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Brg1 promotes liver fibrosis via activation of hepatic stellate cells

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ABSTRACT

Liver fibrosis, an important health concern associated to chronic liver injury that provides a permissive environment for cancer development, is characterized by the persistent deposition of extracellular matrix components mainly derived from activated hepatic stellate cells (HSCs). Brg1, the core subunit of the SWI/SNF chromatin remodeling complex, has been proved to associated with nonalcoholic steatohepatitis which may progress to cirrhosis. Herein, we determined whether Brg1 regulates liver fibrosis and examined its mechanism by focusing on HSCs activation. In this study, we demonstrate that Brg1 is elevated in human and mouse fibrotic liver tissues and Brg1 mediate the profibrotic response in activated HSCs. Our data indicate that Brg1 regulates the activation of HSCs through TGFβ/Smad signal pathway. Moreover, Brg1 deficiency mice displayed decreased HSCs activation in vitro and liver fibrogenesis after chronic damage by CCl_4 administration. In addition, Brg1 expression is positively correlated with liver fibrosis in cirrhotic patients and may be a prognostic factor in HCC. Collectively, we demonstrate that Brg1 promotes liver fibrosis by activating HSCs and may represent a potential target for anti-fibrotic therapies.

1. Introduction

Liver fibrosis, the critical pre-stage in the development of liver cirrhosis, is defined as the accumulation of excessive amounts of extracellular matrix(ECM) in the liver parenchyma [1]. Liver fibrosis develops on the basis of chronic liver injury including hepatitis B and C, alcoholic liver disease or non-alcoholic steatohepatitis [2]. The fibrotic process may lead to hepatic transplantation eventually or promote a favorable microenvironment for cancer development [3]. Recent evidence indicates that liver fibrosis can be reversed even in advanced stage [4]. Thus, a greater understanding of the molecular mechanisms governing liver fibrosis regression is needed to facilitate the development of antifibrotic therapeutic approaches.

Activation of quiescent hepatic stellate cells(HSCs) to myofibroblasts is central to the fibrogenic process in chronic liver disease [5]. In the healthy liver, HSCs are retinoid and lipid-containing stromal cells located in the space of Disse. Liver injuries result in the activation of quiescent HSCs, which undergo dramatic phenotypic changes and transdifferentiate into myofibroblasts with upregulated alpha-smooth muscle actin(α-SMA) expression and ECM production(e.g. collagen, hyaluronic acid and fibronectin) [6]. TGF-β/Smad signal pathway plays a prominent role in the regulation of ECM formation [7]. It has been shown that local induction of TGF-β1 from the autocrine or paracrine pathway is crucial for the activation of HSCs and the production of ECM proteins leading to liver fibrosis [8].

Chromatin remodeling is one of the most important epigenetic mechanisms regulating gene expression. The Swltch/Sucrose Nonfementable (SWI/SNF) nucleosome repositioning complex regulates gene expression using the energy derived from ATP hydrolysis to disrupt histone-DNA interactions, resulting in transcriptional activation or repression [9]. Brg1, the core subunit of the SWI/SNF chromatin remodeling complex, is essential for DNA repair, differentiation and organ development [10,11]. Previous studies have showed that depletion of Brg1 significantly ameliorated hepatic pathology in nonalcoholic steatohepatitis(NASH) mice [12] and NASH is considered to be a potentially health-threatening disease that may progress to cirrhosis in 10–15% of patients [13]. However, the functional role of Brg1 in liver fibrosis and its molecular mechanism remain to be elucidated.

In the present study, we aimed to investigate the contribution of Brg1 for liver fibrosis in vitro and in vivo. Our results revealed that Brg1 is an interesting target to block HSC transformation by inhibiting TGFβ/Smad-α-SMA/Col1a1 signal pathway in vitro and the suppression of Brg1 diminishes experimental liver fibrosis after chronic administration of CCl_4. Thus, Brg1 may represent a potential target for anti-fibrotic strategies.

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2. Materials and methods

2.1. Cell lines and cell culture

Human hepatic stellate cells LX-2 were cultured at 5% CO_2 and 37 °C in Dulbecco's Modified Eagle Medium (DMEM) that is supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA).

2.2. Clinical tissue samples

Normal liver tissues and cirrhotic liver tissues were collected from liver angioma and liver cirrhosis patients, respectively. The informed consent was obtained from all participant before sample collection. This study was approved by the Huazhong University of Science and Technology Research Ethics Committee.

2.3. Western blot analysis and real-time PCR assay

Western bolt analysis and real-time PCR assay was conducted as previously described [14]. The antibodies to Brg1 and GAPDH were purchased from Santa Cruz Company(Santa Cruz, CA, USA) and the antibodies to α-SMA, Col1a1 and SMAD3 were purchased from Cell Signaling Technology(Danvers, Massachusetts, USA). Brg1 inhibitors PFI-3 came from Selleck (Shanghai, China). The primer sequences for RT-PCR were listed in Supplementary Table 1.

