

## Thrombocytopenia Exacerbates Cholestasis-Induced Liver Fibrosis in Mice

TAKAHIRO KODAMA,\* TETSUO TAKEHARA,\* HAYATO HIKITA,\* SATOSHI SHIMIZU,\* WEI LI,\* TAKUYA MIYAGI,\* ATSUSHI HOSUI,\* TOMOHIDE TATSUMI,\* HISASHI ISHIDA,\* SEIJI TADOKORO,<sup>‡</sup> AKIO IDO,<sup>§</sup> HIROHITO TSUBOUCHI,<sup>§</sup> and NORIO HAYASHI\*

\*Department of Gastroenterology and Hepatology and <sup>‡</sup>Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Suita, Osaka; and <sup>§</sup>Digestive Disease and Life-style Related Disease Health Research, Human and Environmental Science, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Kagoshima, Japan

**BACKGROUND & AIMS:** Circulating platelet counts gradually decrease in parallel with progression of chronic liver disease. Thrombocytopenia is a common complication of advanced liver fibrosis and is thought to be a consequence of the destruction of circulating platelets that occurs during secondary portal hypertension or hypersplenism. It is not clear whether thrombocytopenia itself affects liver fibrosis. **METHODS:** Thrombocytopenic mice were generated by disruption of *Bcl-xL*, which regulates platelet life span, specifically in thrombocytes. Liver fibrosis was examined in thrombocytopenic mice upon bile duct ligation. Effect of platelets on hepatic stellate cells (HSCs) was investigated in vitro. **RESULTS:** Thrombocytopenic mice developed exacerbated liver fibrosis, with increased expression of type I collagen  $\alpha 1$  and  $\alpha 2$ , during cholestasis. In vitro experiments revealed that, upon exposure to HSCs, platelets became activated, released hepatocyte growth factor (HGF), and then inhibited HSC expression of the type I collagen genes in a Met signal-dependent manner. In contrast to the wild-type mice, the thrombocytopenic mice did not accumulate hepatic platelets or phosphorylate Met in the liver following bile duct ligation. Administration of recombinant HGF to thrombocytopenic mice reduced liver fibrosis to the levels observed in wild-type mice and attenuated hepatic expression of the type I collagen genes. **CONCLUSIONS: Thrombocytopenia exacerbates liver fibrosis; platelets have a previously unrecognized, antifibrotic role in suppressing type I collagen expression via the HGF-Met signaling pathway.**

**Keywords:** Bcl-2; Apoptosis; Cre; Conditional Knockout.

Cirrhosis followed by chronic liver disease is considered to be a major medical issue worldwide, causing significant morbidity and mortality because it can progress to liver failure or develop into hepatocellular carcinoma. The pathogenesis of cirrhosis is characterized by liver fibrosis, which is defined as excessive production and deposition of several extracellular matrix (ECM) proteins. The accumulation of ECM proteins, as fibrotic scars, gradually distorts liver structure and increases intrahepatic resis-

tance to blood flow, leading to portal hypertension.<sup>1</sup> Among the deposited ECM proteins in the cirrhotic liver, type I collagen is the most prevalent, and it is well known that activated hepatic stellate cells (HSCs) are major collagen-producing cells.<sup>1,2</sup> With fibrosis progression in chronic liver disease, patients often suffer from thrombocytopenia, which promotes a tendency for bleeding and can result in mortal hemorrhagic complications such as variceal bleeding.<sup>3</sup> Multiple factors have been proposed for the pathogenesis of thrombocytopenia in advanced liver fibrosis; they include enhanced destruction of circulating platelets in an enlarged spleen arising because of portal hypertension<sup>4</sup> and reduced production of thrombopoietin (TPO) in the liver.<sup>3</sup> In general, concomitant thrombocytopenia is considered to be a secondary phenomenon caused by liver fibrosis progression. However, whether thrombocytopenia per se affects liver fibrosis has not been thoroughly examined. In the present study, we generated a novel mouse model of severe thrombocytopenia by thrombocyte-specific knockout of *Bcl-xL*, a critical regulator of thrombocyte life span,<sup>5</sup> and found that the mice developed exacerbated liver fibrosis during bile duct ligation (BDL)-induced cholestasis because of an increase in type I collagen gene expression. In vitro study revealed that platelets negatively regulated type I collagen gene expression in activated HSCs via a pathway involving the platelet-derived hepatocyte growth factor (HGF) and its receptor, Met.

**Abbreviations used in this paper:** ALP, alkaline phosphatase;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; BDL, bile duct ligation; BrdU, 5-bromo-2-deoxyuridine; ECM, extracellular matrix; HGF, hepatocyte growth factor; HSCs, hepatic stellate cells; MMP, matrix metalloproteinase; mRNA, messenger RNA; Pf4, platelet factor 4; siRNA, small interfering RNA; T-Bil, total bilirubin; TPO, thrombopoietin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

© 2010 by the AGA Institute  
0016-5085/\$36.00  
doi:10.1053/j.gastro.2010.02.054

## Materials and Methods

### Mice

Thrombocyte-specific Bcl-xL knockout mice (*bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre*) were generated by mating *bcl-x<sup>fllox/fllox</sup>* mice<sup>6,7</sup> and *Pf4-Cre* transgenic mice.<sup>8</sup> They were maintained in a specific pathogen-free facility and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

### BDL Treatment

Wild-type (*bcl-x<sup>fl/fl</sup>*) and knockout (*bcl-x<sup>fl/fl</sup>* *Pf4-Cre*) mice were subjected to BDL as previously reported.<sup>9</sup> Briefly, the common bile duct was ligated 3 times with 5-0 silk sutures and then cut between the ligatures. After 10 days, the animals were killed for the following analyses. For more detailed description of the Materials and Methods used, see the Supplementary Materials and Methods.

## Results

### Thrombocyte-Specific Disruption of Bcl-xL Causes Massive Thrombocytopenia

Previous research has demonstrated that traditional knockout mice lacking a single allele of the *bcl-x* gene develop mild thrombocytopenia.<sup>5</sup> We generated thrombocyte-specific Bcl-xL knockout mice by crossing *floxed bcl-x* mice<sup>6,7</sup> and *Pf4-Cre* transgenic mice.<sup>8</sup> After mating *bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre* mice with *bcl-x<sup>fllox/fllox</sup>* mice, *bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre* mice were born at the expected Mendelian frequency and did not show any developmental abnormality. As expected, *bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre* mice showed severe thrombocytopenia without any phenotypes of other hematopoietic lineages (Figure 1A). Western blot analysis confirmed a substantial decrease in Bcl-xL expression in circulating platelets of *bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre* mice compared with *bcl-x<sup>fllox/fllox</sup>* mice (Figure 1B). CD41 protein, a specific surface receptor expressed in the thrombocyte lineage,<sup>10,11</sup> was used as a loading control of platelets. To demonstrate the thrombocyte-lineage specificity of the Platelet factor 4 (Pf4) promoter that we used, we examined Bcl-xL protein expression in several tissues and hematopoietic cells of *bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice by Western blotting. In all of these tissues and cells, Bcl-xL protein expression was not different between the 2 groups (Figure 1C), indicating that the Pf4 promoter was specific to platelets and their precursors in our mice model. The physiologic liver status was not different between the 2 groups as evidenced by serum biochemistry data for alanine transaminase (ALT), total bilirubin (T-Bil), and alkaline phosphatase (ALP) (Figure 1D) as well as for liver histology (Figure 1E). In the following experiments, *bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre* mice were crossed with *bcl-x<sup>fllox/fllox</sup>* mice, and their offspring, *bcl-x<sup>fllox/fllox</sup>* *Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice, were used as thrombocytopenic mice and control littermates, respectively.

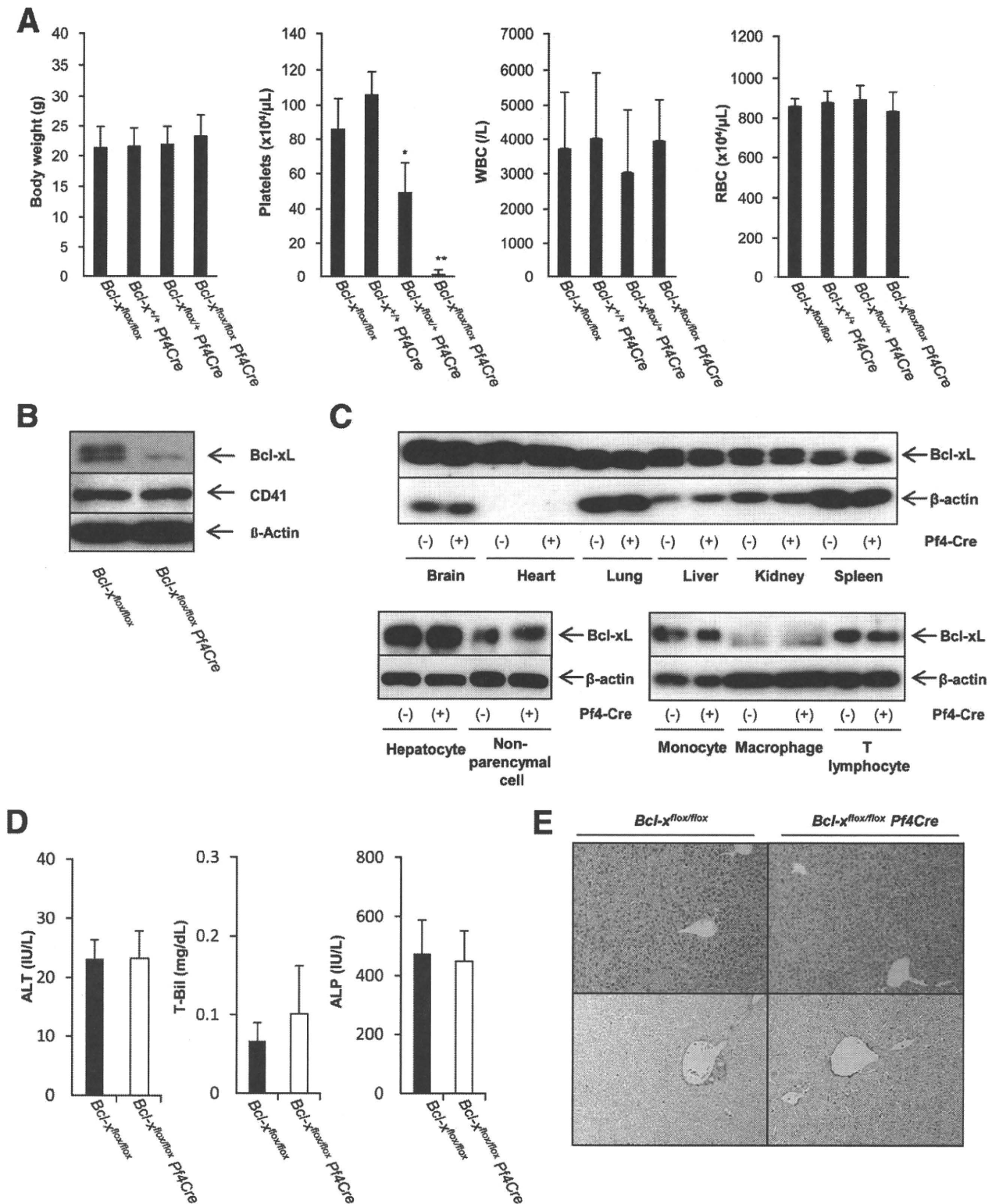
### Thrombocytopenic Mice Display Exacerbation of Cholestasis-Induced Liver Fibrosis

