01$. (B) Correlation analysis was performed between liver stiffness measurement (LSM) and MEF2A expression in serum from patients with PBC. **$p < 0.01$.

in HIBECs. The results showed that TGF-β1 causes an increase in the levels of phosphorylated p38 (p-p38), but not in that of p-JNK or p-ERK, demonstrating activation of p38 MAPK (Fig. 6A). We further examined whether altering the p38 MAPK signaling could modulate the TGF-β1-induced MEF2A expression, EMT, and senescence in HIBECs. Indeed, inhibiting phosphorylation of p38 by its inhibitor SB 203580 was sufficient to prevent the increase of MEF2A, p21, α-SMA and suppress the decrease of E-cadherin induced by TGF-β1 (Fig. 6B). These data suggest that suppressing the TGF-β1-activated p38 MAPK signaling contributes to the anti-EMT and anti-senescence properties of MEF2A inhibition in HIBECs.

3.6 MEF2A Expression Increases in PBC Patients and Positively Correlates with the Extent of Liver Fibrosis

Since MEF2A plays a key role in the fibrogenic response, we aimed to answer whether it contributes to PBC fibrosis. We examined the expression of MEF2A in the serum of patients with PBC and found its levels were significantly high in the serum of patients versus HCs (Fig. 7A). To assess whether serum MEF2A levels were also associated with the extent of liver fibrosis, we determined the liver stiffness measurement (LSM). We used a transient elastographic instrument for the noninvasive evaluation of liver

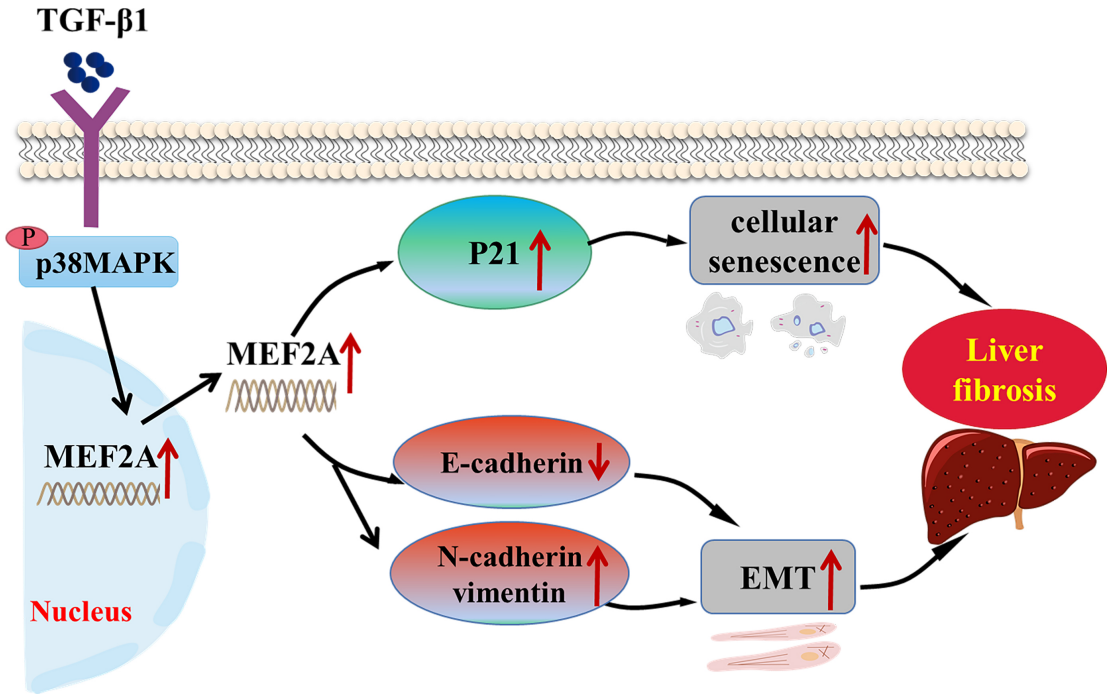

Fig. 8. Schematic representation of the role of MEF2A in the pathogenesis of cholestatic liver fibrosis. TGF-β1 stimulated MEF2A expression and cytoplasm translocation via p38MAPK pathway in HIBECs. Increased MEF2A promoted EMT and cellular senescence in HIBECs, and eventually led to liver fibrosis.

