Thrombocytopenia Exacerbates Cholestasis-Induced Liver Fibrosis in Mice

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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 caricinoma. 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 resistance to blood flow, leading to portal hypertension.[1] 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.[1,2] 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.[3] 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[4] and reduced production of thrombopoietin (TPO) in the liver.[3] 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,[5] 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; α-SMA, α-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 metalloprotease; 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.

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Materials and Methods

Mice

Thrombocyte-specific Bcl-xL knockout mice (*bcl-x$^{flox/flox}$ Pf4-Cre*) were generated by mating *bcl-x$^{flox/flox}$* mice[6,7] and *Pf4-Cre* transgenic mice.[8] 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$^{fl/fl}$*) and knockout (*bcl-x$^{fl/fl}$ Pf4-Cre*) mice were subjected to BDL as previously reported.[9] 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.[5] We generated thrombocyte-specific Bcl-xL knockout mice by crossing *floxed bcl-x* mice[6,7] and *Pf4-Cre* transgenic mice.[8] After mating *bcl-x$^{flox/flox}$Pf4-Cre* mice with *bcl-x$^{flox/flox}$* mice, *bcl-x$^{flox/flox}$Pf4-Cre* mice were born at the expected Mendelian frequency and did not show any developmental abnormality. As expected, *bcl-x$^{flox/flox}$Pf4-Cre* mice showed severe thrombocytopenia without any phenotypes of other hematopoietic lineages (Figure 1*A*). Western blot analysis confirmed a substantial decrease in Bcl-xL expression in circulating platelets of *bcl-x$^{flox/flox}$Pf4-Cre* mice compared with *bcl-x$^{flox/flox}$* mice (Figure 1*B*). CD41 protein, a specific surface receptor expressed in the thrombocyte lineage,[10,11] was used as a loading control of platelets. To demonstrate the thrombocyte-lineage specificity of the Platelet factor 4 (Pf4) promotor that we used, we examined Bcl-xL protein expression in several tissues and hematopoietic cells of *bcl-x$^{flox/flox}$ Pf4-Cre* mice and *bcl-x$^{flox/flox}$* mice by Western blotting. In all of these tissues and cells, Bcl-xL protein expression was not different between the 2 groups (Figure 1*C*), indicating that the Pf4 promotor 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 1*D*) as well as for liver histology (Figure 1*E*). In the following experiments, *bcl-x$^{flox/flox}$ Pf4-Cre* mice were crossed with *bcl-x$^{flox/flox}$* mice, and their offspring, *bcl-x$^{flox/flox}$ Pf4-Cre* mice and *bcl-x$^{flox/flox}$* 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,[9] and examined 10 days later. Cholestasis was similarly induced in both groups as evidenced by serum levels of alkaline phosphatase and T-Bil (Figure 2*A*). Both oncotic necrosis, also known as bile infarcts, and apoptosis are characteristic features of liver injury in the BDL model.[12] Although serum ALT levels were slightly lower in the thrombocytopenic mice than in the control littermates (Figure 2*A*), 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 2*B*). The number of accumulating neutrophils, which are known to play a major role in liver inflammation induced by cholestasis,[12] did not differ between the 2 groups as assessed by chloroacetate esterase staining of the liver sections (Figure 2*C*). 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 2*D*).

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 2*E*). Similarly, the hepatic hydroxyproline content, a biochemical marker of collagen accumulation,[6] in the thrombocytopenic mice was elevated to a level significantly higher than in the control littermates (Figure 2*E*). The major form of collagen in cirrhosis is known to be type I collagen composed of 2 α1 and 1 α2 chains. After BDL, hepatic expression of type I collagen α1 and α2 genes, *col1a1* and *col1a2*, sharply rose in both groups and was significantly higher in the thrombocytopenic mice than in the control littermates (Figure 2*F*). 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 2*F*). 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