2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assay were performed in accordance with the manufacturer's protocols (EpiQuik Chromatin Immunoprecipitation Kit, Epigentek Group Inc.). To examine changes in SMAD3-binding activity at the α-SMA and Col1a1 promoter, ChIP assays were conducted with the anti-SMAD3 antibody(9523, Cell Signaling Technology). The primer sequences for ChIP are listed in Supplementary Table 1.

2.5. Luciferase assays

Luciferase activity assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), in accordance with the manufacturer's protocol. LX-2 cells were transfected with α-SMA and Col1a1 promoter luciferase reporter constructs and siBrg1 or siControl. Luciferase assays were performed 72 h after transfection using the Dual-Luciferase Assay System. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity.

2.6. IHC analysis

IHC analysis was simultaneously conducted by two pathologists using a multiple viewing microscope to evaluate the staining of Brg1, α-SMA, Col1a1. Staging of fibrosis was assessed according to Ishak [15] on the sections with Sirius red. The quantification of IHC staining was evaluated by an IRS system as previously described [16]. Brg1, α-SMA and Col1a1 were determined as low (IRS:0–4) and high (IRS:6–12).

2.7. RNA interference and adenovirus system

siRNA duplexes targeting the human *Brg1* gene(siBrg1) and nonsense control siRNA were synthesized and purified by RiboBio (Ribobio, Guangzhou, China). RNA oligonucleotides were transfected using Lipofectamine RNAiMAX Regent (Invitrogen, Carlsbad, CA, USA) and the expression level of Brg1 was quantified 72 h after transfection. Adenovirus-expression Brg1-targeted short hairpin RNA (shBrg1) and control adenovirus (shCon) was previously constructed in our laboratory.

2.8. Administration of AAV vectors in mice

AAV8 vectors encoding murine Brg1-targeted shRNA(shBrg1) or GFP were purchased from Vigene Biosciences (Jinan, Shandong, China). AAV8-shBrg1 or AAV8-GFP vectors were administered by tail vein injection at a dose of 10×10^{11} vector genomes(vg)/mice in a total volume of 200ul.

2.9. Chronic carbon tetrachloride(CCl_4) liver injury model

C57BL/6 mice were treated intraperitoneal injected twice weekly for 4 weeks with CCl_4 at 2 μl/g body weight or olive oil vehicle. The mice were divide into four groups and every group had 5 mice. Mice were sacrificed and the tissues were harvested 48 h after the final CCl_4 injection.

2.10. Determination of ALT and HA levels

Serum mouse ALT levels and HA levels were determined using ALT Test Kit (Huili Biotech, Changchun, Jilin, China) and Hyaluronan Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA) following the manufacturer's instructions.

2.11. Statistical analysis

Collagen distribution was analyzed by Image Pro Plus software (Media Cybernetics, Bethesda, MD) to quantify the area of tissue occupied by positive staining. Data were presented as mean ± S.D. and two groups of data were statistically analyzed by two-tailed *t*-tests using Graphpad Prism 5 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Brg1 is overexpressed in liver fibrosis both in human and mice model

To investigate the expression of Brg1 in liver fibrosis, we performed the NCBI Gene Expression Omnibus (GEO) dataset parameters in normal liver tissues and cirrhosis tissues. The result showed that Brg1 was significantly higher in cirrhosis than in normal tissues (Fig. 1A). Interestingly, we found that in hepatocellular carcinoma (HCC), the most common progression of cirrhosis, the expression level of Brg1 was much higher than cirrhosis. Furthermore, the expression of Brg1 increased with the stage of HCC increased (Supplementary Figure 1A and B). It reveals that Brg1 overexpression seems to be early features of cirrhosis and continuing increase of Brg1 contributes to HCC.

To further investigate the relationship between the expression of Brg1 and liver fibrosis, We used the chronic administration of CCl_4 as a model to generate liver damage and fibrogenesis in mice. C57BL/6 mice were administered twice weekly i.p. CCl_4 for 4 weeks followed by tissue harvests 48 h after the final CCl_4 injection (Fig. 1B). Hepatic fibrosis was assessed by morphometric analysis of Sirius Red and α-SMA (Fig. 1C and D). The results showed that Brg1 was overexpressed both in protein and mRNA levels in liver fibrosis mice model. Taken together, Brg1 was overexpressed during progression of liver fibrosis.

3.2. Brg1 mediates the profibrotic response in activated HSCs

Considering the role of Brg1 in NASH in previous study [12] and the expression of Brg1 in our liver fibrosis mice, we have reasons to hypothesize that Brg1 may play an important role in the regulation of liver fibrosis progression. To validate this hypothesis, depletion or overexpression of Brg1 was performed to investigate the profibrotic response in LX-2 cells, a human-activated HSC cell line. siRNA-mediated depletion of Brg1 resulted in a significantly reduction of α-SMA (a marker for activated HSCs) and collagen type1(Col1a1, a prominent

