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Conditional loss of heparin-binding EGF-like growth factor results in enhanced liver fibrosis after bile duct ligation in mice

Takayo Takemura [a,1], Yuichi Yoshida [a,1], Shinichi Kiso [a,*], Takashi Kizu [a], Kunimaro Furuta [a], Hisao Ezaki [a], Mina Hamano [a], Mayumi Egawa [a], Norihiro Chatani [a], Yoshihiro Kamada [a], Yasuharu Imai [b], Shigeki Higashiyama [c], Ryo Iwamoto [d], Eisuke Mekada [d], Tetsuo Takehara [a]

[a] Department of Gastroenterology and Hepatology, Osaka University, Graduate School of Medicine, Osaka, Japan
[b] Department of Gastroenterology, Ikeda Municipal Hospital, Ikeda, Osaka, Japan
[c] Department of Biochemistry and Molecular Genetics, Ehime University, Graduate School of Medicine and Department of Cell Growth and Tumor Regulation, Proteo-Medicine Research Center (ProMRes), Ehime University, Shitsukawa, Toon, Ehime, Japan
[d] Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

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ABSTRACT

Our aims were to evaluate the involvement of heparin-binding EGF-like growth factor (HB-EGF) in liver fibrogenesis of humans and mice and to elucidate the effect of HB-EGF deficiency on cholestatic liver fibrosis using conditional HB-EGF knockout (KO) mice. We first demonstrated that gene expression of HB-EGF had a positive significant correlation with that of collagen in human fibrotic livers, and was increased in bile duct ligation (BDL)-induced fibrotic livers in mouse. We then generated conditional HB-EGF knockout (KO) mice using the interferon inducible *Mx-1* promoter driven Cre recombinase transgene and wild type (WT) and KO mice were subjected to BDL. After BDL, KO mice exhibited enhanced liver fibrosis with increased expression of collagen, compared with WT mice. Finally, we used mouse hepatic stellate cells (HSCs) to examine the role of HB-EGF in the activation of these cells and showed that HB-EGF antagonized TGF-β-induced gene expression of collagen in mouse primary HSCs. Interestingly, HB-EGF did not prevent the TGF-β-induced nuclear accumulation of Smad3, but did lead to stabilization of the Smad transcriptional co-repressor TG-interacting factor. In conclusion, our data suggest a possible protective role of HB-EGF in cholestatic liver fibrosis.

1. Introduction

Chronic liver injury and wound healing response result in the accumulation of extracellular matrix (ECM) and the replacement of normal parenchyma by scar tissue, causing liver fibrosis and cirrhosis [1,2]. In fibrotic livers, hepatic stellate cells (HSCs) are known to be a key player in producing ECM [1,2]. The activation of HSCs has been shown to be caused by a variety of cytokines or growth factors, including TGF-β or PDGF, during the liver fibrosis process [1,2]. Among these factors, the role of epidermal growth factor receptor (EGFR) ligands in this process has been poorly understood.

The heparin-binding epidermal growth factor-like growth factor (HB-EGF) belongs to the EGF family of growth factors and associates with and stimulates EGF receptors (EGFR) and EerbB4 [3,4]. HB-EGF has been shown to be synthesized as a type 1 transmembrane protein (pro HB-EGF) [3,5]. Cleavage of membrane anchored pro HB-EGF at the juxtamembrane domain results in the shedding of mature HB-EGF (soluble HB-EGF) [6]. Soluble HB-EGF has a mitogenic effect on several cell types, such as vascular smooth muscle cells, fibroblasts and keratinocytes [3,4]. Previously, we reported that HB-EGF has a capacity to stimulate hepatocyte proliferation both *in vitro* and *in vivo* [7,8]. We have also reported that HB-EGF was induced during liver regeneration after partial hepatectomy [9] and that hepatocyte-specific HB-EGF transgenic mouse showed accelerated proliferation of hepatocytes during liver regeneration after partial hepatectomy [10]. Because abnormal liver regeneration is known to be associated with liver fibrosis, we hypothesized that loss of HB-EGF may affect liver fibrosis during chronic liver injury. Recently, it has been reported that traditional

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; BDL, bile duct ligation; EGF, epidermal growth factor; H&E, hematoxylin and eosin; HB-EGF, heparin-binding EGF-like growth factor; HSCs, hepatic stellate cells; KO, knockout; poly I:C, polyinosinic:polycytidylic acid; RT-PCR, reverse-transcription polymerase chain reaction; T.Bil, total bilirubin; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; WT, wild type.