To investigate the effect of thrombocytopenia on liver fibrosis, these mice were subjected to BDL, a well-established model of liver fibrosis,<sup>9</sup> and examined 10 days later. Cholestasis was similarly induced in both groups as evidenced by serum levels of alkaline phosphatase and T-Bil (Figure 2A). Both oncotic necrosis, also known as bile infarcts, and apoptosis are characteristic features of liver injury in the BDL model.<sup>12</sup> Although serum ALT levels were slightly lower in the thrombocytopenic mice than in the control littermates (Figure 2A), the area of oncotic necrosis as well as the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive cells in the liver was not significantly different between the 2 groups (Figure 2B). The number of accumulating neutrophils, which are known to play a major role in liver inflammation induced by cholestasis,<sup>12</sup> did not differ between the 2 groups as assessed by chloroacetate esterase staining of the liver sections (Figure 2C). Similarly, the T lymphocyte and macrophage population in the liver did not differ between the 2 groups as determined by real-time reverse-transcription polymerase chain reaction (Supplementary Figure 1). Upon BDL treatment, compensatory regeneration occurred, but there was no significant difference between the 2 groups as determined by the count of 5-bromo-2-deoxyuridine (BrdU)-positive cells (Figure 2D).

To assess liver fibrosis, hepatic collagen deposition was evaluated by picrosirius red staining of liver sections. Collagen deposition increased following BDL treatment in both groups and was significantly higher in the thrombocytopenic mice than in the control littermates (Figure 2E). Similarly, the hepatic hydroxyproline content, a biochemical marker of collagen accumulation,<sup>6</sup> in the thrombocytopenic mice was elevated to a level significantly higher than in the control littermates (Figure 2E). The major form of collagen in cirrhosis is known to be type I collagen composed of 2  $\alpha$ 1 and 1  $\alpha$ 2 chains. After BDL, hepatic expression of type I collagen  $\alpha$ 1 and  $\alpha$ 2 genes, *col1a1* and *col1a2*, sharply rose in both groups and was significantly higher in the thrombocytopenic mice than in the control littermates (Figure 2F). Western blot analysis confirmed that the hepatic expression of type I collagen protein was higher in the thrombocytopenic mice than in the control littermates (Figure 2F). These results indicated that thrombocytopenia enhanced collagen synthesis in the liver and exacerbated liver fibrosis without affecting liver inflammation, apoptosis, and regeneration.

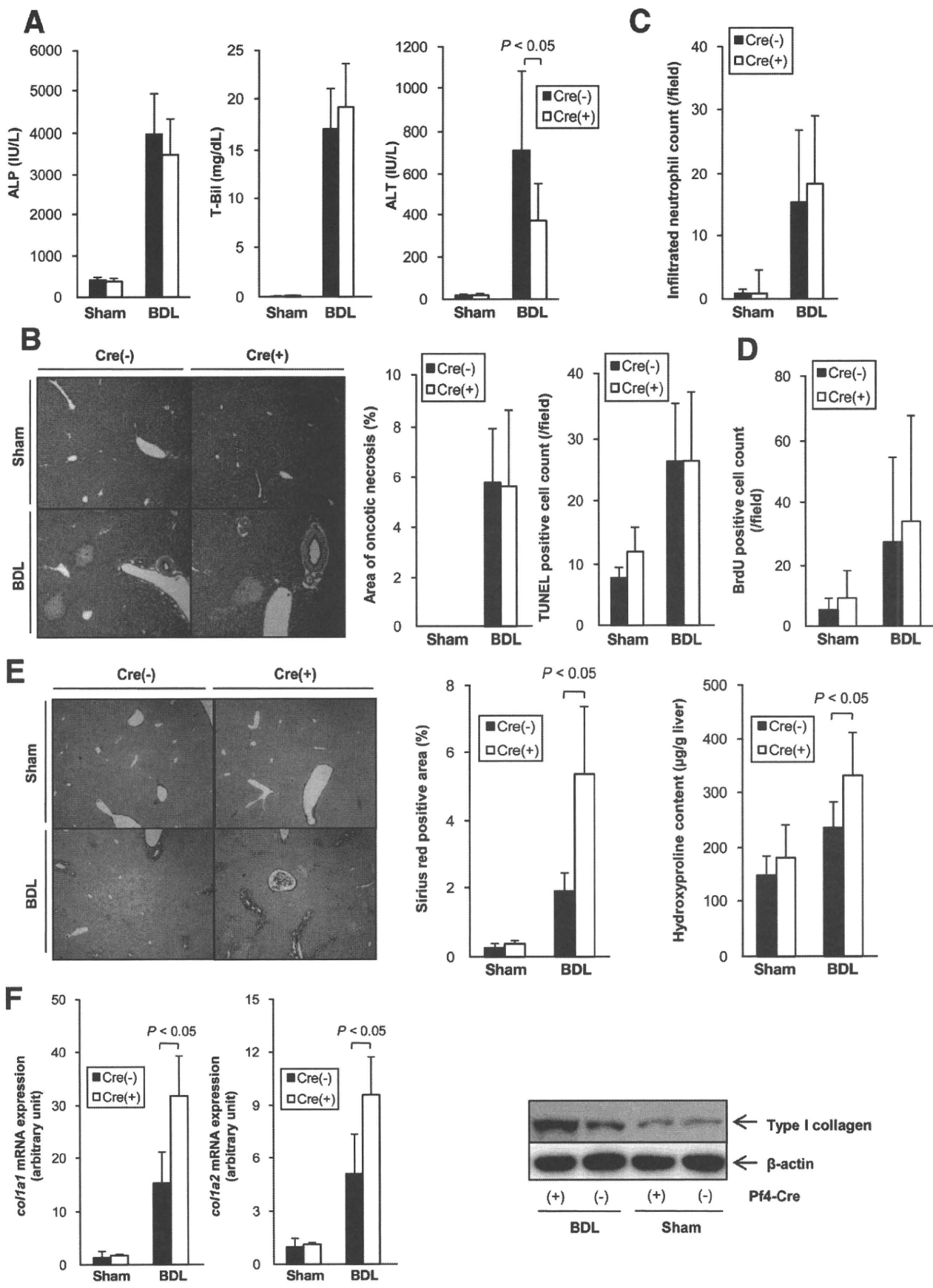
### Platelets Become Activated and Inhibit Collagen Synthesis in Activated HSCs In Vitro

To explore the underlying mechanisms of increased collagen synthesis after BDL in the liver of the thrombocytopenic mice, we tested the hypothesis that



**Figure 1.** Thrombocyte-specific Bcl-xL knockout mice show massive thrombocytopenia. (A) Body weight and circulating blood cell counts of offspring from mating of *bcl-x<sup>fllox/+</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/+</sup>* mice; 7–11 mice per group; \**P* < .05 vs the other 3 groups, \*\**P* < .05 vs the other 3 groups. (B) Expression of Bcl-xL and CD41 protein in circulating platelets by Western blot analysis. β-Actin is included as a control. (C) Expression of Bcl-xL in indicated tissues and cells by Western blot analysis. Heart tissue lysates were equally loaded between the 2 groups confirmed by expression of glyceraldehyde-3-phosphate dehydrogenase, although the data are not shown. Pf4-Cre(+) and Pf4-Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. (D) Serum levels of alanine aminotransferase (ALT), total bilirubin (T-Bil), and alkaline phosphatase (ALP). (E) H&E staining (upper panel) and picrosirius red staining (lower panel) of liver sections.

BASIC-LIVER, PANCREAS, AND BILIARY TRACT



BASIC-LIVER, PANCREAS, AND BILIARY TRACT



platelets would suppress collagen production in activated HSCs, which are known as the main collagen-producing cells in the injured liver.<sup>13</sup> We isolated HSCs from C57BL/6J mice and cultured them for 7 to 10 days, leading to their transdifferentiation from quiescent cells to activated myofibroblast-like cells.<sup>13</sup> These culture-activated HSCs were then cocultured with platelets isolated from C57BL/6J mice. Expression of *col1a1* and *col1a2* messenger RNA (mRNA) in HSCs was clearly inhibited upon addition of platelets (Figure 3A). After a few passages, similar suppression of type I collagen gene expression was observed in these cells, appearing in a platelet dose-dependent manner (Figure 3B). Type I collagen protein in HSCs also decreased upon coculture with platelets as determined by Western blot analysis (Figure 3C). Platelets generally execute their biologic effects through activation that is associated with their shape change and granule secretion represented by P-selectin (CD62P) translocation from the  $\alpha$ -granule to the outer surface.<sup>14,15</sup> To find whether or not platelets are activated upon exposure to HSCs, platelets from cocultures with HSCs were analyzed by flow cytometry, which revealed their dynamic shape change and the surface translocation of P-selectin (Figure 3D). The levels of soluble P-selectin, which are also known to reflect platelet activation,<sup>15</sup> were significantly higher in the coculture medium with platelets and HSCs than those in the medium with platelets alone (Figure 3E). These results demonstrated that platelets were activated upon exposure to HSCs and inhibited collagen synthesis in activated HSCs.