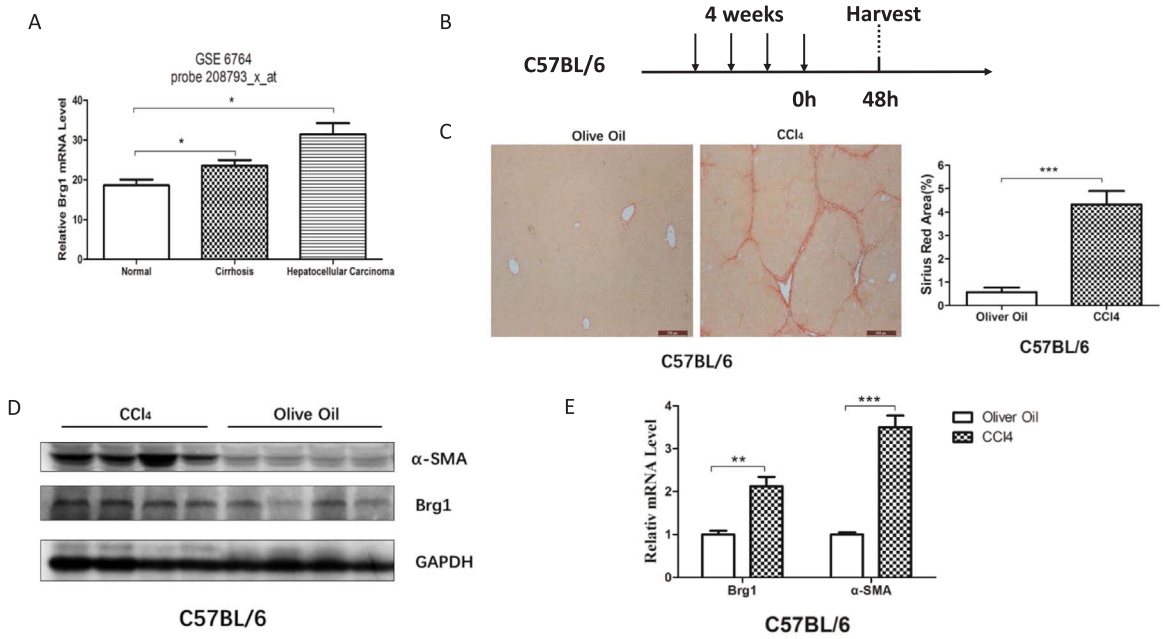

Fig. 1. Brg1 is overexpressed in liver fibrosis both in human and mice model. A. Expression of Brg1 in normal liver tissue (10 samples), cirrhosis (13 samples) and hepatocellular carcinoma (35 samples) in GEO dataset(GSE6764). $*p < 0.05$. B. Schematic illustration of CCl_4-induced liver fibrosis. C. Histological characterization of liver fibrosis in Olive oil or CCl_4-treated mice by Sirius Red staining. $**p < 0.001$. D and E. Expression of Brg1 and α-SMA in liver tissues of Olive oil or CCl_4-treated mice in protein and mRNA levels. $**p < 0.01$, $**p < 0.001$.