* Corresponding author. Address: Department of Gastroenterology and Hepatology, Osaka University, Graduate School of Medicine, 2-2 K1 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 3629.
E-mail address: kiso@gh.med.osaka-u.ac.jp (S. Kiso).

[1] These authors equally contributed to this paper.

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HB-EGF KO mice showed enhanced liver fibrosis in chemical-induced liver fibrosis [11]. However, the role of HB-EGF in cholestatic liver fibrosis remains unclear.

Because HB-EGF null mice die immediately after birth due to severe heart failure [12], we recently generated conditional HB-EGF knockout mice using the interferon-inducible Mx1-Cre transgene and examined the role of HB-EGF during toxin-induced acute liver injury [13]. In the present study, we investigated the role of HB-EGF in cholestatic liver fibrosis using these conditional HB-EGF KO mice.

2. Materials and methods

2.1. Human liver tissues

Non-tumorous liver tissues were obtained from 16 patients undergoing partial liver section at Ikeda Municipal Hospital, Japan. The degree of liver fibrosis was classified as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis), or F4 (cirrhosis). Detailed information of the patients is presented in the Supplemental Information. Informed consent was obtained from all patients and the study was approved by the Ethical Committees of Osaka University Hospital and Ikeda Municipal Hospital.

2.2. Mice

Generation of C57BL/6 mice carrying the HB-EGF gene flanked by loxP sites (HB-EGF$^{lox/lox}$ mice) was described previously [12]. HB-EGF$^{lox/lox}$ mice were further bred with Mx1-Cre transgenic (TG) C57BL/6 mice (Jackson Laboratories, West Grove, PA, USA) to generate Mx1-Cre HB-EGF$^{lox/lox}$ mice. To delete HB-EGF from the liver, Mx1-Cre HB-EGF$^{lox/lox}$ (KO) mice and HB-EGF$^{lox/lox}$ (WT) mice were injected with 250 μg of polyinosinic:polycytidylic acid (poly I:C) (P1530 Sigma, St. Louis, MO) intraperitoneally three times at 2-day intervals as previously described [13]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University, Graduate School of Medicine.

2.3. Bile duct ligation model

To induce liver fibrosis, male 8–10-week-old WT and KO mice underwent bile duct ligation (BDL) [14]. The BDL procedure was performed as previously described. In brief, the common bile duct was doubly ligated under anesthesia via laparotomy. The sham procedure was performed via similar laparotomy without BDL.

2.4. Histological analysis

To evaluate liver fibrosis, liver sections were stained in 0.1% Sirius red F3B (Sigma–Aldrich, St. Louis, MO, USA) in saturated aqueous picric acid (Sigma–Aldrich). The hematoxylin and eosin (H&E) stained liver sections were also used to evaluate oncotic necrosis. The relative fibrotic or oncotic necrosis area was calculated using ImageJ software. To evaluate macrophage infiltration in the injured livers, liver sections were stained with anti F4/80 antibody (Abcam, Cambridge, MA).

2.5. Real time RT-PCR

Total RNA was extracted from whole liver using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as previously described [15]. Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR were performed as previously described [15]. The Quantitect gene assay kit (Qiagen, Hilden, Germany) was used for analysis of human HB-EGF, human Col1α1, human Col1α2, human Acta2, human TGF-β, human GAPDH, mouse HB-EGF, mouse Col1α1, mouse Col1α2, mouse Acta2, mouse TGF-β, mouse PDGF, mouse MMP13 mouse Timp1, mouse F4/80, mouse CD68, mouse TNF-α, mouse IL-6, mouse MCP-1, mouse IL-1β, and mouse GAPDH. The relative gene expression was quantified using GAPDH as an internal control.

2.6. Isolation and culture of mouse hepatic stellate cells

Mouse HSCs were isolated from male C57BL6 mice by in situ collagenase perfusion and Nycodenz (Sigma–Aldrich) density gradients as previous described [16]. Isolated HSCs were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle medium containing 10% FCS. Activated HSCs after a few passages were used for the experiments in this study. To assess the effect of HB-EGF on the activation of HSCs, serum-starved HSCs were stimulated with 1 ng/ml of TGF-β (100 21, PeproTech EC, Rocky Hill, CT) with or without 20 ng/ml of HB-EGF (259-HE-050, R&D Systems Inc. Minneapolis, MN) for 24 h and fibrosis related genes were examined by real time RT-PCR.