#### ***Soluble Factors Released From Activated Platelets Are Involved in the Inhibition of Collagen Synthesis in HSCs***

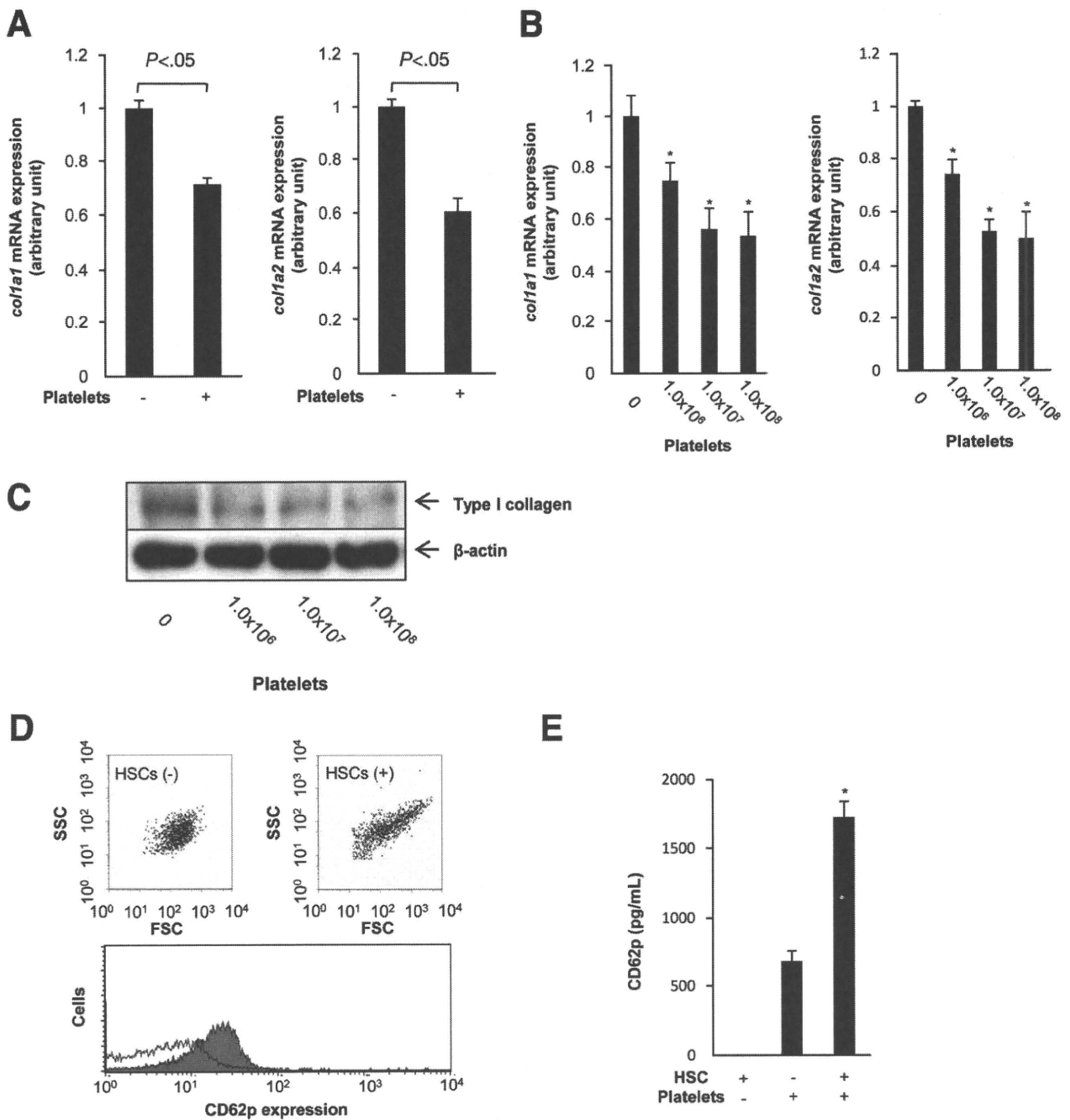
Once activated, platelets are known to affect many other cells via secreted soluble factors or direct interaction with surface molecules. Platelet activation and secretion can be triggered artificially by a variety of strong agonists such as thrombin.<sup>14</sup> To examine whether soluble factors secreted from activated platelets are involved in the suppression of collagen synthesis in HSCs, we stimulated platelets with or without thrombin and applied the supernatant to HSCs. Thrombin induced clear platelet activation as evidenced by shape changes and P-selectin translocation (Figure 4A). The levels of soluble P-selectin were also significantly higher in the supernatant of thrombin-stimulated platelets than in the supernatant

of unstimulated platelets (Figure 4B). The supernatant of thrombin-stimulated platelets suppressed type I collagen gene expression in HSCs but that of unstimulated platelets did not (Figure 4C), indicating that soluble factors derived from activated platelets were involved in suppressing collagen production in HSCs.

#### ***HGF in Platelet Granules Contributes to the Inhibition of Collagen Synthesis in HSCs***

To identify the platelet-derived soluble factors that contribute to the suppression of collagen synthesis in HSCs, we focused on HGF, a pleiotropic growth factor,<sup>16,17</sup> which is known to exist in platelets.<sup>18</sup> We hypothesized that, in our *in vitro* study, HGF may be secreted from activated platelets and inhibit collagen synthesis in HSCs. Administration of recombinant HGF to HSCs inhibited *col1a1* and *col1a2* gene expression (Figure 5A). Consecutively, murine platelets were capable of releasing HGF upon exposure to thrombin (Figure 5B). Importantly, the levels of HGF were significantly higher in the coculture supernatant of HSCs and platelets than in that of platelets alone (Figure 5C). We next examined whether HGF secreted from activated platelets is actually involved in suppressing collagen synthesis in HSCs. The multiple biologic activities of HGF are mediated by Met, a transmembrane tyrosine kinase receptor, which transduces the effects of HGF upon phosphorylation.<sup>19</sup> Western blot analysis showed that the Met protein of HSCs was phosphorylated at multiple sites after coculture with platelets (Figure 5D) and proteins of its downstream pathways such as Erk1/2, Akt, and stat3 were phosphorylated as well (Figure 5D). To assess the involvement of this activated signaling in the inhibition of collagen production in HSCs, we performed small interfering RNA (siRNA)-mediated knockdown of *met*. Transfection of *met* siRNA into HSCs resulted in a substantial decrease in Met expression (Figure 5E) and blunted HGF-induced suppression of type I collagen gene expression (Figure 5F). Under these conditions, *met* knockdown abolished platelet-induced suppression of type I collagen gene expression in HSCs (Figure 5F). This result clearly demonstrated that HGF/Met signaling was indispensable for platelet-mediated inhibition of the collagen synthesis in activated HSCs.

**Figure 2.** Thrombocytopenic mice show exacerbated liver fibrosis following BDL treatment. *bcl-x<sup>fllox/fllox</sup> Pfl4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice were sham operated or subjected to BDL and analyzed 10 days later (8–12 mice per group). Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pfl4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. (A) Serum levels of alkaline phosphatase (ALP), total bilirubin (T-Bil), and alanine aminotransferase (ALT). (B) Oncotic necrosis and hepatocyte apoptosis were evaluated by H&E staining and TUNEL staining of liver sections, respectively. (C) Infiltrated neutrophil count was evaluated by chloroacetate esterase staining of liver sections. (D) Liver regeneration was evaluated by 5-bromo-2-deoxyuridine (*BrdU*) staining of liver sections. (E) Liver fibrosis was evaluated by picrosirius red staining of liver sections and total liver hydroxyproline levels. (F) *col1a1* and *col1a2* mRNA levels in the liver were determined by real-time reverse-transcription polymerase chain reaction. Expression of type I collagen protein in the liver was assessed by Western blotting.



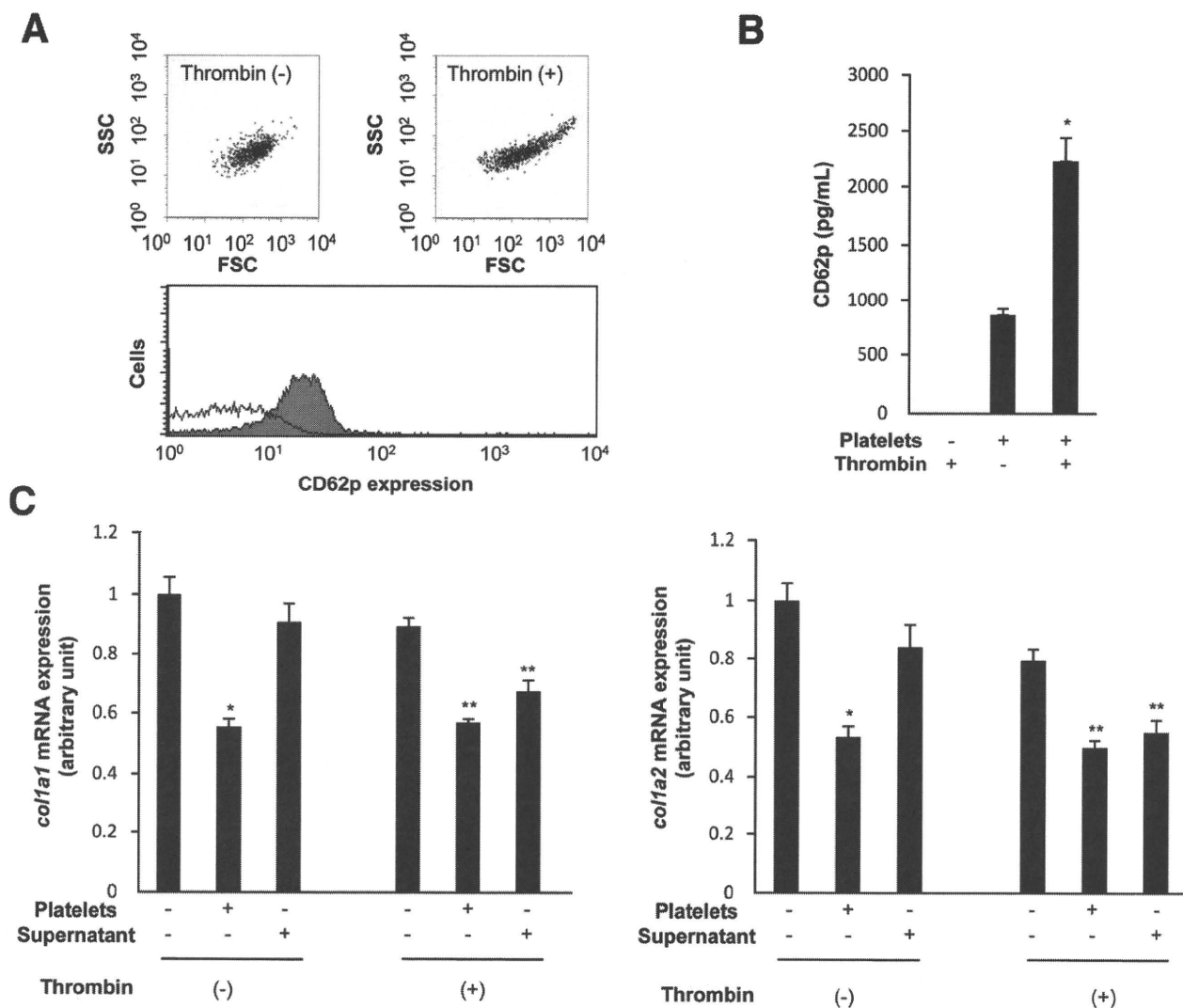
**Figure 3.** Platelets become activated and inhibit collagen synthesis in activated HSCs in vitro. (A and B) *col1a1* and *col1a2* mRNA levels in HSCs by real-time reverse-transcription polymerase chain reaction. Primary isolated HSCs were cocultured with  $1.0 \times 10^8$  platelets for 6 hours (A),  $n = 3$ /group. HSCs ( $1.0 \times 10^5$ ) were cocultured with indicated dosages of platelets for 6 hours (B),  $n = 3$ /group,  $*P < .05$  vs HSCs with control group. (C) Expression of type I collagen protein in HSCs determined by Western blot analysis. HSCs ( $5.0 \times 10^5$ ) were cocultured with indicated dosages of platelets for 14 hours. (D and E) Activation of platelets on exposure to HSCs. Platelets ( $1.0 \times 10^7$ ) were cocultured with or without  $1.0 \times 10^5$  HSCs for 1 hour. Shape change and P-selectin surface expression of platelets were analyzed by flow cytometry (D); representative data are shown; note that FSC increased with addition of HSCs; closed histograms and open histograms indicate P-selectin surface expression of platelets cocultured with or without HSCs, respectively. Soluble P-selectin levels of the culture supernatants were determined by ELISA (E),  $n = 3$ /group,  $*P < .05$  vs the other 2 groups.