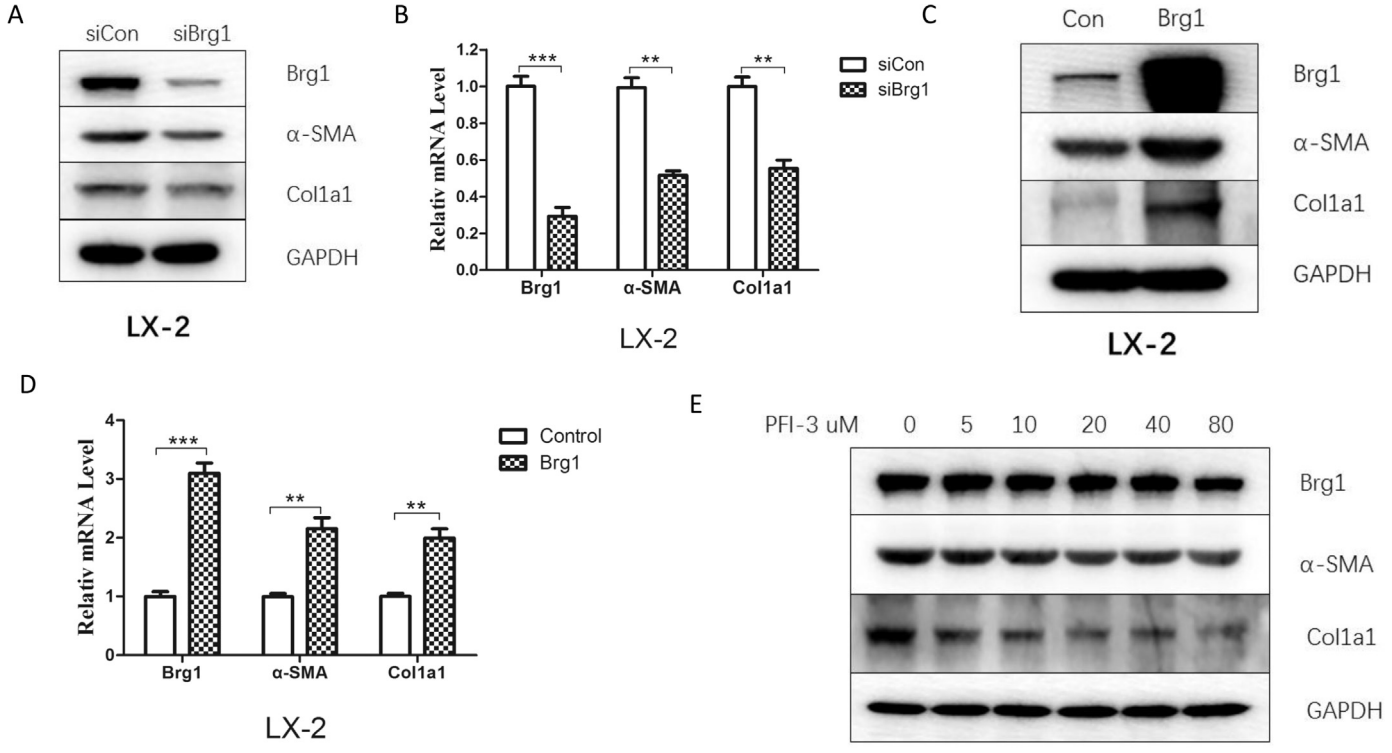

Fig. 2. Brg1 mediates the profibrotic response in activated HSCs. A and B. Effect of Brg1 knockdown on levels of α-SMA and Col1a1. LX-2 cells were treated with siControl or siBrg1 for 72 h, then α-SMA and Col1a1 levels were examined by western blot(A) and Real-time PCR(B). $**p < 0.01$, $**p < 0.001$. C and D. Effect of Brg1 overexpression on levels of α-SMA and Col1a1. LX-2 cells were treated with recombinant adenovirus (Ad-Brg1) and control adenovirus (Ad-Con) for 48 h. then α-SMA and Col1a1 levels were examined by western blot(C) and Real-time PCR(D). $**p < 0.01$, $**p < 0.001$. E. Western blot analysis of Brg1, α-SMA and Col1a1 expression in the presence of PFI-3(0uM, 5uM, 10uM,20uM,40uM,80uM) for 48 h in the indicated concentration in LX-2 cells.

ECM component of tissue fibrosis) both in protein and mRNA level (Fig. 2A and B). On the contrary, overexpression of Brg1 resulted in a significantly upregulation of α-SMA and Col1a1(Fig. 2C and D). To further explore the role of Brg1 in fibrosis, we adopted the structurally distinct Brg1 bromodomain inhibitor, PFI-3 [17], in LX-2 cells. Remarkably, the inhibitor suppressed profibrotic gene expression (Fig. 2E). All these data revealed that Brg1 played a critical role in mediating the profibrotic response in activated HSCs.