fibrosis called Fibroscan [28]. Next, we analyzed the correlation of serum MEF2A expression with LSM in patients with PBC. Interestingly, MEF2A expression in serum from PBC patients positively correlated with LSM (Fig. 7B). These data indicate that MEF2A expression in serum of patients with PBC positively correlates with the extent of liver fibrosis.

4. Discussion

Chronic cholestatic liver diseases, including PSC and PBC, mainly affecting intrahepatic bile ducts, usually lead to liver fibrosis, cirrhosis, and failure. Cholangiocytes are primary targets during the pathogenesis of cholestatic liver diseases [29]. It is demonstrated that MEF2A implicated in liver fibrosis, but whether and how cholangiocytes-derived MEF2A affect cholestatic liver fibrosis are still poorly understood. This study revealed that MEF2A expression increases in BDL mice and PBC patients, and positively correlates with the extent of liver fibrosis. Silencing MEF2A in HIBECs suppresses TGF-β1-induced EMT, senescence, and fibrosis. Moreover, MEF2A is a downstream effector of TGF-β1 signals in HIBECs, whose expression depends on the p38 MAPK pathway. Thus, the TGF-β1-p38 MAPK-MEF2A axis is an essential mechanism that may contribute to EMT, senescence, and fibrosis in cholestasis (Fig. 8).

MEF2A belongs to the myocyte enhancer factor 2 (MEF2) family. It is critical for activating genetic programs that control cell differentiation, morphogenesis, proliferation, and apoptosis of numerous cell types [30]. The implication of MEF2A in diverse human cancers, such as prostate, breast, gastrointestinal, and liver cancer, was discovered. In gastric cancer, the p38 MAPK pathway phosphorylates MEF2A, promoting tumor proliferation and metastasis [31]. Furthermore, MEF2A promotes colorectal cancer proliferation and metastasis by activating the Wnt pathway [32]. It also contributes to the apoptosis of HepG2 hepatocellular carcinoma cells [33]. In neurons, MEF2A defines oxytocin-induced morphological effects and regulates mitochondrial functions [34]. In addition, it is a nonredundant regulator of the inflammatory epigenome in macrophages [35]. However, only a few reports investigated its roles in fibrotic diseases. For instance, blockade of MEF2A prevented hyperglycemia-induced extracellular matrix accumulation via suppressing Akt and TGF-β1/Smad activation in cardiac fibroblasts [36]. MEF2A protein expression is high in liver sections obtained from patients with liver cirrhosis and increases during culture-induced activation of primary human HSCs [19]. In this study, we confirmed the role of MEF2A in cholangiocytes and cholestatic liver fibrosis.

EMT has been implicated in several types of chronic fibrotic diseases. It is a process where epithelial cells obtain mesenchymal features, contributing to the fibrogenic process [37,38]. It consists of two crucial steps. The first involves the loss of epithelial cell adhesion and degradation of junction proteins, including E-cadherin. The second includes the enhancement of cytoskeletal proteins from the

mesenchymal lineage, including N-cadherin and vimentin [39]. Knockdown of vimentin reduces EMT in cholangiocytes and leads to decreased liver fibrosis in PSC [40]. MicroRNA-34a accelerates EMT in HIBECs and advances liver fibrosis in PBC by regulating the TGF-β1/Smad pathway [41]. However, the role of cholangiocyte EMT in liver fibrosis remains controversial. Biliary epithelial cell EMT is the key pathogenetic process identified in a study of a PBC patient after transplantation [42]. We found low expression of E-cadherin and augmented expression of N-cadherin, vimentin, and α-SMA upon treating HIBECs with TGF-β1, in line with previous studies [43]. Silencing MEF2A reversed the above EMT steps and fibrosis induced by TGF-β1. Our results indicate that elevated MEF2A is a trigger for TGF-β1-induced EMT and a profibrotic factor in HIBECs.