**HGF Administration Alleviates Liver Fibrosis in Thrombocytopenic Mice to the Level in the Control Littermates After BDL**

To investigate the involvement of platelets in cholestasis-induced liver fibrosis in vivo, we examined platelet

kinetics upon BDL treatment. To find whether platelets accumulate in the liver, we examined the expression of CD41 protein. Western blot analysis revealed that CD41 expression in the liver was up-regulated upon BDL treatment in the control littermates but not in the thrombocy-

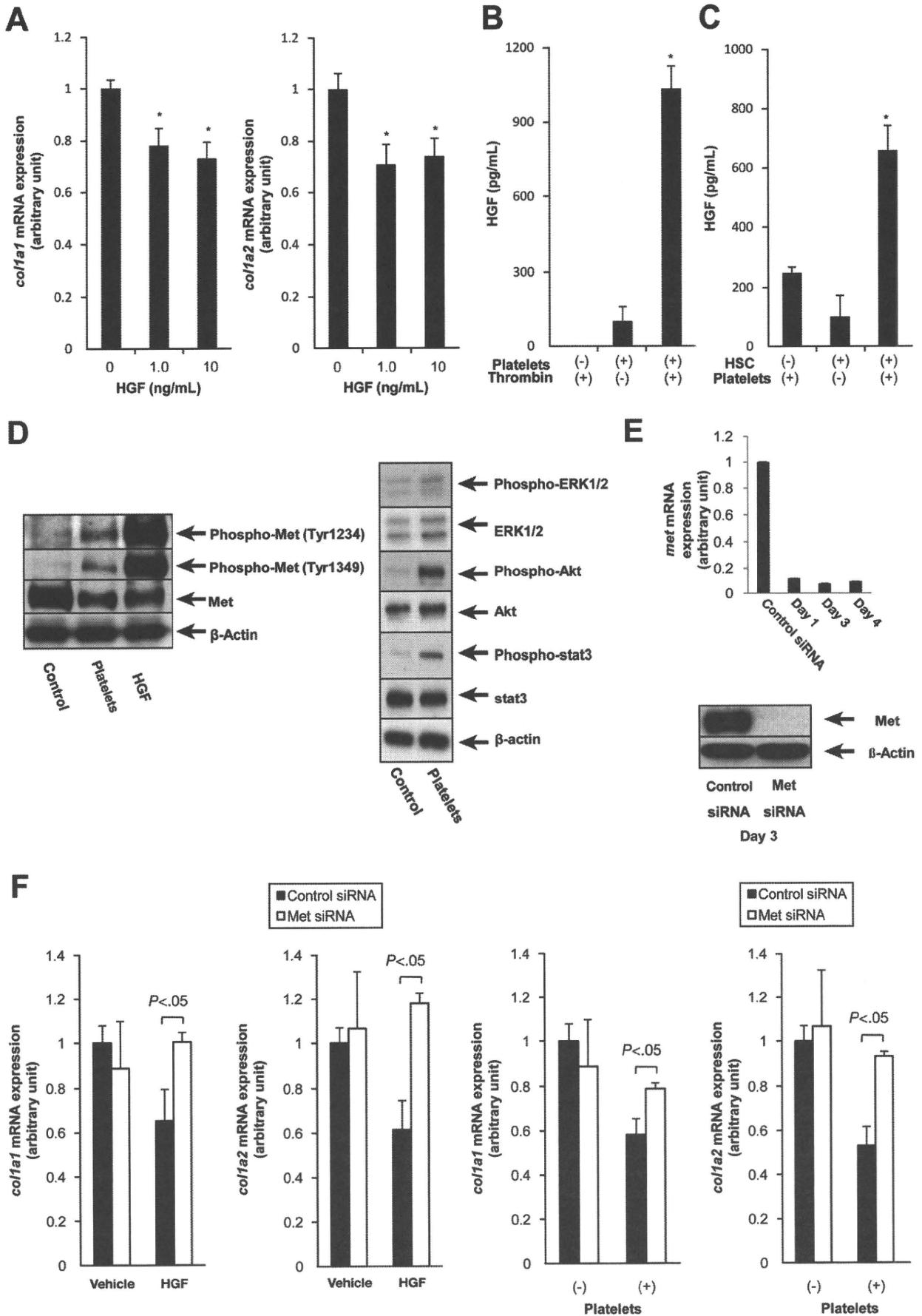
BASIC-LIVER, PANCREAS, AND BILIARY TRACT



**Figure 4.** Soluble factors released from activated platelets are involved in the inhibition of collagen synthesis in HSCs. (A and B) Activation of platelets stimulated with thrombin. Platelets ( $1.0 \times 10^7$ ) were stimulated with or without thrombin (1 U/mL) for 15 minutes. Shape change and P-selectin surface expression of platelets were analyzed by flow cytometry (A); representative data are shown; note that FSC increased with addition of thrombin; closed histograms and open histograms indicate P-selectin surface expression of platelets stimulated with or without thrombin, respectively. Soluble P-selectin levels of the culture supernatants were determined by ELISA (B),  $n = 3/\text{group}$ , \* $P < .05$  vs the other 2 groups. (C) *col1a1* and *col1a2* mRNA levels in HSCs treated with the supernatant of activated or quiescent platelets by real-time reverse-transcription polymerase chain reaction. HSCs were cocultured with or without  $1.0 \times 10^7$  platelets for 6 hours in the presence or absence of thrombin (1 U/mL). In parallel, HSCs were cultured for 6 hours with the supernatants of platelets, which had been stimulated with or without thrombin (1 U/mL) for 15 minutes,  $n = 3/\text{group}$ . \* $P < .05$  vs HSC with control group and HSC with platelet supernatant group. \*\* $P < .05$  vs HSC with thrombin group.

topenic mice (Figure 6A). Furthermore, phosphorylation of Met protein in the liver occurred upon BDL treatment, but it was weaker in the thrombocytopenic mice than in the control littermates (Figure 6B). Similar attenuation of Met phosphorylation in the thrombocytopenic mice was also observed at 3 days after BDL (Supplementary Figure 2). These results indicated that BDL-induced cholestasis led to intrahepatic platelet accumulation and activated the Met signal in the liver. In contrast, both were attenuated in the liver of the thrombocytopenic mice. Furthermore, plasma HGF levels in the thrombocytopenic mice did not increase upon BDL and were evidently lower than in the control littermates (Figure 6C). Finally, to investigate whether at-

tenuation of Met activation in the liver of the thrombocytopenic mice was involved in the exacerbation of liver fibrosis, we tested the hypothesis that administration of HGF, known to exert an antifibrotic effect,<sup>20-22</sup> would alleviate liver fibrosis in the thrombocytopenic mice more than in the control littermates. These mice were treated with either vehicle or recombinant HGF following BDL. As expected, HGF administration alleviated liver fibrosis in the thrombocytopenic mice to the level found in the control littermates (Figure 6D). Notably, elevated hepatic expression of type I collagen genes in the thrombocytopenic mice was also attenuated to a level comparable with that in the control littermates by the HGF therapy (Figure 6E).



BASIC-LIVER,  
PANCREAS, AND  
BILIARY TRACT

## Discussion

Platelets are circulating blood cells with the daily job of handling hemostasis and thrombosis.<sup>14</sup> On the other hand, they are also involved in inflammation,<sup>23</sup> angiogenesis, and tissue repair. Platelets have been shown to accumulate in the liver under some pathologic conditions such as acute viral hepatitis<sup>24</sup> and cholestasis.<sup>25</sup> Previous work on such situations has focused on platelets as a producer of inflammatory cytokines and on their proinflammatory role. However, a recent study has demonstrated a new role for platelets in the liver: that of platelet-derived serotonin mediating liver regeneration.<sup>26</sup> Moreover, it has been reported that TPO-induced thrombocytosis attenuates progression of liver fibrosis and accelerates liver fibrolysis.<sup>27,28</sup> However, the mechanisms remain obscure, and the extrathrombotic effect of TPO could not be excluded from their study results. In the present study, we were able to clearly demonstrate that platelets serve as antifibrotic cells in the liver via the HGF/Met pathway and offer the novel finding that thrombocytopenia exacerbates liver fibrosis in vivo.