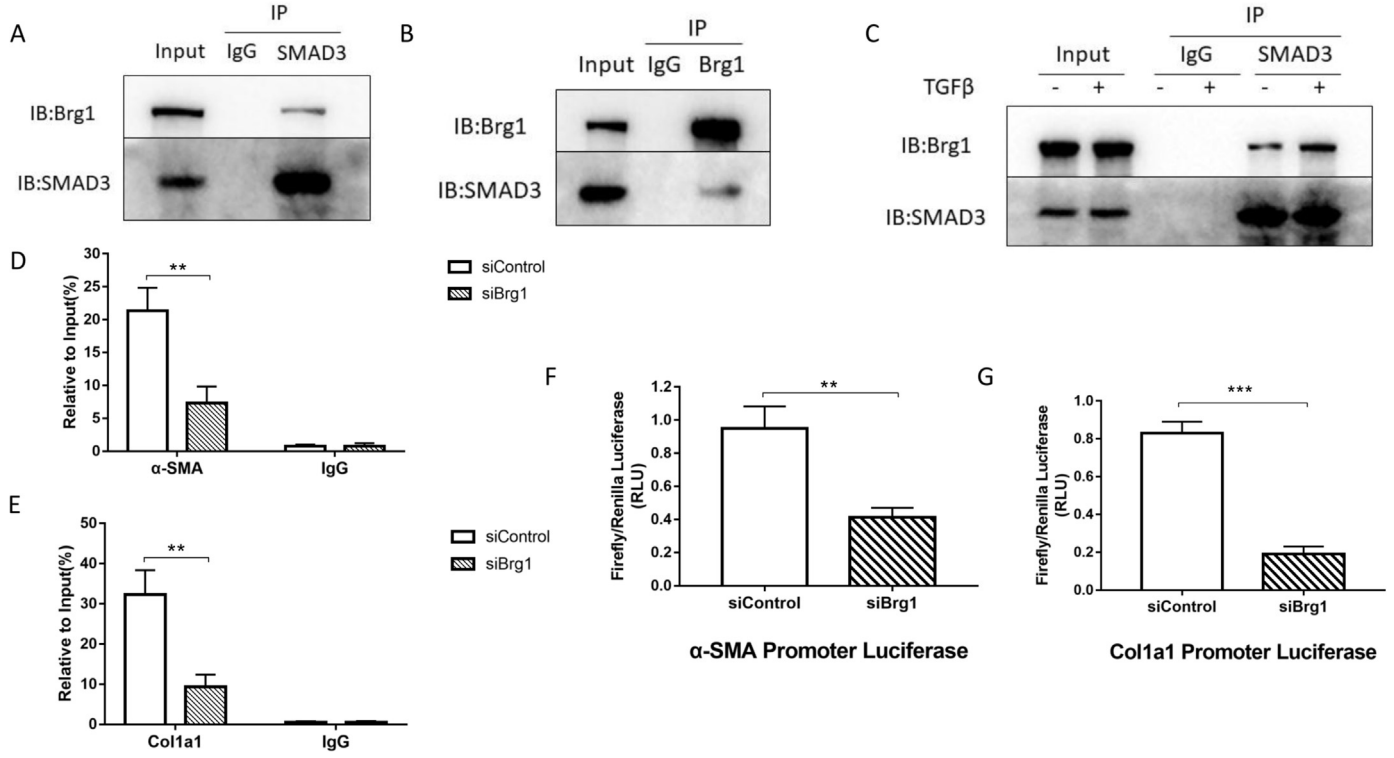

Fig. 3. Brg1 regulates the activation of HSCs through TGFβ/Smad-α-SMA/Col1a1 signal pathway. A and B. The interaction between Brg1 and Smad3 was verified by immunoprecipitation (IP) with anti-Smad3(A) or anti-Brg1(B), followed by western immunoblotting(IB) with anti-Brg1 or anti-Smad3 in LX-2 cells. C. The interaction between Brg1 and Smad3 was verified by IP with anti-Smad3 of TGFβ-treated (5 ng/ml, 30 min) or untreated LX-2 cells. D and E. ChIP assay and Real-time PCR analysis showed the effect on the recruitment of Smad3 to the promoter of α-SMA and Col1a1 at the deficient of Brg1 in LX-2 cells. $**p < 0.01$. F and G. Relative luciferase activity analysis showed the effect of Smad3 in the activity of the promoter of α-SMA and Col1a1 at the deficient of Brg1 in LX-2 cells. $**p < 0.01$, $***p < 0.001$.

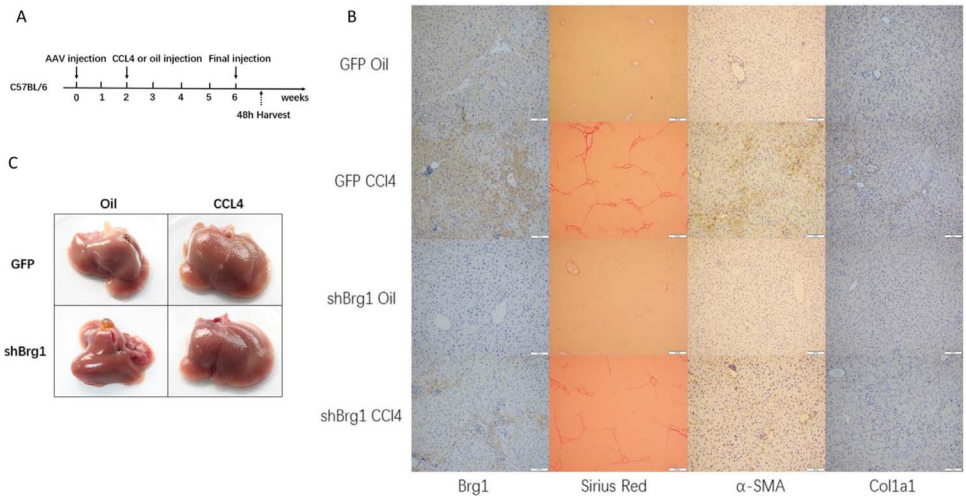

Fig. 4. Brg1 deficiency attenuates liver fibrosis induced by CCl_4. A. Experimental plan: AAV8-shBrg1 and AAV8-GFP vectors were administered by tail vein injection. After 2 weeks, mice were administered with CCl_4 or olive oil twice weekly for 4 weeks. Mice were sacrificed and the liver tissues were harvested 48 h after the final CCl_4 injection. B. Liver tissues were harvested and stained using anti-Brg1, anti-α-SMA, anti-Col1a1 antibodies and Sirius Red. C. All mice were sacrificed and the representative livers were collected and shown.

3.3. Brg1 regulates the activation of HSCs through TGF-β/Smad-α-SMA/Col1a1 signal pathway

The TGF-β/Smad signal pathway plays a prominent role in the activation of HSCs and the regulation of the production, degradation, and accumulation of ECM proteins [18]. Smad proteins mediate intracellular TGF-β signaling. Whereas both Smad2 and Smad3 are strongly activated in liver fibrosis, only Smad3 appears to be a key element in the signal transduction pathways responsible for fibrosis [19]. Smad3 directly binds to DNA sequences that regulate fibrogenic genes(collagens and α-SMA) [20,21]. To further investigate the mechanism of Brg1 in the regulation of profibrotic response in activated HSCs, we focused on Smad3. We verified the interaction between Brg1 and Smad3 by means of coimmunoprecipitation in LX-2 cells (Fig. 3A and B). The interaction between endogenous Brg1 and Smad3 was increased by TGFβ stimulation (Fig. 3C). In response to TGFβ, Smad3 accumulated in the nucleus [22], which is where Brg1 reside [23]. The deficient of Brg1 diminished the recruitment of Smad3 to the promoter of α-SMA and Col1a1 (Fig. 3D and E) by Smad3 ChIP and depressed the activity of the promoter of α-SMA and Col1a1(Fig. 3F and G) by luciferase reporter assays. The results suggested that Brg1 could have effect on the recruitment of Smad3 to the promoter of α-SMA and Col1a1 by the interaction with Smad3.