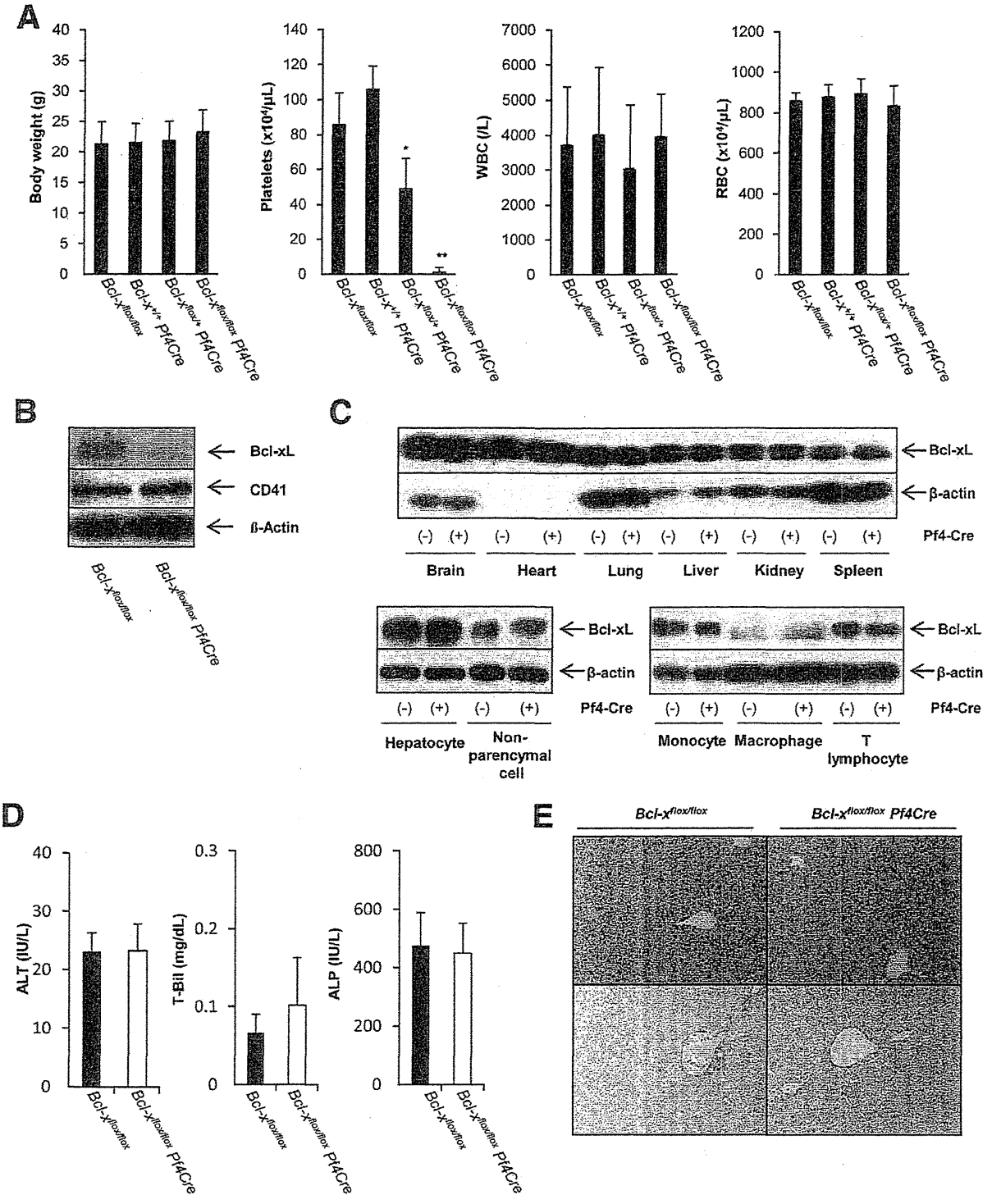