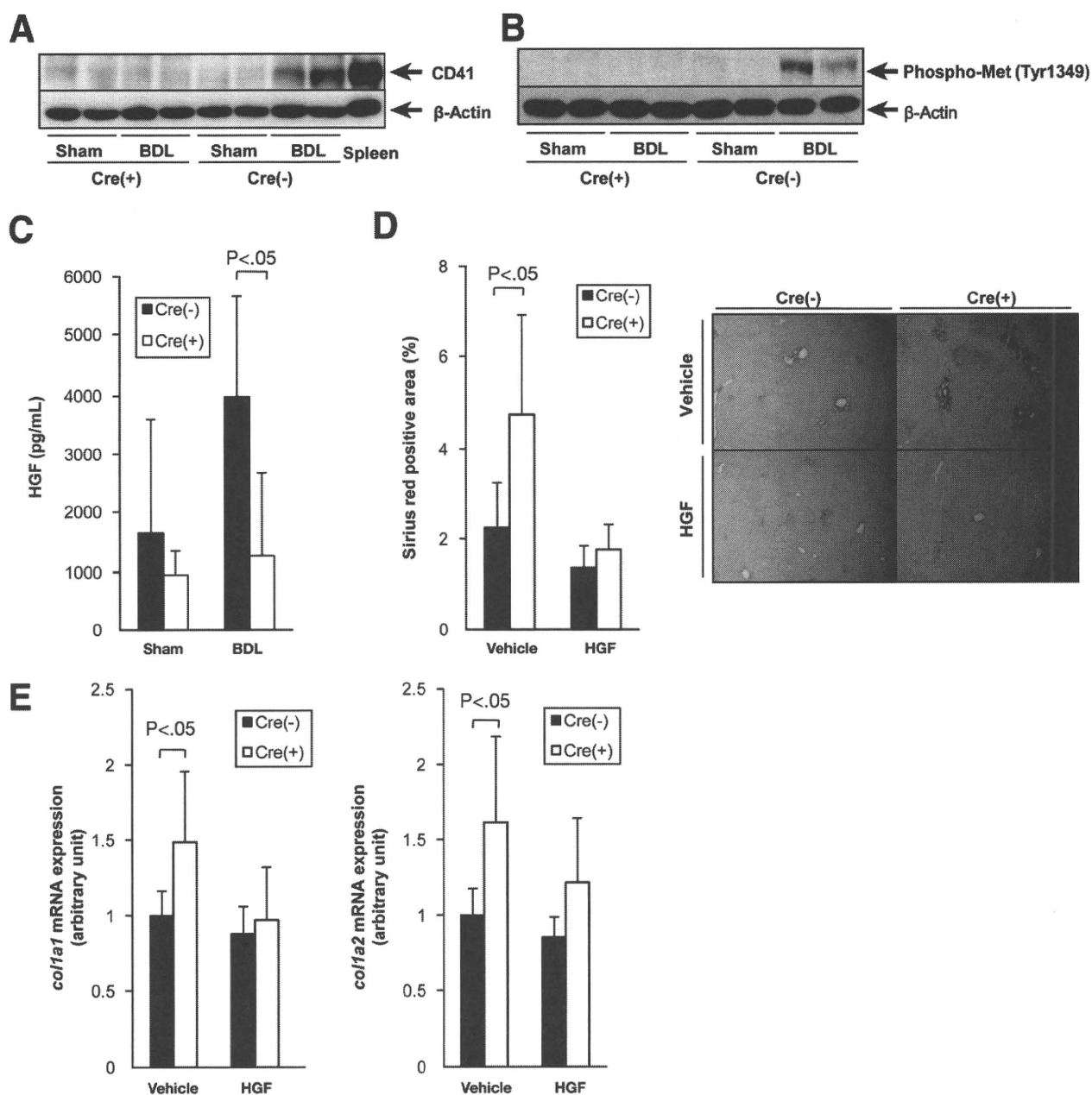
To examine the impact of thrombocytopenia in liver fibrosis, we generated a novel mouse model of severe thrombocytopenia. Previous research has shown that platelets are genetically programmed to die in an apoptotic manner and that their life span is regulated by a fine balance between antiapoptotic Bcl-xL and proapoptotic Bak; mice lacking a single allele of the *bcl-x* gene develop mild thrombocytopenia, which is attenuated with a *bak* knockout background.<sup>5</sup> However, traditional knockout of both alleles of the *bcl-x* gene leads to embryonic lethality.<sup>6</sup> To develop severe thrombocytopenic mice without phenotype expression in other organs caused by Bcl-xL deficiency, we generated thrombocyte-specific Bcl-xL knockout mice by crossing floxed *bcl-x* mice<sup>6,7</sup> and transgenic mice expressing the Cre-recombinase under regulation of the promoter of the Pf4 gene.<sup>8</sup> The expression of Pf4 promoter is reported to be specific to thrombocytes,<sup>8</sup> and its specificity was also confirmed in our generated mice. The mice displayed severer thrombocytopenia than the single allele knockout mice, at as early as 4 weeks of age, and it persisted for a longer time (Supplementary Figure 3).

Thrombocytopenic mice did not develop any liver pathology under physiologic conditions but developed exacerbated liver fibrosis upon BDL. Similar exacerbation of liver fibrosis was found in another liver fibrosis model induced by chronic injection of carbon tetrachloride (Supplementary Figure 4). Following BDL, thrombocytopenic mice showed lower ALT levels than the control mice. Because there was no significant difference in histologic necrosis and hepatocyte apoptosis between the 2 groups, the significance of this difference is obscure. Research has revealed proinflammatory roles of platelets in the liver under some experimental conditions.<sup>23-25</sup> Thus, thrombocytopenia might have led to modest reduction of liver injury in our experiment without any histologic differences. Even if that were the case, modest decline of liver injury could not explain the exacerbation of liver fibrosis in thrombocytopenic mice. It is well known that the liver has the unique capacity to regenerate in response to partial hepatectomy or some types of liver injury.<sup>29</sup> Recent research has shown that platelets mediate liver regeneration after partial hepatectomy.<sup>26</sup> In our experiment, modest compensatory regeneration did occur following BDL, but we could not find any difference in liver regeneration between the 2 groups. Following two thirds' partial hepatectomy, most hepatocytes in the remaining liver enter an active state of cell cycle progression,<sup>29</sup> whereas only a relatively small number of them may do so following liver injury. That may explain why liver regeneration did not differ in our models. Taken together, we considered that liver fibrosis is the primary and most prominent difference between the thrombocytopenic mice and the control littermates after BDL.

With regard to the underlying mechanisms of exacerbated liver fibrosis in thrombocytopenic mice upon BDL, we first took particular notice of the increase in collagen gene expression. In fact, liver fibrosis is known to be regulated by a fine balance between fibrogenesis and fibrolysis.<sup>1,2</sup> A variety of matrix metalloproteases (MMPs), such as MMP-2, MMP-9, and MMP-14, which may be involved in fibrolysis, were also up-regulated in the liver in thrombocytopenic mice compared with control mice (Supplementary Figure 5A). In addition, gene expression of platelet-derived growth factor, D polypeptide, transforming

**Figure 5.** The HGF/Met pathway is involved in platelet-mediated inhibition of collagen synthesis in HSCs in vitro. (A) *co1a1* and *co1a2* mRNA levels in HSCs stimulated with murine HGF for 6 hours by real-time reverse-transcription polymerase chain reaction,  $n = 3/\text{group}$ . \* $P < .05$  vs control. (B) Secretion of HGF from activated platelets. Platelets ( $1.0 \times 10^7$ ) were stimulated with or without thrombin (1 U/mL) for 15 minutes, and the levels of HGF in the culture supernatant were determined by ELISA,  $n = 3/\text{group}$ . \* $P < .05$  vs other 2 groups. (C) Production of HGF in platelet/HSC coculture. HSCs ( $1.0 \times 10^5$ ) were cocultured with  $5.0 \times 10^7$  of platelets for 3 hours, and the levels of HGF in the culture medium were determined by ELISA,  $n = 3/\text{group}$ . \* $P < .05$  vs the other 2 groups. (D) Activation of Met and its downstream pathways in platelet/HSC coculture. HSCs ( $5.0 \times 10^5$ ) were cocultured with or without  $5.0 \times 10^7$  platelets or with 20 ng/mL HGF as a positive control for 1 hour. Western blot analysis of phosphorylated Met protein at indicated position of tyrosine (left panel) and phosphorylated Erk1/2, Akt, and stat3 proteins (right panel). (E) Real-time reverse-transcription polymerase chain reaction (upper panel) and Western blot analysis (lower panel) of Met expression in HSCs transfected with *met* siRNA or control siRNA. (F) *co1a1* and *co1a2* mRNA levels in HSCs treated with *met* siRNA by real-time reverse-transcription polymerase chain reaction. HSCs were transfected with *met* siRNA or control siRNA for 3 days and then cultured for 6 hours with or without 20 ng/mL HGF (left) or with or without  $1.0 \times 10^7$  platelets (right),  $n = 3/\text{group}$ .





**Figure 6.** HGF administration prevents the exacerbation of liver fibrosis in the thrombocytopenic mice after BDL. (A–C) Platelet accumulation and Met activation in the liver following BDL. *bcl-x<sup>fl</sup>/x<sup>fl</sup> Pf4-Cre* mice and *bcl-x<sup>fl</sup>/x<sup>fl</sup>* mice were sham operated or subjected to BDL and analyzed 10 days later (4–7 mice per group). Cre(+) stands for *bcl-x<sup>fl</sup>/x<sup>fl</sup> Pf4-Cre* mice and Cre(–) for *bcl-x<sup>fl</sup>/x<sup>fl</sup>* mice. Platelet accumulation in the liver was assessed by Western blotting of CD41 (A); lysate of spleen was used as a positive control. Phosphorylation of Met protein in the liver was determined by Western blot analysis (B). Plasma HGF levels were determined by ELISA (C). (D and E) Attenuation of liver fibrosis by HGF therapy in thrombocytopenic mice following BDL. *bcl-x<sup>fl</sup>/x<sup>fl</sup> Pf4-Cre* mice and *bcl-x<sup>fl</sup>/x<sup>fl</sup>* mice were subjected to BDL, followed by intraperitoneal injection of recombinant human HGF or vehicle 2 times per day and analyzed 10 days later (5–7 mice per group). Liver fibrosis was evaluated by picrosirius red staining of liver sections (D). *col1a1* and *col1a2* mRNA in the liver were determined by real-time reverse-transcription polymerase chain reaction (E).

growth factor- $\beta$ , and tumor necrosis factor- $\alpha$ , which are also known as cytokines involved in fibrosis, was not different between the 2 groups (Supplementary Figure 5B). These gene expression profiles suggest that increase of collagen gene expression may have a causative role in exacerbated liver fibrosis and that MMP up-regulation may be a compensatory phenomenon.

In general, according to the transdifferentiation of quiescent HSCs into activated HSCs, these myofibroblast-like cells express myogenic markers such as  $\alpha$ -smooth muscle actin (SMA) and cause a parallel increase in collagen synthesis.<sup>1,2</sup> However, instead of increased collagen synthesis in thrombocytopenic mice,  $\alpha$ -SMA positive cells were similarly induced in thrombocytopenic mice and control

mice upon BDL (Supplementary Figure 6). Thereafter, we expected that the levels of collagen gene expression per cell would increase in thrombocytopenic mice. Previous research has demonstrated that, even in activated HSCs, the level of type I collagen mRNA can be modulated by the change of collagen mRNA stabilization, which is regulated by interaction with a specific protein such as  $\alpha$  complex protein.<sup>30</sup> Furthermore, it has recently been demonstrated that HGF suppresses *col1a2* promoter activation by inhibiting nuclear accumulation of Smad3 in activated HSCs.<sup>31</sup> We used activated HSCs for in vitro experiments and found that coculture with platelets did not affect mRNA expression of  $\alpha$ -SMA (Supplementary Figure 7) but did suppress the *col1a1* and *col1a2* genes in activated HSCs. This suggests that platelets regulate type I collagen gene expression in each cell without affecting the activation status of HSCs, which agrees with our in vivo findings.

Platelet suppression of collagen gene expression was clearly associated with platelet activation as evidenced by the shape change and P-selectin translocation and shedding. Moreover, the supernatant of thrombin-activated platelets was capable of inhibiting collagen gene expression, although the supernatant of quiescent platelets could not. These results strongly suggest that platelet activation is indispensable for the inhibition of collagen synthesis in vitro. It should be noted that our results also suggest that, once activated, platelets are capable of releasing soluble factor(s) residing in platelet granules such as HGF and thereby suppress collagen synthesis, which is independent of how platelets are activated. Therefore, although platelets could be activated upon contact with HSCs in vitro, this contact may not be a requisite for platelet inhibition of HSCs in vivo. Indeed, platelets are well known to be activated by cell-to-cell contact with a variety of cells and soluble factors in injured organs.<sup>10,14</sup> We also observed that platelets could be activated upon contact with murine hepatocytes or macrophages in vitro, even though activated platelets did not affect type I collagen synthesis in these cells (data not shown).