2.7. Western blotting

For extraction of nuclear protein, subcellular fractionation was performed using the NE-PERTM nuclear extraction kit (Pierce, Rockford, IL). Western blotting was performed as previously described [15]. To examine the effect of HB-EGF on the nuclear accumulation of Smad3 in HSCs, serum-starved HSCs were stimulated with 1 ng/ml of TGF-β and increasing amount of HB-EGF (0–100 ng/ml) for 40 min. To examine the effect of HB-EGF on phosphorylation of TGIF in HSCs, serum-starved HSCs were stimulated with 20 ng/ml of HB-EGF for 0–120 min. We used primary antibodies specific for Smad3 (9513, Cell Signaling, Danvers, MA), TGIF (sc-9084, Santa Cruz Biotechnology, Santa Cruz, CA), and Histone H3 (4499, Cell Signaling, Danvers, MA).

2.8. Transient reporter gene assay

HSCs were transfected with 1 μg of reporter plasmid (pGL3-$(CAGA)_9$-luc) and 100 ng of control plasmids (pGL4-RLtk) using FuGENE6 (1815091, Roche, Basel Switzerland) in 6-well plates. After incubation for 6 h, the cells were serum-starved overnight, then stimulated with 1 ng/ml of TGF-β with or without 20 ng/ml of HB-EGF for 24 h. Luciferase activity was measured with a luminometer using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). pGL3-$(CAGA)_9$-luc [17] was gifted by Dr. S. Ehata and Dr. K. Miyazono (University of Tokyo).

2.9. Statistical analysis

The results are presented as mean ± SE. Differences between two groups were examined for statistical significance using the Mann–Whitney *U* test and multiple comparisons were made by the Bonferroni/Dunn test. A p value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Increased expression of HB-EGF is associated with liver fibrosis in humans and mice

To investigate the involvement of HB-EGF in the process of liver fibrosis, we examined the gene expression of HB-EGF in the livers of patients with chronic liver disease. Real time RT-PCR analysis