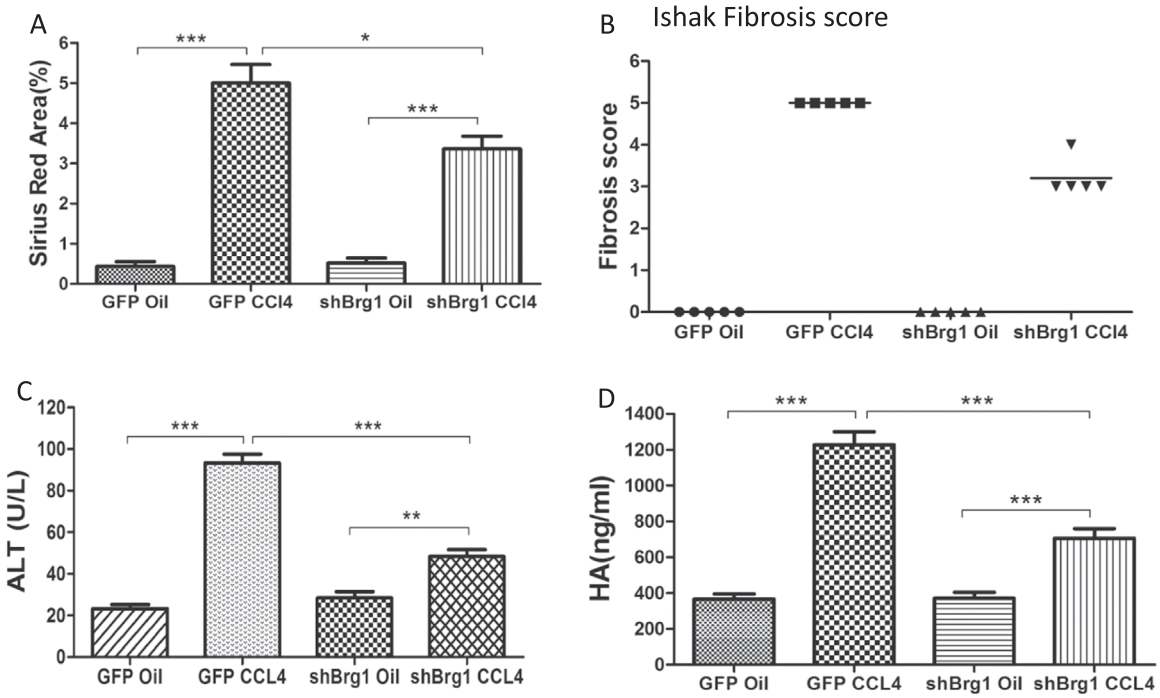

Fig. 5. Brg1 deficiency reduces CCl_4-induced liver fibrosis. A. Sirius Red quantification of liver slides using Image Pro Plus software in three random sections from each animal. $*p < 0.05$, $***p < 0.001$. B. Fibrosis scores expressed as individual data and mean mice according to the Ishak fibrosis scale. (n = 5). C and D. Serum ALT(C) and HA(D) levels are shown. $**p < 0.01$, $***p < 0.001$.

3.4. Brg1 deficiency attenuates liver fibrosis

To further identify the role of Brg1 in liver fibrosis, we used Brg1 deficient mice by AAV vectors of serotype 8(AAV8), a well-documented combination that restricts transgene expression to hepatocytes [24]. C57BL/6 mice were administered two times weekly i.p. CCl_4 or Olive oil for 4 weeks after intravenous injection of AAV8-shBrg1 or AAV8-GFP vectors for 2 weeks and liver tissue were harvested 48 h after the final CCl_4 injection (Fig. 4A). After CCL_4 treatment mice exhibited increased areas stained for Sirius Red as well as α-SMA and Col1a1 levels compared to oil-treated mice in the AAV8-GFP groups (Fig. 4B). Brg1 deficiency led to an impaired accumulation of α-SMA and Col1a1 under the treatment of CCl_4 in AAV8-shBrg1 group compared with the GFP CCl_4 group(Fig. 4B). The scar tissues were also reduced in the AAV8-shBrg1 group (Fig. 4C). Mice treated with CCl_4 in AAV8-shBrg1 group showed a significant improvement in the percentage of Sirius Red area (Fig. 5A) and the Ishak fibrosis pathology score (Fig. 5B) which was indicative of a therapeutic blockade of fibrosis progression. We could observe the similar result assessed by serum alanine aminotransferase (ALT) and hyaluronic acid (HA) levels (Fig. 5C and D). The results showed Brg1 depletion could attenuate liver fibrosis induced by CCl_4.