We have demonstrated that platelet-derived HGF plays a critical role in platelet suppression of type I collagen gene expression in cultured HSCs. BDL-induced cholestasis led to intrahepatic platelet accumulation and Met phosphorylation in the liver of control mice, but both were attenuated in thrombocytopenic mice. Furthermore, plasma HGF levels in thrombocytopenic mice were lower than in control mice after BDL. Despite the lack of intrahepatic platelet accumulation, HGF administration could alleviate cholestasis-induced liver fibrosis in thrombocytopenic mice to the same level found in control mice. These findings implied that the lack of platelet-derived HGF signaling in the liver was involved in exacerbated liver fibrosis in thrombocytopenic mice. It is not clear, in the present study, whether cholestasis-induced Met phos-

phorylation in the liver originates from activated HSCs or just hepatocytes. Therefore, it is quite possible that HGF administration alleviated liver fibrosis by stimulating both hepatocytes and HSCs in thrombocytopenic mice. However, marked suppression of hepatic type I collagen gene expression by HGF therapy in thrombocytopenic mice suggests that attenuated Met phosphorylation and the subsequent increase of collagen synthesis in activated HSCs may be involved in exacerbated liver fibrosis in thrombocytopenic mice because collagen production is mainly mediated by activated HSCs in the injured liver.

HGF was first identified as a potent mitogen for primary hepatocytes after being purified from the plasma of a patient with fulminant hepatic failure<sup>16,17</sup> and also from rat platelets.<sup>18</sup> HGF is known to be a multifunctional growth factor that shows mitogenic, motogenic, morphogenic, and antiapoptotic activities in a variety of cells.<sup>16,17</sup> Increasing evidence indicates that HGF has an antifibrotic effect in several experimental models, especially when administered exogenously.<sup>20,21</sup> Although platelets are known to contain HGF in their granules,<sup>18</sup> the functional role of platelet-derived HGF has remained unknown. Because human platelets contain a smaller amount of HGF than rodent platelets,<sup>18</sup> it is obscure whether the same mechanisms observed in rodents are operative in humans. However, the present study, for the first time, sheds light on HGF derived from platelets serving as an endogenous negative regulator for HSC expression of collagen genes and liver fibrosis under pathologic conditions.

Thrombocytopenia is a common complication of advanced chronic liver disease and is generally considered to be a secondary phenomenon via associated portal hypertension or reduced production of TPO in the liver.<sup>3,4</sup> Our study indicates a causal link of thrombocytopenia with progression of liver fibrosis, suggesting a complicated interaction between liver fibrosis and thrombocytopenia. However, the mice we generated show extremely severe thrombocytopenia, which does not exactly mimic the thrombocytopenia usually seen in patients with cirrhosis. Moreover, the components of platelet granules may differ between the human and the mouse. Therefore, we cannot directly conclude from our findings that thrombocytopenia in patients with cirrhosis exacerbates liver fibrosis. However, in addition to the fact that liver fibrosis progresses in parallel with the decrease of platelet count, several studies on human patients have shown that splenectomy or partial splenic embolization can improve the liver function of cirrhotic patients in parallel with elevation of platelet count.<sup>32,33</sup> Therefore, further clinical study is important in order to elucidate whether an increase in platelet count is beneficial for preventing the progression of liver fibrosis in thrombocytopenic patients with advanced liver disease.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2010.02.054.

### References

- Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115:209–218.
- Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;134:1655–1669.
- Afdhal N, McHutchison J, Brown R, et al. Thrombocytopenia associated with chronic liver disease. *J Hepatol* 2008;48:1000–1007.
- Aster RH. Pooling of platelets in the spleen: role in the pathogenesis of “hypersplenic” thrombocytopenia. *J Clin Invest* 1966;45:645–657.
- Mason KD, Carpinelli MR, Fletcher JI, et al. Programmed anuclear cell death delimits platelet life span. *Cell* 2007;128:1173–1186.
- Takehara T, Tatsumi T, Suzuki T, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. *Gastroenterology* 2004;127:1189–1197.
- Hikita H, Takehara T, Shimizu S, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. *Hepatology* 2009;50:1217–1226.
- Tiedt R, Schomber T, Hao-Shen H, et al. Pf4-Cre transgenic mice allow generating lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood* 2007;109:1503–1506.
- Tsukamoto H, Matsuoka M, French SW. Experimental models of hepatic fibrosis: a review. *Semin Liver Dis* 1990;10:56–65.
- Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil interactions: linking hemostasis and inflammation. *Blood Rev* 2007;21:99–111.
- Bennet JS. Structure and function of the platelet integrin  $\alpha$ IIb $\beta$ 3. *J Clin Invest* 2005;115:3363–3369.
- Gujral JS, Liu J, Farhood A, et al. Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. *Hepatology* 2004;40:998–1007.
- Friedman SL, Roll FJ, Boyles J, et al. Maintenance of differentiated phenotype of cultured rat hepatic lipocytes by basement membrane matrix. *J Biol Chem* 1989;264:10756–10762.
- Holmsen H. Physiological functions of platelets. *Ann Med* 1989;21:23–30.
- Dunlop LC, Skinner MP, Bendall LJ, et al. Characterization of GMP-140 (P-selectin) as a circulating plasma protein. *J Exp Med* 1992;175:1147–1150.
- Gohda E, Tsubouchi H, Nakayama H, et al. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. *J Clin Invest* 1988;81:414–419.
- Miyazawa K, Tsubouchi H, Naka D, et al. Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. *Biochem Biophys Res Commun* 1989;163:967–973.
- Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989;342:440–443.
- Tulasne D, Foveau B. The shadow of death on the MET tyrosine kinase receptor. *Cell Death Differ* 2008;15:427–434.
- Ueki T, Kaneda Y, Tsutsui H, et al. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat Med* 1999;5:226–230.
- Li Z, Mizuno S, Nakamura T. Antinecrotic and antiapoptotic effects of hepatocyte growth factor on cholestatic hepatitis in a mouse model of bile-obstructive diseases. *Am J Physiol Gastrointest Liver Physiol* 2007;292:G639–G646.
- Giebeler A, Boekschoten MV, Klein C, et al. c-Met confers protection against chronic liver tissue damage and fibrosis progression after bile duct ligation in mice. *Gastroenterology* 2009;137:297–308.
- Iannacone M, Sitia G, Isogawa M, et al. Platelets mediate cytotoxic T lymphocyte-induced liver damage. *Nat Med* 2005;11:1167–1169.
- Lang PA, Contaldo C, Georgiev P, et al. Aggravation of viral hepatitis by platelet-derived serotonin. *Nat Med* 2008;14:756–761.
- Laschke MW, Dold S, Menger MD, et al. Platelet dependent accumulation of leukocytes in sinusoids mediates hepatocellular damage in bile duct ligation-induced cholestasis. *Br J Pharmacol* 2008;153:148–156.
- Lesurtel M, Graf R, Aleil B, et al. Platelet-derived serotonin mediates liver regeneration. *Science* 2006;312:104–107.
- Murata S, Hashimoto I, Nakano Y, et al. Single administration of thrombopoietin prevents progression of liver fibrosis and promotes liver regeneration after partial hepatectomy in cirrhotic rats. *Ann Surg* 2008;248:821–828.
- Watanabe M, Murata S, Hashimoto I, et al. Platelets contribute to the reduction of liver fibrosis in mice. *J Gastroenterol Hepatol* 2009;24:78–89.
- Fausto N, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology* 2006;43:S45–S53.
- Stefanovic B, Hellebrand C, Holcik M, et al. Posttranscriptional regulation of collagen  $\alpha$ 1(I) mRNA in hepatic stellate cells. *Mol Cell Biol* 1999;17:5201–5209.
- Inagaki Y, Higashi K, Kushida M, et al. Hepatocyte growth factor suppresses profibrogenic signal transduction via nuclear export of smad3 with galectin-7. *Gastroenterology* 2008;134:1180–1190.
- Murata K, Ito K, Yoneda K, et al. Splenectomy improves liver function in patients with liver cirrhosis. *Hepatogastroenterology* 2008;55:1407–1411.
- Lee CM, Leung EK, Wang HJ, et al. Evaluation of the effect of partial splenic embolization of platelet values for liver cirrhosis patients with thrombocytopenia. *World J Gastroenterol* 2007;13:619–622.

Received September 4, 2009. Accepted February 24, 2010.

### Reprint requests

Address requests for reprints to: Norio Hayashi, MD, PhD, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. e-mail: hayashin@gh.med.osaka-u.ac.jp; fax: (81) 6-6879-3629.

### Acknowledgments

The authors thank Radek Skoda (University Hospital Basel) and Lothar Hennighausen (National Institute of Health) for providing the Pf4-Cre mice and the floxed bcl-x mice, respectively.

T. Kodama and T. Takehara contributed equally to this work and share first authorship.

### Conflicts of interest

The authors disclose no conflicts.

### Funding

Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Takehara), and a Grant-in-Aid from the Ministry of Health, Labour, and Welfare of Japan.

## Supplementary Materials and Methods

### Hematologic Analyses

Blood was collected from the inferior vena cava of mice. Complete blood cell counts were determined using an Automated Cell Counter (Sysmex, Kobe, Japan).

### Histologic Analyses

The liver sections were stained with H&E or picrorius red. The percentage of oncotic necrosis or fibrotic area was calculated using image analysis software (win-ROOF visual system; Mitani Co, Tokyo, Japan). To assess intrahepatic neutrophil accumulation, liver sections were stained with chloroacetate esterase, which is a specific marker of neutrophils,<sup>1</sup> using a Naphthol-ASD Chloroacetate Esterase Kit (Sigma-Aldrich, St. Louis, MO). To detect apoptotic cells, the liver sections were also subjected to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining as previously reported.<sup>2</sup> To assess regenerative status, nuclear 5-bromo-2-deoxyuridine incorporation was evaluated as previously described.<sup>3</sup>

### Determination of Liver Hydroxyproline Content

Hydroxyproline content was determined essentially as described previously.<sup>4</sup> Results are expressed as micrograms of hydroxyproline per gram of wet liver.

### Isolation and Culture of Mouse Hepatic Stellate Cell

Hepatic stellate cell (HSCs) were isolated from C57BL/6J mice by 2-step collagenase-pronase perfusion of mouse liver followed by density gradient centrifugation with 8.2% Nycodenz (Sigma-Aldrich) as previously described.<sup>5</sup> Isolated HSCs were maintained at 37°C under 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Activated HSCs after a few passages were used for the experiments unless otherwise indicated.

### Cell Isolation

Monocytes and T lymphocytes were isolated from spleens of *bcl-x<sup>flax/flax</sup> Pff4-Cre* mice and *bcl-x<sup>flax/flax</sup>* mice by magnetic cell sorting using magnetic beads (MACS; Miltenyi Biotec, Gladbach, Germany) with CD11b and CD90.2 antibodies according to the manufacturer's protocol. Abdominal macrophages were collected from these mice 5 days after intraperitoneal injection of 50  $\mu$ L/g body weight thioglycollate broth (Sigma-Aldrich) by peritoneal lavage. Hepatocytes and nonparenchymal cells were isolated from those mice by collagenase perfusion of mouse liver followed by centrifugation.