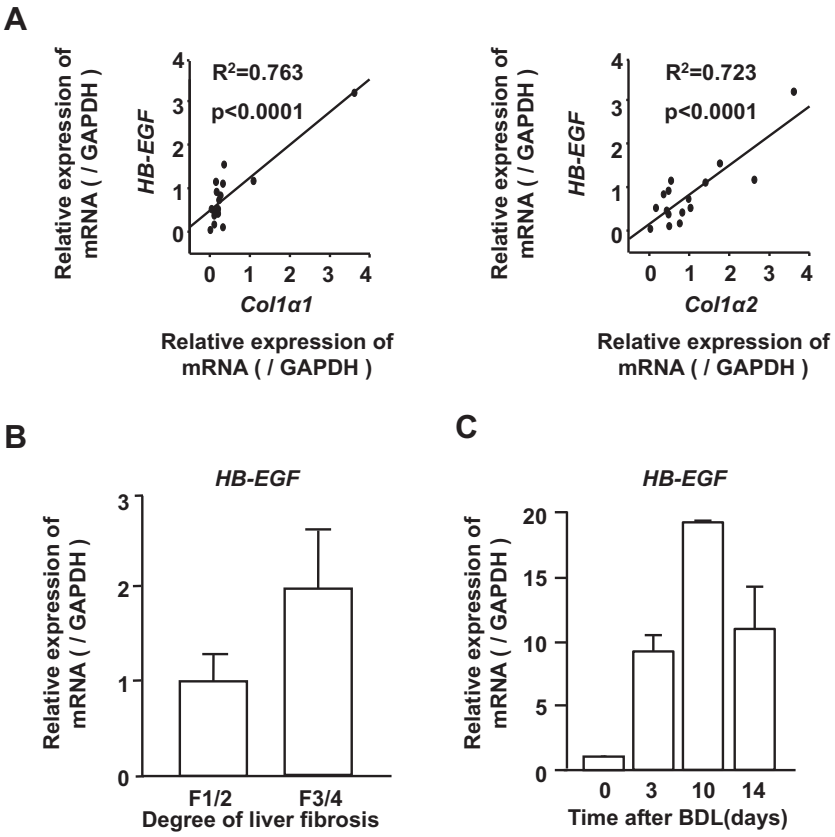

Fig. 1. Increased expression of HB-EGF gene during liver fibrosis in humans and mice. (A) Correlation between hepatic gene expression of HB-EGF and that of fibrosis-related genes, such as Col1α1 and Col1α2 in human samples with chronic liver disease. (B) Hepatic HB-EGF expression was increased as the fibrosis developed in human samples with chronic liver disease. (C) Increased expression of HB-EGF in murine livers during liver fibrosis after BDL.

showed that the expression of HB-EGF mRNA was increased in fibrotic livers and was positively correlated with that of fibrosis-related genes, such as collagen type I alpha 1 (Col1α1) and collagen type I alpha 2 (Col1α2) (Fig. 1A). We also revealed that the gene expression of HB-EGF was increased during the development of liver fibrosis in these patients (Fig. 1B) and that its expression was induced by bile duct ligation (BDL) in the mouse model (Fig. 1C). These results indicated the possible involvement of HB-EGF in liver fibrogenesis.

3.2. HB-EGF KO mice show enhanced liver fibrosis after BDL

To investigate the role of HB-EGF in liver fibrosis, we performed BDL in conditional HB-EGF knockout (KO) and wild type (WT) mice. Real time RT-PCR analysis confirmed that the expression of HB-EGF mRNA was reduced in KO mice compared with that of WT mice (Fig. 2A). KO mice exhibited about a 1.5-fold increase in the fibrosis area as evaluated by Sirius red staining compared with WT mice after BDL (Fig. 2B and C). This enhanced fibrosis in KO mice was associated with increased expression of fibrosis-related genes, such as Col1α1, Col1α2 or TIMP1, as assessed by real time RT-PCR analysis, compared with WT mice (Fig. 2D). These data indicated that loss of HB-EGF caused enhanced liver fibrosis during chronic liver injury.

3.3. HB-EGF KO mice display enhanced oncotic necrosis and macrophage infiltration in the liver after BDL

We next characterized the effect of HB-EGF deletion on the liver injury after BDL by H&E staining of liver sections. KO mice displayed significantly more oncotic necrosis after BDL than WT mice (Fig. 3A). Quantification of H&E staining showed about a 2.6-fold increase in the oncotic necrotic area in KO mice compared with WT mice after BDL (Fig. 3B).

Kupffer cells, or resident hepatic macrophages, are known to be involved in the development of liver fibrosis [2,18]. We therefore examined the effect of HB-EGF deletion on macrophage infiltration in the liver after BDL by F4/80 staining of the injured livers after BDL (Fig. 3D). KO mice showed increased infiltration of F4/80 positive macrophages in the injured livers compared with WT mice (Fig. 3D). Consistent with this, KO mice showed increased expression of F4/80 and CD68 in the injured livers compared with WT mice (Fig. 3E and F). However, there was no statistically significant difference in the hepatic expression of pro-inflammatory genes, such as TNF-α, IL-6, MCP-1 or IL-1β, between the WT and KO mice (Fig. 3G–J). These data indicate that the enhanced liver fibrosis in KO mice might not be associated with the cytokine production of macrophages in the injured livers induced by BDL.

3.4. HB-EGF attenuates TGF-β-induced expression of fibrosis-related genes in murine hepatic stellate cells

Because hepatic stellate cells (HSC) are known to play a major role in the development of liver fibrosis [2,18], we investigated the role of HB-EGF on the activation of mouse primary HSCs *in vitro*. HB-EGF treatment significantly reduced the gene expression of Col1α1 or Col1α2 in the HSCs activated by TGF-β (Fig. 4A). HB-EGF treatment also significantly suppressed TGF-β-dependent transcription and this effect was cancelled by addition of U0126, ERK1/2 inhibitor in HSCs (Fig. 4B). We therefore examined the effect of HB-EGF treatment on the nuclear accumulation of Smad3 induced by TGF-β in HSCs (Fig. 4C). However, Western