Cellular senescence is a cell cycle arrest occurring when cells under stress stimulation, such as DNA damage, dysfunctional telomeres, and oncogenic mutations [44]. Decreased cholangiocytes senescence may alleviate liver fibrosis. For instance, remission of liver fibrosis in NK-1R$^{-/-}$ mice with BDL surgery is associated with reduced senescence of cholangiocytes and enhanced senescence of HSCs [25]. Knocking out secretin receptor alleviates liver fibrosis in Mdr2$^{-/-}$ mice by diminishing cholangiocyte senescence [6]. In this study, we demonstrated that knocking down MEF2A suppresses TGF-β1-induced senescence of HIBECs, suggesting that MEF2A participates in the pathological process of PBC fibrosis by partially promoting senescence in these cells.

We also discovered that MEF2A is a central mediator linking EMT and senescence in TGF-β1-induced liver fibrosis. To further evaluate the functions of MEF2A under TGF-β1 stimulation *in vitro*, we examined its subcellular localization in HIBECs and showed that TGF-β1 induces MEF2A expression and cytoplasm translocation. Several pathways mediate the TGF-β1 signal in various cell types, including MAPK and Smad. In addition, MEF2A is a nuclear target of the p38 MAPK signaling pathway [45]. Our study revealed that inhibiting p38 MAPK decreases MEF2A expression in HIBECs under TGF-β1 stimulation. Thus, we propose the TGF-β1-p38 MAPK-MEF2A axis is an essential mechanism that contributes to cholangiocyte EMT, senescence, and fibrosis in cholestatic liver fibrosis.

We show that MEF2A silencing may protect against liver fibrosis by regulating cholangiocyte EMT and senescence in cholangiopathies. The underlying mechanism likely involves the expression regulation and cytoplasm translocation of MEF2A via interactions with the p38 MAPK pathway. In summary, our study sheds new light on the role of MEF2A in the pathogenesis of cholestatic liver fibrosis. We believe that inhibiting MEF2A could be a valuable strategy for managing liver fibrosis in cholestatic liver diseases. Intriguingly, the positive correlation of serum level of MEF2A with fibrosis degree of PBC patients suggests that MEF2A may also serve as a potential biomarker for predicting the risk of fibrogenesis in cholestatic liver diseases. However, further research should be done in PSC patients and using more animal models for cholestatic liver fibrosis, for example Mdr2$^{-/-}$ mouse model. Moreover, we are establishing cholangiocyte-specific MEF2A KO mice, which may better clarify the role of cholangiocyte-derived MEF2A in cholestatic liver fibrosis.

5. Conclusions

In conclusion, we have shown here in an *in vitro* BDL mouse model, PBC patients and human intrahepatic biliary epithelial cells that MEF2A may involve in the pathogenesis of cholestatic liver fibrosis through regulating TGF-β1-induced cholangiocyte EMT and senescence. These data identify MEF2A as a novel inducer of cholestatic liver fibrosis and targeting MEF2A may serve as a potential therapeutic approach for cholestatic liver fibrosis.

Availability of Data and Materials

The data and material underlying this article are all available in the article.

Author Contributions

FZ conceived and designed the experiments. GZ and FH performed the experiments. HH, YX and YW analyzed the data. GZ, XC and FZ wrote the manuscript. All authors discussed and revised the manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board for Clinical Research of the Affiliated Hospital of Jining Medical University. Written informed consent has been obtained from the patients to publish this paper (Ethic approval number: 2019-FY-066).

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Conflict of Interest

The authors declare no conflict of interest.

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