### Platelet Isolation

Platelets were isolated as described previously.<sup>6</sup> Briefly, whole blood collected from the inferior vena cava

of C57BL/6J mice was mixed with one fourth volume of citrate phosphate dextrose (Sigma-Aldrich). Platelet-rich plasma was obtained by centrifugation at 100g for 15 minutes at room temperature without braking. After incubation with 1  $\mu$ mol/L prostaglandin E<sub>1</sub> (Sigma-Aldrich) and 1 U/mL apyrase (Sigma-Aldrich), the platelets were isolated by centrifugation at 200g at room temperature for 15 minutes.

### Western Blot Analysis

Western blotting was performed as previously described.<sup>2</sup> A detailed description of the antibodies used is provided in Supplementary Table 1.

### Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA extracted from the liver tissue and HSCs were reverse transcribed and subjected to real-time reverse-transcription polymerase chain reaction as previously described.<sup>2</sup> mRNA expression of the specific genes was quantified using TaqMan Gene Expression Assays (Applied Biosystems Inc, Foster City, CA). Assay IDs of the specific genes are provided in Supplementary Table 2. Transcript levels are presented as fold induction.

### Small Interfering RNA-Mediated Knockdown

Cultured HSCs were transfected with small interfering RNA against *met* (Stealth RNAi, Oligo ID:MSS206635) (Invitrogen, Carlsbad, CA) using lipofectamine RNAi-MAX (Invitrogen) according to the manufacturer's protocol. Stealth RNA: Negative Control Low GC Duplex (Invitrogen) was used as the control.

### Flow Cytometry

Isolated platelets were surface-stained with a fluorescein isothiocyanate-conjugated rat anti-mouse CD62p (P-selectin) antibody (BD Biosciences, Franklin Lakes, NJ). Samples were analyzed with a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences), and the data were processed with the CELLQuest software (BD Biosciences).

### Enzyme-Linked Immunosorbent Assay

Mouse HGF and soluble CD62p (P-selectin) levels in plasma and culture supernatant were measured by using DuoSet enzyme-linked immunosorbent assay mouse hepatocyte growth factor (HGF) and CD62p (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's protocol.

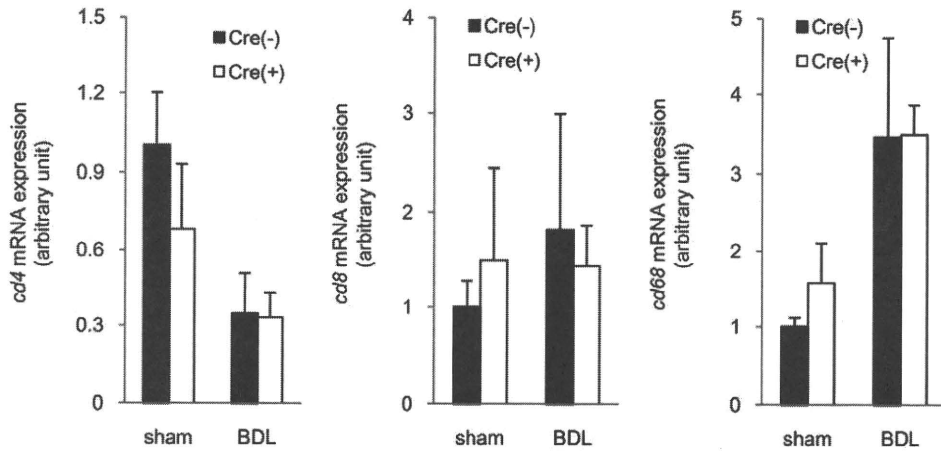
### HGF Treatment

Wild-type (*bcl-x<sup>fl/fl</sup>*) and knockout (*bcl-x<sup>fl/fl</sup> Pff4-Cre*) mice were subjected to bile duct ligation, followed by intraperitoneal injection of recombinant human HGF (500  $\mu$ g/kg) or vehicle every 12 hours for 10 days and then killed to sample the liver tissues.

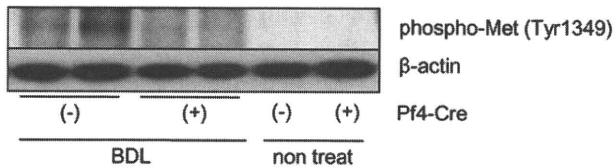
### References

1. Gujral JS, Liu J, Farhood A, et al. Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. *Hepatology* 2004;40:998–1007.
2. Hikita H, Takehara T, Shimizu S, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing an adult murine liver. *Hepatology* 2009;50:1217–1226.
3. Lesurtel M, Graf R, Aleil B, et al. Platelet-derived serotonin mediates liver regeneration. *Science* 2006;312:104–107.
4. Takehara T, Tatsumi T, Suzuki T, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. *Gastroenterology* 2004;127:1189–1197.
5. Seki E, De Minicis S, Osterreicher CH, et al. TLR4 enhances TGF- $\beta$  signaling and hepatic fibrosis. *Nat Med* 2007;13:1324–1332.
6. Iannacone M, Sitia G, Isogawa M, et al. Platelets mediate cytotoxic T lymphocyte-induced liver damage. *Nat Med* 2005;11:1167–1169.

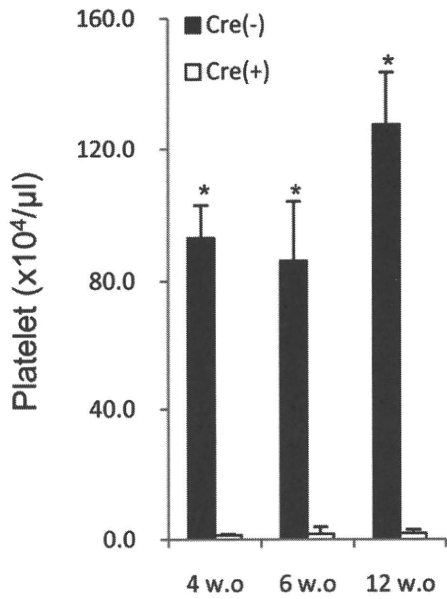




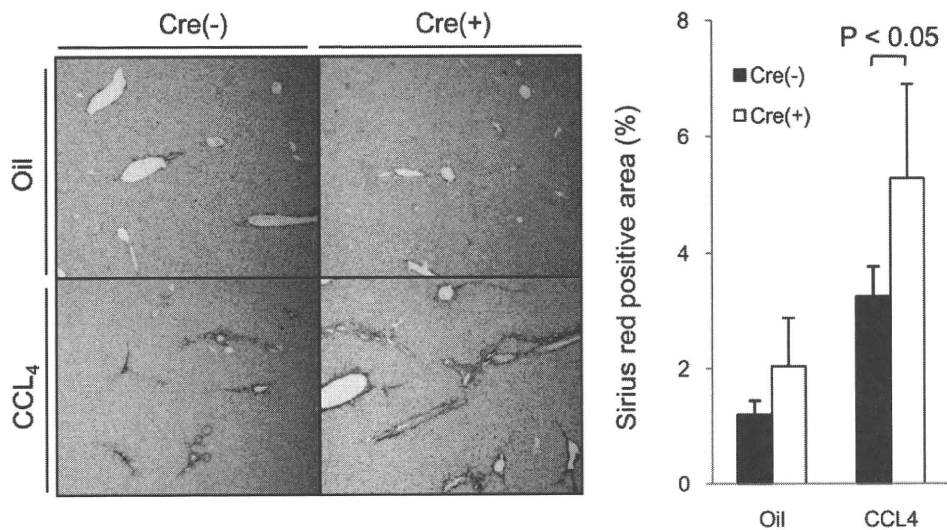
**Supplementary Figure 1.** Intrahepatic cell fractions upon bile duct ligation (BDL) treatment are not different between the thrombocytopenic mice and the control littermates. *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice were sham operated or subjected to BDL and analyzed 10 days later (4–6 mice per group). Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. *cd4*, *cd8*, And *cd68* messenger RNA levels in the liver were determined by real-time reverse-transcription polymerase chain reaction.



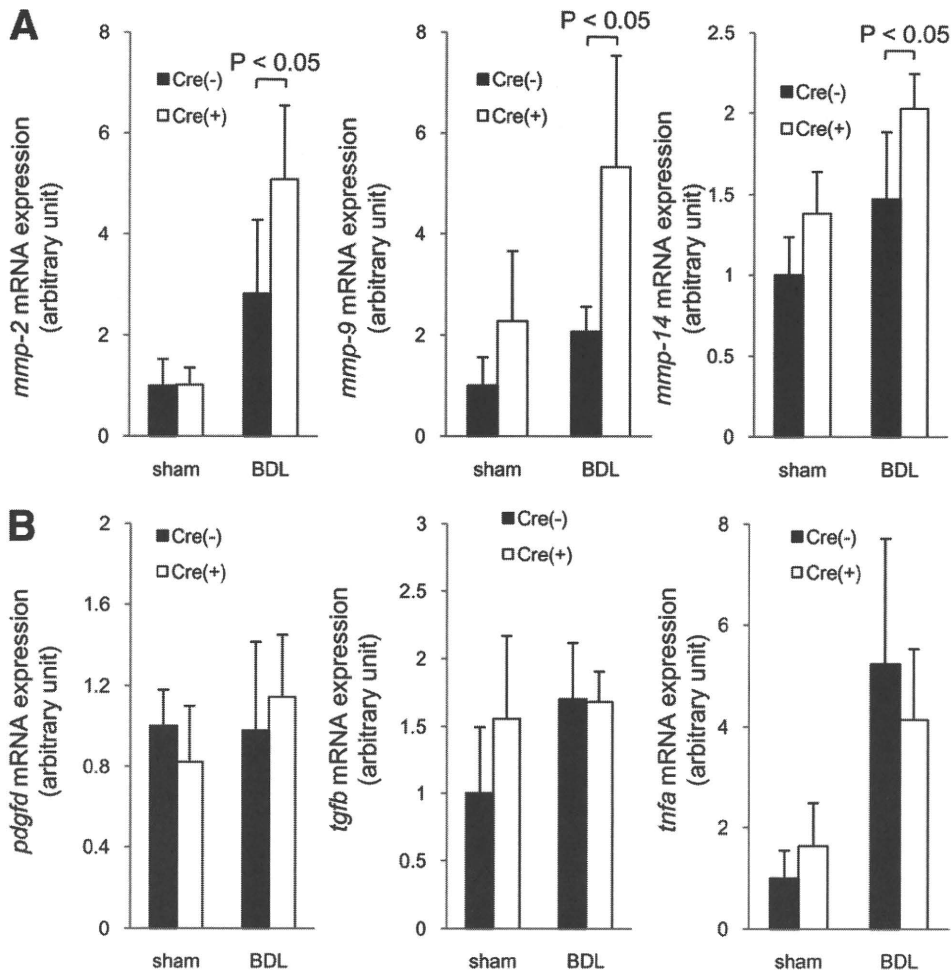
**Supplementary Figure 2.** Phosphorylation of Met protein in the liver is stronger in the control littermates than in the thrombocytopenic mice at 3 days after bile duct ligation (BDL) treatment. *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice were subjected to BDL and analyzed 3 days later. Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. Phosphorylation of Met protein in the liver was determined by Western blotting.