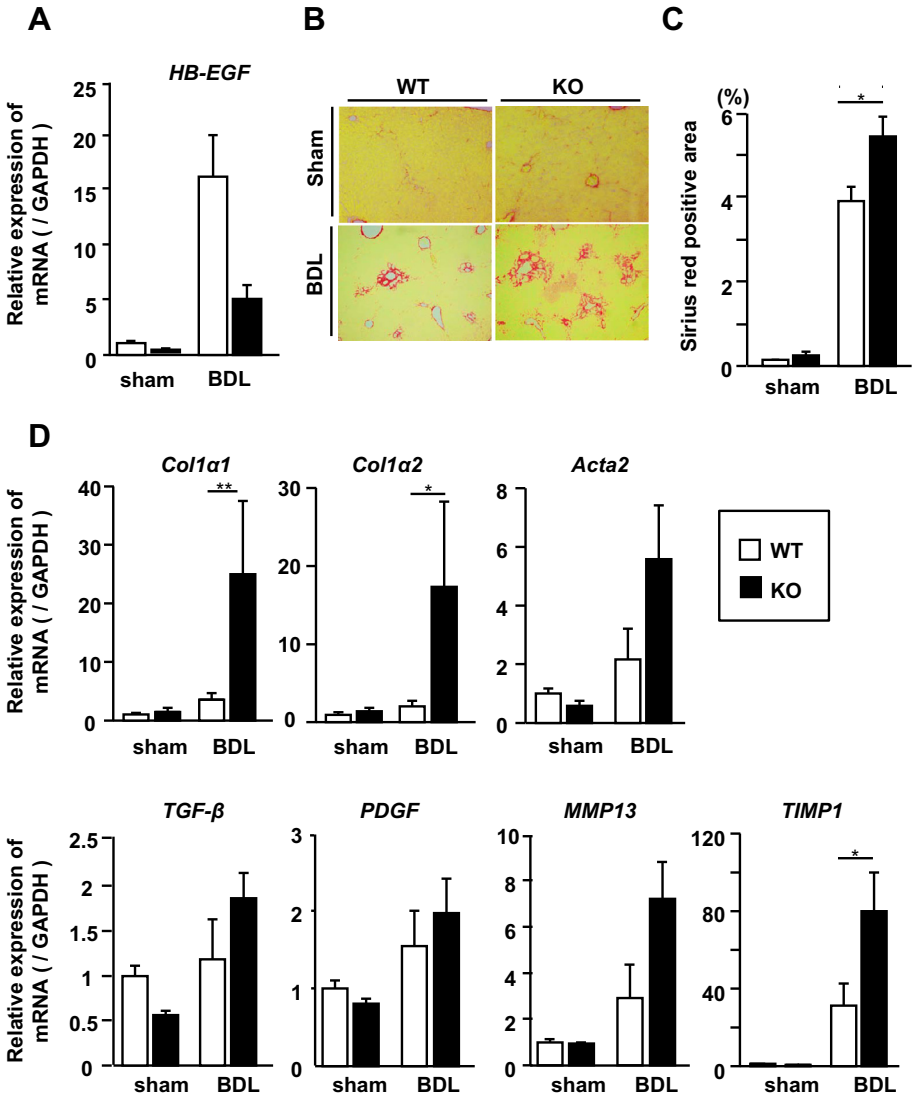

Fig. 2. Enhanced live fibrosis in conditional HB-EGF KO mice after BDL. WT and KO mice were subjected to BDL or sham operation (sham). (A) Gene expression of HB-EGF in the liver at 10 days after BDL ($n = 6$ for WT, $n = 5$ for KO) or sham ($n = 6$ for WT, $n = 5$ for KO) in WT and KO mice. (B) Representative views of Sirius red staining of the liver sections at 14 days after BDL ($n = 7$ for WT, $n = 6$ for KO) or sham (original magnification × 200). (C) Quantification of Sirius red staining positive area in the injured livers of WT and KO mice at 14 days after BDL or sham (data are mean ± SE, $^{*}p < 0.05$). (D) Gene expression of Collα1, Collα2, Acta2, TGF-β, PDGF, MMP13 and Timp1 in the liver at 10 days after BDL or sham in WT and KO mice (data are mean ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$).

blotting analysis showed that HB-EGF did not decrease TGF-β-dependent nuclear accumulation of Smad3 in HSCs (Fig. 4C). Finally, we demonstrated that HB-EGF treatment caused the stabilization of Smad transcriptional co-repressor TG-interacting factor (TGIF) of HSCs (Fig. 4D). These results indicate that HB-EGF treatment may suppress the activation of HSCs through the inhibition of TGF-β signaling.