3.5. Brg1 expression is positively correlated with liver fibrosis in cirrhotic patients and may be a prognostic factor in HCC

Finally, we sought to investigate whether the observations in mice could be verified in liver fibrosis patients. The expression of Brg1 was measured in normal liver tissues and cirrhotic liver tissues which were collected from liver angioma and liver cirrhosis patients in our hospital, respectively. The result showed Brg1 was significantly overexpressed in cirrhosis specimens (Fig. 6A). In order to further validate our results, we performed tissue microarray (US Biomax Inc, Rockville, MD, USA) to disclose the Brg1 expression in normal liver tissues and cirrhotic liver tissues. It was gratifying that Brg1 was high expression in cirrhotic liver tissues (Fig. 6B and Supplementary Figure 1C). HCC is the most rapidly increasing cause of cancer-related mortality in the United States [25]. Because of the lack of viable treatment options for HCC, prevention in high-risk patients has been proposed as an alternative strategy. The main risk factor for HCC is cirrhosis [26]. Considering the high expression of Brg1 both in cirrhosis and HCC, we hypothesized whether Brg1 could be a prognostic factor in HCC. We performed gene expression profile analysis in TCGA database which were followed up for a median of 10 years. The result showed that high Brg1 expression group had poor outcomes (Fig. 6C, $p = 0.0397$). Even though there was no statistical significance between high and low group in another GEO database (GSE 45114), we could observe a significantly poor tendency in high Brg1 expression group (Fig. 6D, $p = 0.159$). These results indicated that Brg1 expression is positively correlated with liver fibrosis in cirrhotic patients and may be a prognostic factor in HCC.

4. Discussion

Fibrotic diseases contribute to 45% mortalities in developed countries and represent a significant burden on public health systems [27]. Liver fibrosis, the important component of fibrotic diseases, is driven by activation of quiescent of HSCs and characterized by the accumulation of ECM. Unabated fibrosis eventually progresses to liver failure and results in the major cause of HCC [28]. Despite recent progress in understanding the pathogeny of liver fibrosis, the mechanisms are not yet fully known. In our study, Brg1 was discovered to accumulate in liver tissue during the progression of fibrosis. Blocking the expression of Brg1 suppressed the profibrotic response in activated HSCs and attenuated liver fibrosis progression in mice model. It was found Brg1 promoted the activation of HSCs through TGF-β/Smad signaling pathway. Moreover, Brg1 expression levels were significantly elevated in cirrhotic patients and may be a prognostic factor in HCC.

This is the first study to investigate the biological function of Brg1 in liver fibrosis. Brg1, as a chromatin remodeling factor, is correlated with oncogenesis and organ development [29]. Brg1 was usually reported as a tumor suppressor [30]. Depletion of Brg1 promoted tumor metastasis in colorectal cancer via the miR-550/RNF43 pathway, indicating that Brg1 is a potential tumor suppressor [31]. However, the role of Brg1

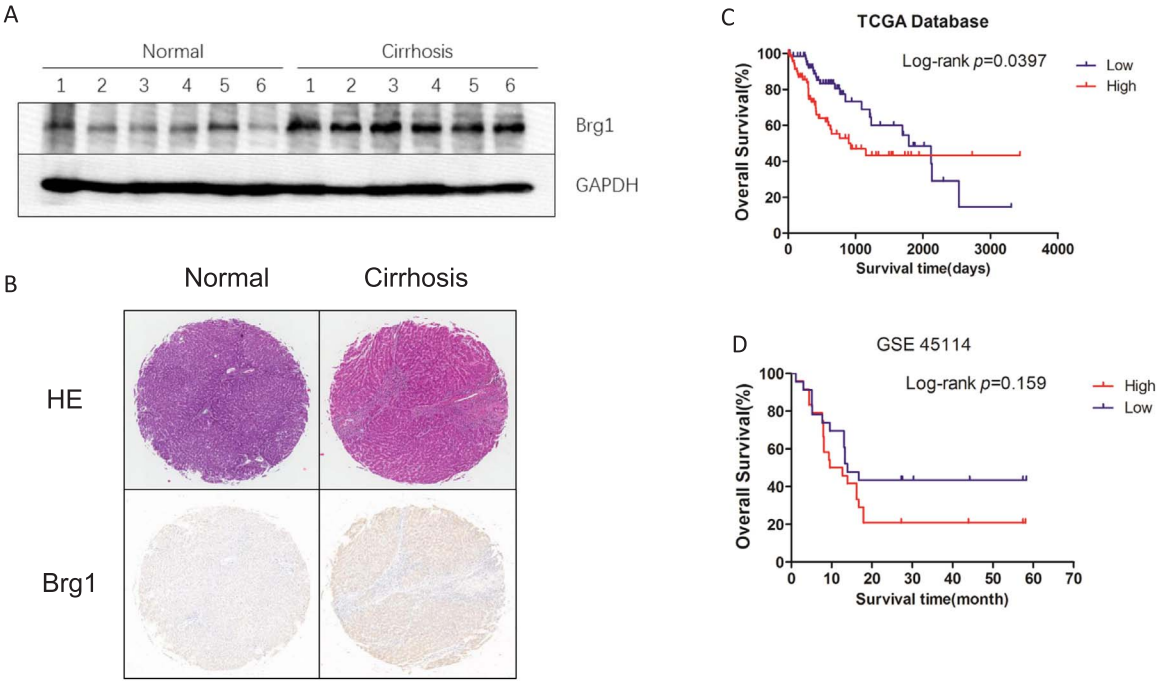

Fig. 6. Brg1 expression is positively correlated with liver fibrosis in cirrhotic patients and may be a prognostic factor in HCC. A. Western blot analysis for indicated protein expression levels in normal liver tissues and cirrhotic liver tissues. B. Tissue Microarray (US Biomax Inc, LV805c, including 30 normal liver tissue specimens and 40 cirrhotic liver tissue specimens) was stained using anti-Brg1 antibodies. C. Kaplan-Meier survival curve of Brg1 expression levels in 144 HCC patients from TCGA database. $p = 0.0397$. D. Kaplan-Meier survival curve of Brg1 expression levels in 45 HCC patients from GEO database(GSE45114). $p = 0.159$.