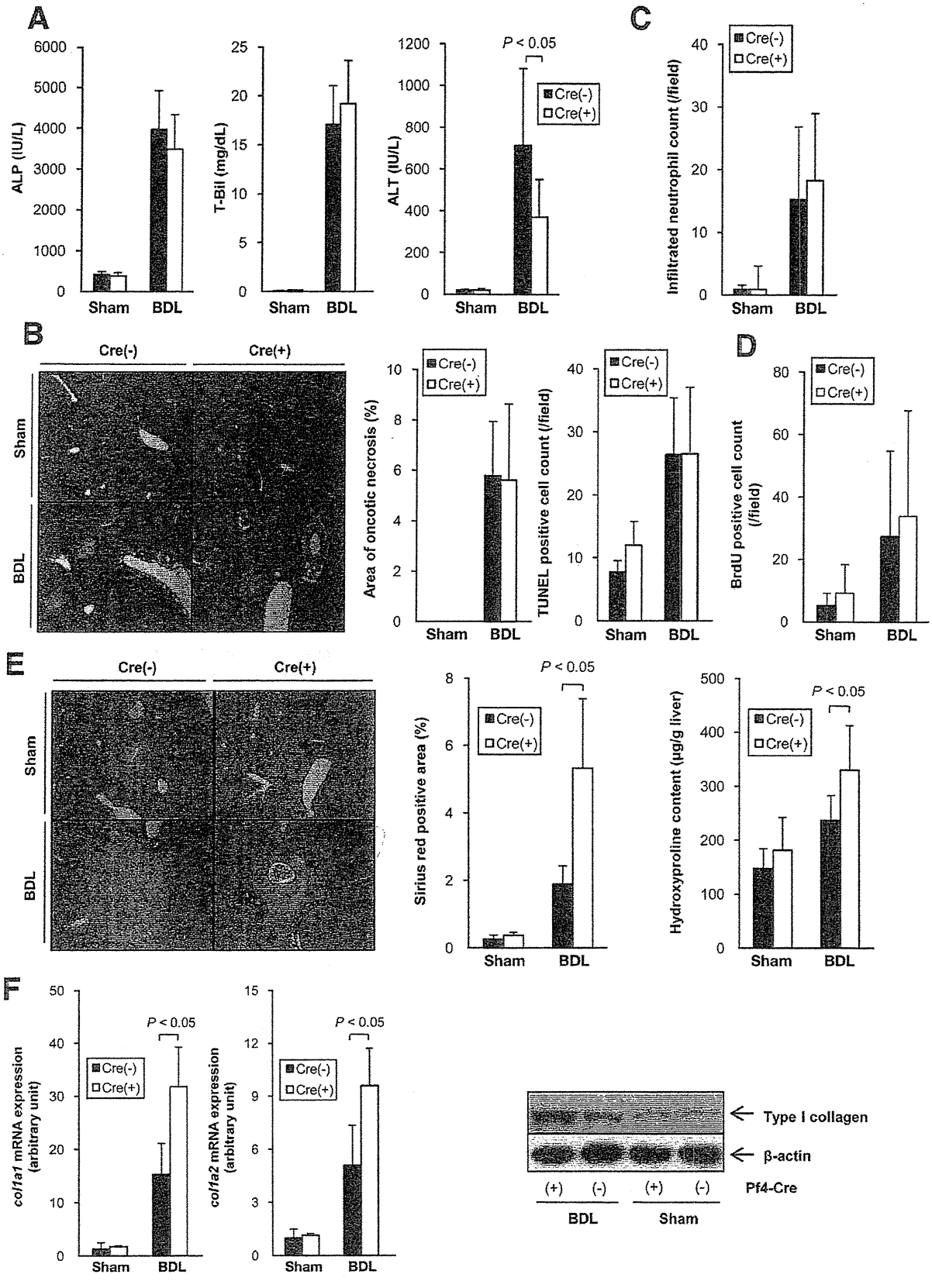