4. Discussion

EGFR ligands are known to stimulate hepatocyte proliferation and promote liver regeneration during acute liver injury [19,20]. However, their role in liver fibrogenesis remains elusive. In this study, we investigated whether conditional loss of HB-EGF could affect liver fibrosis after BDL, a well-established murine model of cholestatic liver fibrosis, using conditional HB-EGF KO mice. We demonstrated that conditional loss of HB-EGF accelerates liver fibrosis during cholestatic liver injury. Our results suggest that HB-EGF plays a protective role during cholestatic liver fibrosis.

Using liver samples from patients with chronic liver disease, we first demonstrated that the expression of HB-EGF had a positive correlation with that of fibrosis-related genes and was increased as liver fibrosis developed. The expression of HB-EGF was also increased during liver fibrosis after BDL in mice. Collectively, one explanation for our results is that the increased expression of HB-EGF during chronic liver injury might serve as a self-defense mechanism to suppress an excess fibrosis response. Consistent with our observation, previous publications also showed that hepatocyte growth factor (HGF) and its receptor, c-Met system play a protective role for liver fibrosis, although the expression of HGF increases during this process [21–23].

To investigate the molecular mechanism underlying the enhanced liver fibrosis in KO mice, we examined the liver injury and macrophage infiltration in the injured livers. Histological examinations showed larger areas of oncotic necrosis in the livers of KO mice than WT mice. This result was consistent with the result of our recent publication demonstrating that conditional HB-EGF KO mice show the increased liver injury after a single administration of CCl_4 [13]. Real time RT-PCR also showed