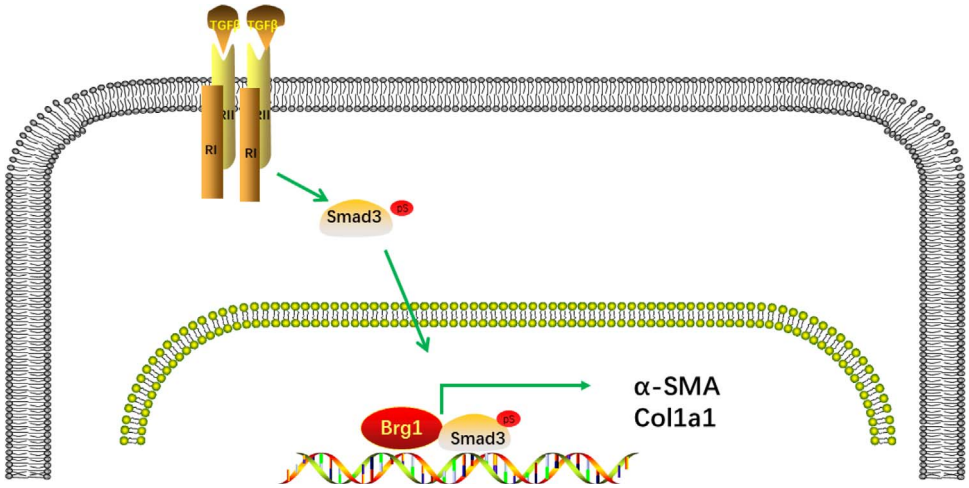

Fig. 7. A hypothetical representation of the regulatory pathway underlying BRG1-induced liver fibrosis.

remains controversial. Brg1 is correlated with tumor proliferation in gastric cancer [32] and loss of Brg1 induces cell senescence in colorectal cancer, indicating Brg1 promotes oncogenesis [16]. Fibrosis has many similarities to cancer including alterations in cell phenotype and dysregulation of cell cycle, which promotes the proliferation of myofibroblasts and spread of fibrosis [33,34]. To date there has been little attention focused on the function of Brg1 in liver fibrosis. Previous study reported a Brg1-dependent pathway that connected the epigenetic regulation of proinflammatory genes to the pathogenesis of NASH [12]. It should be noted that NASH has been recognized as a major cause of liver fibrosis [35]. Therefore, it was reasonable to investigate the function of Brg1 in liver fibrosis. It was gratifying that we demonstrated depletion of Brg1 attenuated liver fibrosis progression, an unreported biological function for Brg1.

HSCs transform during chronic liver injury from a quiescent state into a myofibroblast-like phenotype, which represents a crucial cell reprogramming event in the progression of liver fibrosis [5]. Activated HSCs frequently accumulate in the parenchymal tissues close to injured areas. During the fibrogenic process, TGF-β1 stimulates the activation of HSCs and ultimately causes the acceleration of liver fibrosis. In HSCs, TGF-β1 can potentiate extracellular matrix deposition and suppress collagenase activity [36]. Our data demonstrate that Brg1 could have effect on the recruitment of Smad3 to the promoter of α-SMA and Col1a1 by the interaction with Smad3 in LX-2 cells. It may be plausible that Brg1 plays regulatory roles by the amplification of the TGF-β1/Smad signaling pathway. We propose a model that suggests Brg1 have effect on the recruitment of Smad3 to the promoter of α-SMA and Col1a1 by the interaction with Smad3 (Fig. 7).

The lack of sensitive and reproducible markers for liver fibrosis has been a major limitation not only for research and clinical management of liver diseases, but also for the development of anti-fibrotic drugs [37,38]. Our data showed that Brg1 expression is positively correlated

with cirrhosis and HCC patients. Moreover, high expression of Brg1 indicated a poor prognosis in HCC. The results here suggest that Brg1 may be an ideal candidate for predicting the progression of liver fibrosis and HCC. Thus, Brg1 down-regulation or inhibition may be a candidate anti-fibrotic therapy in the future.

Acknowledgements

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Author contribution

Zhixin cao and Xuelai Luo designed this project. Haijie Li, Jingqin Lan, Caishun Han, Kaixuan Guo, Guihua wang, Junbo Hu and Jianping Gong performed the experiments.

Conflict of interest

There is no conflict of interest.

Compliance with ethical standards

Ethical approval Research involving animals: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Research Involving human participants: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2018.02.003.

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