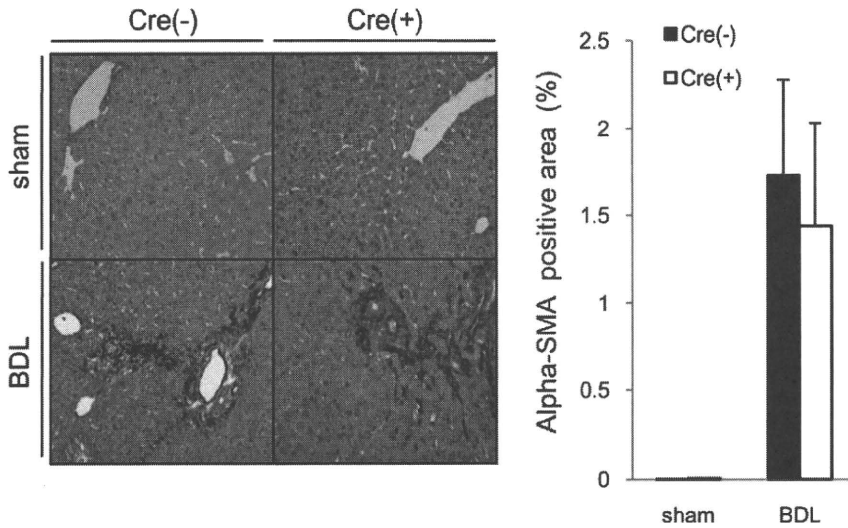
**Supplementary Figure 3.** *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice exhibit severe thrombocytopenia at as early as 4 weeks of age, and it persists for a longer time. Circulating platelet counts of *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice at the age of 4, 6, and 12 weeks. Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. \**P* < .05 vs Cre(+). 5–8 Mice per group.



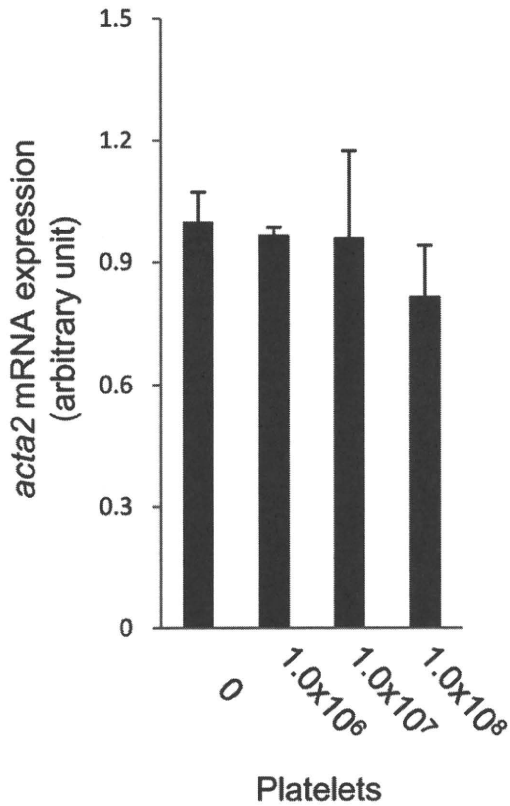
**Supplementary Figure 4.** Thrombocytopenia exacerbates liver fibrosis induced by chronic CCl<sub>4</sub> administration. *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice were administered intraperitoneal injection of CCl<sub>4</sub> (0.3 mL/kg) or oil 2 times per week and examined 6 weeks later (7 mice per group). Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. Liver fibrosis was evaluated by picosirius red staining of liver sections.



**Supplementary Figure 5.** (A) Gene expression of matrix metalloproteases is up-regulated in thrombocytopenic mice upon bile duct ligation (BDL) treatment. *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice were sham operated or subjected to BDL and analyzed 10 days later (4–6 mice per group). Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. *mmp-2*, *mmp-9*, and *mmp-14* messenger RNA levels in the liver were determined by real-time reverse-transcription polymerase chain reaction. (B) Gene expression of fibrosis-related cytokines in the liver is not different between the thrombocytopenic mice and their control littermates. *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice were sham operated or subjected to BDL and analyzed 10 days later (4–6 mice per group). Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. *pdgfd*, *tgfb*, and *tnfa* messenger RNA levels in the liver were determined by real-time reverse-transcription polymerase chain reaction.



**Supplementary Figure 6.** HSCs are similarly activated in the thrombocytopenic mice and the control mice upon bile duct ligation (BDL). *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* mice and *bcl-x<sup>fllox/fllox</sup>* mice were sham operated or subjected to BDL and analyzed 10 days later (4 or 5 mice per group). Cre(+) and Cre(-) stand for *bcl-x<sup>fllox/fllox</sup> Pf4-Cre* and *bcl-x<sup>fllox/fllox</sup>*, respectively. To assess HSC activation, liver sections were stained with monoclonal anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Dako, Glostrup, Denmark).



**Supplementary Figure 7.** Coculture with platelets does not affect messenger RNA expression of  $\alpha$ -SMA in activated HSCs. HSCs ( $1.0 \times 10^5$ ) were cocultured with indicated dosages of platelets for 6 hours. *acta2* Messenger RNA levels in HSCs were determined by real-time reverse-transcription polymerase chain reaction. N = 3/group.

**Supplementary Table 1.** Antibodies Used for Western Blotting

Antibody	Manufacturer
Rabbit polyclonal antibody to Bcl-xL	Santa Cruz Biotechnology, Santa Cruz, CA
Rat monoclonal antibody to mouse integrin- $\alpha$ 2B/CD41	R&D Systems, Minneapolis, MN
Mouse monoclonal antibody to Met	Cell Signaling Technology, Beverly, MA
Rabbit monoclonal antibody to phospho-Met (Tyr1234)	Cell Signaling Technology
Rabbit monoclonal antibody to phospho-Met (Tyr1349)	Cell Signaling Technology
Mouse monoclonal antibody to $\beta$ -actin	Sigma-Aldrich, St Louis, MO
Rabbit polyclonal antibody to type I collagen	Rockland, Gilbertsville, PA
Rabbit polyclonal antibody to GAPDH	Trevigen, Gaithersburg, MD
Rabbit monoclonal antibody to stat3	Cell Signaling Technology
Rabbit monoclonal antibody to Erk1/2	Cell Signaling Technology
Rabbit monoclonal antibody to Akt	Cell Signaling Technology
Rabbit monoclonal antibody to phospho-stat3	Cell Signaling Technology
Rabbit monoclonal antibody to phospho-Ekr1/2	Cell Signaling Technology
Rabbit monoclonal antibody to phospho-Akt	Cell Signaling Technology

**Supplementary Table 2.** Clinicopathologic Features of HCC Patients

Target gene	Assay ID
<i>col1a1</i>	Mm00801666_g1
<i>col1a2</i>	Mm01165187_m1
<i>met</i>	Mm01156980_m1
<i>mmp-2</i>	Mm00439506_m1
<i>mmp-9</i>	Mm00600164_g1
<i>mmp-14</i>	Mm01318969_g1
<i>acta2</i>	Mm01546133_m1
<i>actb</i>	Mm02619580_g1
<i>cd4</i>	Mm01182108_m1
<i>cd8</i>	Mm00442754_m1
<i>cd68</i>	Mm03047343_m1
<i>Tnfa</i>	Mm01178820_m1
<i>tgfb</i>	Mm00546829_m1
<i>pdgfd</i>	Mm01135193_m1



## Absence of invariant natural killer T cells deteriorates liver inflammation and fibrosis in mice fed high-fat diet

Takuya Miyagi · Tetsuo Takehara · Akio Uemura · Kumiko Nishio · Satoshi Shimizu · Takahiro Kodama · Hayato Hikita · Wei Li · Akira Sasakawa · Tomohide Tatsumi · Kazuyoshi Ohkawa · Tatsuya Kanto · Naoki Hiramatsu · Norio Hayashi

Received: 28 November 2009 / Accepted: 31 May 2010 / Published online: 2 July 2010  
© Springer 2010

### Abstract

**Background** Invariant natural killer T (iNKT) cells have been suggested to play critical roles in a wide range of immune responses by acting in a proinflammatory or anti-inflammatory manner. Nonalcoholic steatohepatitis (NASH) is a chronic liver disease progressing to advanced cirrhosis and hepatocellular carcinoma. Despite the abundance of iNKT cells in the liver, their role in the pathogenesis of NASH remains obscure. Here, we investigated their role in the development of diet-induced steatosis/steatohepatitis.

**Methods** We used BALB/c wild-type mice and  $J\alpha 18$ -deficient (KO) mice lacking iNKT cells fed either a normal diet or a high-fat diet (HFD). The liver and blood were collected from these mice to examine liver inflammation, steatosis, and fibrosis at the indicated time points.

**Results** KO mice fed the HFD, compared with control mice fed the HFD, exhibited a clearly higher serum alanine aminotransferase level and a greater number of hepatic inflammatory foci, although there was no significant difference in hepatic lipid retention between these groups of mice. The HFD enhanced messenger RNA expression of inflammatory cytokines and chemokines in KO but not in control mice. The HFD also increased the proportion of hepatic CD4 T cells and

CD8 T cells that composed hepatic inflammatory foci in KO mice, but not in the controls. Prolonged feeding with the HFD augmented liver fibrosis in KO but not in control mice.

**Conclusions** These findings indicate that iNKT cells play a protective role against liver inflammation progressing to fibrosis, but not against steatosis, enhanced by dietary excess fat, suggesting a key role of these cells in NASH pathogenesis.

**Keywords** iNKT cells · Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Cytokine · Chemokine

### Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
iNKT	Invariant natural killer T
NK	Natural killer
TCR	T cell receptor
Th	T helper
IFN	Interferon
IL	Interleukin
WT	Wild type
ND	Normal diet
HFD	High-fat diet
KO	$J\alpha 18$ -deficient
ALT	Alanine aminotransferase
RT-PCR	Reverse transcription polymerase chain reaction
H&E	Hematoxylin–eosin
SEM	Standard error of the mean
TNF	Tumor necrosis factor
CCL	Chemokine (C–C motif) ligand
CXCL	Chemokine (C–X–C motif) ligand

T. Miyagi · T. Takehara · A. Uemura · K. Nishio · S. Shimizu · T. Kodama · H. Hikita · W. Li · A. Sasakawa · T. Tatsumi · K. Ohkawa · T. Kanto · N. Hiramatsu · N. Hayashi (✉)  
Department of Gastroenterology and Hepatology,  
Osaka University Graduate School of Medicine,  
2-2 Yamada-oka, Suita, Osaka 565-0871, Japan  
e-mail: hayashin@gh.med.osaka-u.ac.jp