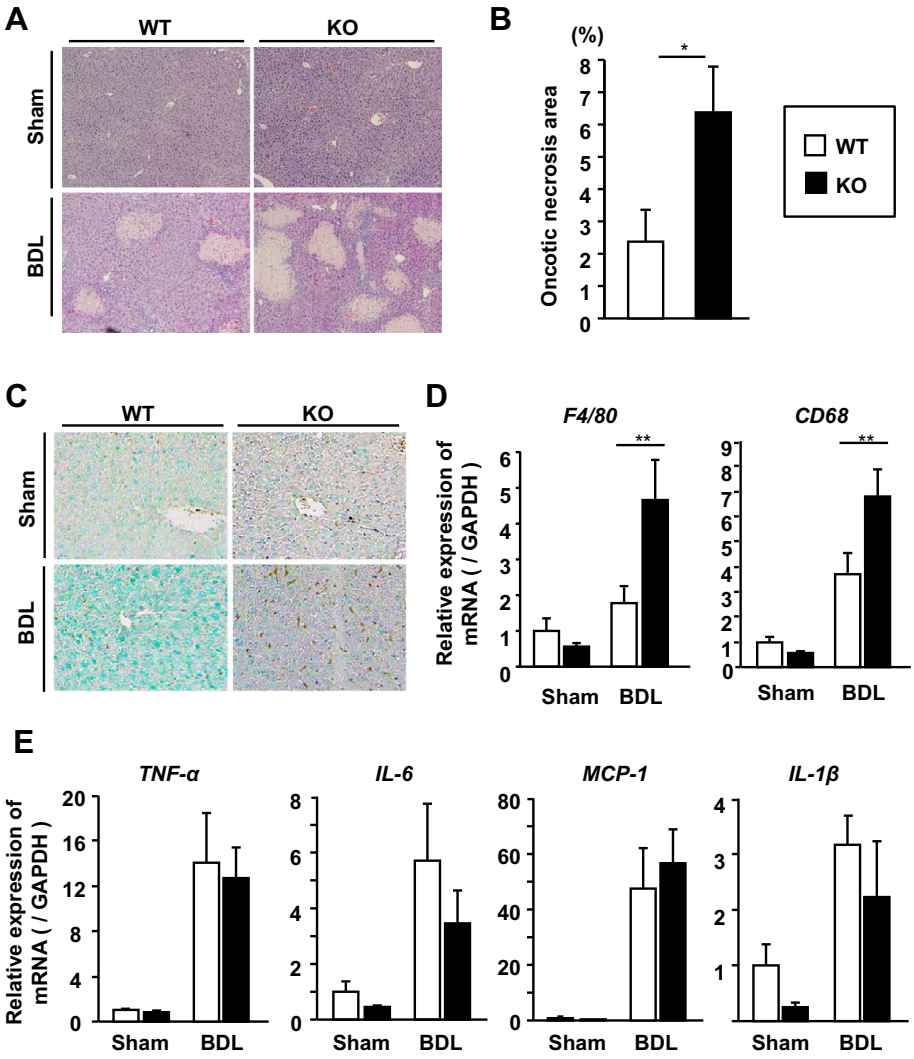

Fig. 3. Enhanced hepatic necrosis and macrophage infiltration in conditional HB-EGF KO mice after BDL. (A) Representative H&E staining of the livers of WT and KO mice at 10 days after BDL ($n = 6$ for WT, $n = 5$ for KO) or sham ($n = 6$ for WT, $n = 5$ for KO) (magnification × 100). (B) Quantification of the area of onocotic necrosis in injured livers of WT and KO mice at 10 days after BDL. (C) Representative F4/80 staining of the livers of WT and KO mice at 10 days after BDL or sham (magnification × 400). (D) Gene expression of F4/80 and CD68 in the liver at 10 days after BDL or sham in WT and KO mice. (E) Gene expression of TNF-α, IL-6, MCP-1, and IL-1β at 10 days after BDL or sham in WT and KO mice (data are mean ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$).

increased expression of F4/80 or CD68, surface markers for macrophages, in the injured livers of KO mice compared with WT mice. Because non-parechymal cells, such as Kupffer cells or sinusoidal endotherial cells, were the main source of HB-EGF production in the liver, these cells might play a protective role against inflammation by the secretion of HB-EGF. However, we could not detect any difference in the gene expression of pro-inflammatory cytokines, such as TNF-α or IL-1β or that of pro-inflammatory chemokine, MCP-1. These findings raise the possibility that the loss of HB-EGF itself might affect the activation of HSCs during liver fibrosis.

Finally, we examined the effects of direct interaction of HB-EGF with HSCs, the major ECM-producing cells in the liver. We demonstrated that HB-EGF could reduce TGF-β-induced expression of Col1α1 or Col1α2 in HSCs. We also demonstrated that HB-EGF could suppress TGF-β-dependent transcription via the ERK dependent pathway in HSCs. It is interesting to note that HB-EGF did not affect the nuclear accumulation of TGF-β induced Smad3, but caused stabilization of the Smad transcriptional co-repressor TGIF of these cells. TGIF was originally identified as a Smad2 co-repressor in the TGF-β/Nodal/Activin signaling pathways [24]. EGF has been shown to phosphorylate TGIF via Ras-MAP kinase pathways, leading to the stabilization of TGIF [25]. In lung fibroblasts, EGF also has been shown to antagonize TGF-β-induced expression of tropoelastin, a component of elastic fibers, via stabilization of TGIF without preventing the nuclear accumulation of Smad2/3 by TGF-β [26]. Considering these findings and ours suggests that HB-EGF might antagonize TGF-β-induced expression fibrosis-related genes through an EGFR-TGIF signaling pathway in HSCs.

Previous work has been demonstrated that over-expression of HB-EGF in pancreatic islets resulted in pancreatic fibrosis and epithelial metaplasia [27]. These reports indicated that HB-EGF has a pro-fibrogenic potential in the pancreas. Pancreatic fibrosis is known to be associated with the activation of pancreatic stellate cells as in the case of the liver fibrosis [28]. In our study, HB-EGF suppressed the activation of HSCs, and conditional loss of HB-EGF resulted in enhanced liver fibrosis in murine cholestatic fibrosis models. It is interesting to note that the role of HB-EGF in tissue fibrosis is completely different between the liver and the pancreas and further investigation will be needed to elucidate this.

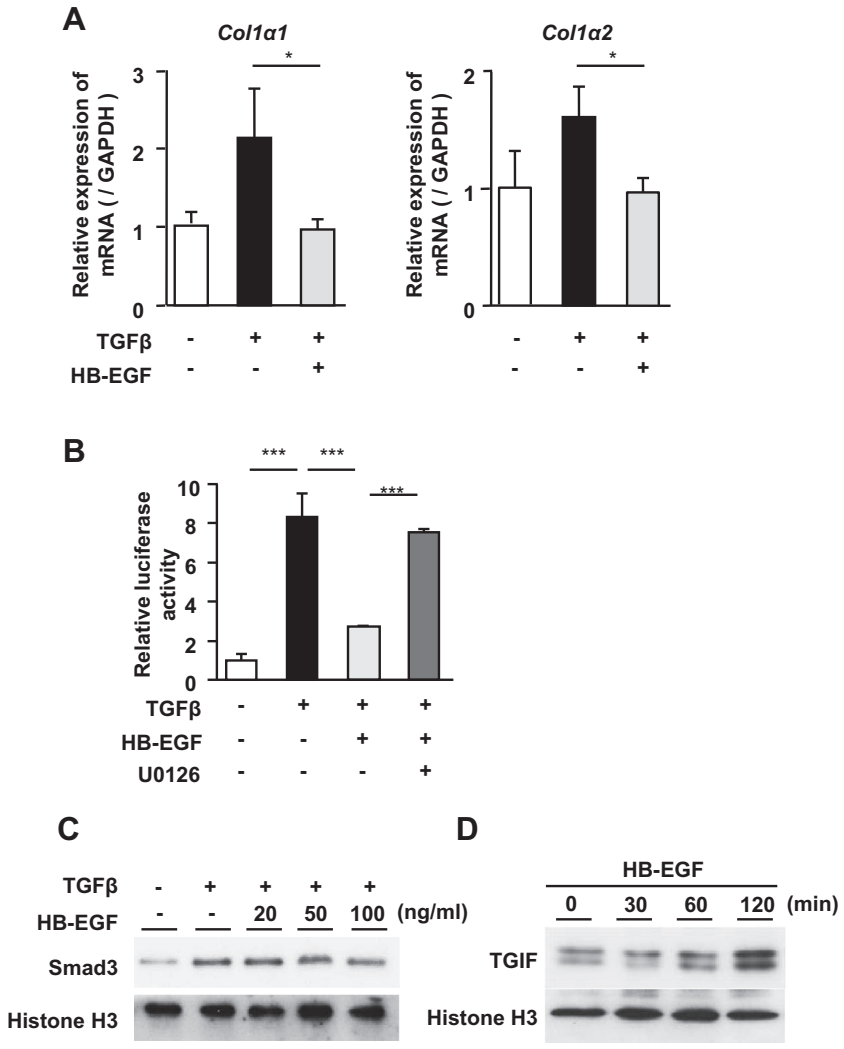

Fig. 4. HB-EGF attenuates TGF-β-induced expression of fibrosis-related genes in murine hepatic stellate cells. (A) Gene expression of Col1α1 and Col1α2 in HSCs treated with HB-EGF (data are mean ± SE, $^{*}p < 0.05$). (B) Effect of HB-EGF on TGF-β-dependent transcription in HSCs (data are mean ± SE, $^{***}p < 0.001$). (C) Effect of HB-EGF on the nuclear accumulation of Smad3 in HSCs. (D) Effect of HB-EGF on the stabilization of TGIF in HSCs.

Recently, Huang et al. published their work about the role of HB-EGF on liver fibrosis using different mouse lines and models [11]. Although they used the TAA or CCl_4-induced liver fibrosis model instead of the BDL model, our results were generally in agreement with theirs on the role of HB-EGF during liver fibrosis. In our study, we developed conditional KO mice to suppress the expression of HB-EGF in the liver because traditional HB-EGF KO mice die immediately after birth due to severe heart failure [12]. According to the original paper regarding KO mice, which they used in their fibrosis study, most of these traditional HB-EGF KO mice die before weaning and survivors have been shown to have enlarged, dysfunctional hearts and reduced life spans [29]. Thus, our conditional HB-EGF KO mice might be more suitable for examining the role of HB-EGF during liver fibrosis than traditional HB-EGF KO mice. Moreover, our results could be used to describe the inhibitory role of HB-EGF on the TGF-β-induced activation of HSCs.

In summary, we demonstrated in the present study that conditional loss of HB-EGF resulted in enhanced liver fibrosis in the murine cholestatic model and that HB-EGF suppresses the activation of HSCs. Our findings further suggest that HB-EGF might be a potential therapeutic target against liver fibrosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.097.

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