Figure 1. Thrombocyte-specific Bcl-xL knockout mice show massive thrombocytopenia. (*A*) Body weight and circulating blood cell counts of offspring from mating of *bcl-x*$^{flox/+}$ *Pf4-Cre* mice and *bcl-x*$^{flox/+}$ mice; 7–11 mice per group; **P* < .05 vs the other 3 groups, ***P* < .05 vs the other 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*$^{flox/flox}$ *Pf4-Cre* and *bcl-x*$^{flox/flox}$, 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.


A
ALP (IU/L)
6000
5000
4000
3000
2000
1000
0
Sham
BDL
T-Bil (mg/dL)
25
20
15
10
5
0
Sham
BDL
ALT (IU/L)
1200
1000
800
600
400
200
0
P < 0.05
Cre(-)
Cre(+)
Sham
BDL
C
Infiltrated neutrophil count (/field)
40
30
20
10
0
Cre(-)
Cre(+)
Sham
BDL
B
Cre(-)
Cre(+)
Sham
BDL
Area of oncotic necrosis (%)
10
8
6
4
2
0
Cre(-)
Cre(+)
Sham
BDL
TUNEL positive cell count (/field)
40
30
20
10
0
Cre(-)
Cre(+)
Sham
BDL
D
BrdU positive cell count (/field)
80
60
40
20
0
Cre(-)
Cre(+)
Sham
BDL
E
Cre(-)
Cre(+)
Sham
BDL
Sirius red positive area (%)
8
6
4
2
0
P < 0.05
Cre(-)
Cre(+)
Sham
BDL
Hydroxyproline content (μg/g liver)
500
400
300
200
100
0
P < 0.05
Cre(-)
Cre(+)
Sham
BDL
F
col1a1 mRNA expression (arbitrary unit)
50
40
30
20
10
0
P < 0.05
Cre(-)
Cre(+)
Sham
BDL
col1a2 mRNA expression (arbitrary unit)
15
12
9
6
3
0
P < 0.05
Cre(-)
Cre(+)
Sham
BDL
Type I collagen
β-actin
(+) (-) (+) (-)
Pf4-Cre
BDL
Sham

platelets would suppress collagen production in activated HSCs, which are known as the main collagen-producing cells in the injured liver.[13] 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.[13] 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 3*A*). 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 3*B*). Type I collagen protein in HSCs also decreased upon coculture with platelets as determined by Western blot analysis (Figure 3*C*). 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 α-granule to the outer surface.[14,15] 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 3*D*). The levels of soluble P-selectin, which are also known to reflect platelet activation,[15] were significantly higher in the coculture medium with platelets and HSCs than those in the medium with platelets alone (Figure 3*E*). 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.[14] 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 4*A*). 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 4*B*). The supernatant of thrombin-stimulated platelets suppressed type I collagen gene expression in HSCs but that of unstimulated platelets did not (Figure 4*C*), 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,[16,17] which is known to exist in platelets.[18] 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 5*A*). Consecutively, murine platelets were capable of releasing HGF upon exposure to thrombin (Figure 5*B*). Importantly, the levels of HGF were significantly higher in the coculture supernatant of HSCs and platelets than in that of platelets alone (Figure 5*C*). 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.[19] Western blot analysis showed that the Met protein of HSCs was phosphorylated at multiple sites after coculture with platelets (Figure 5*D*) and proteins of its downstream pathways such as Erk1/2, Akt, and stat3 were phosphorylated as well (Figure 5*D*). 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 5*E*) and blunted HGF-induced suppression of type I collagen gene expression (Figure 5*F*). Under these conditions, *met* knockdown abolished platelet-induced suppression of type I collagen gene expression in HSCs (Figure 5*F*). 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$^{flox/flox}$ Pf4-Cre* mice and *bcl-x$^{flox/flox}$* 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$^{flox/flox}$ Pf4-Cre* and *bcl-x$^{flox/flox}$*, 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*) *co1a1* 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.

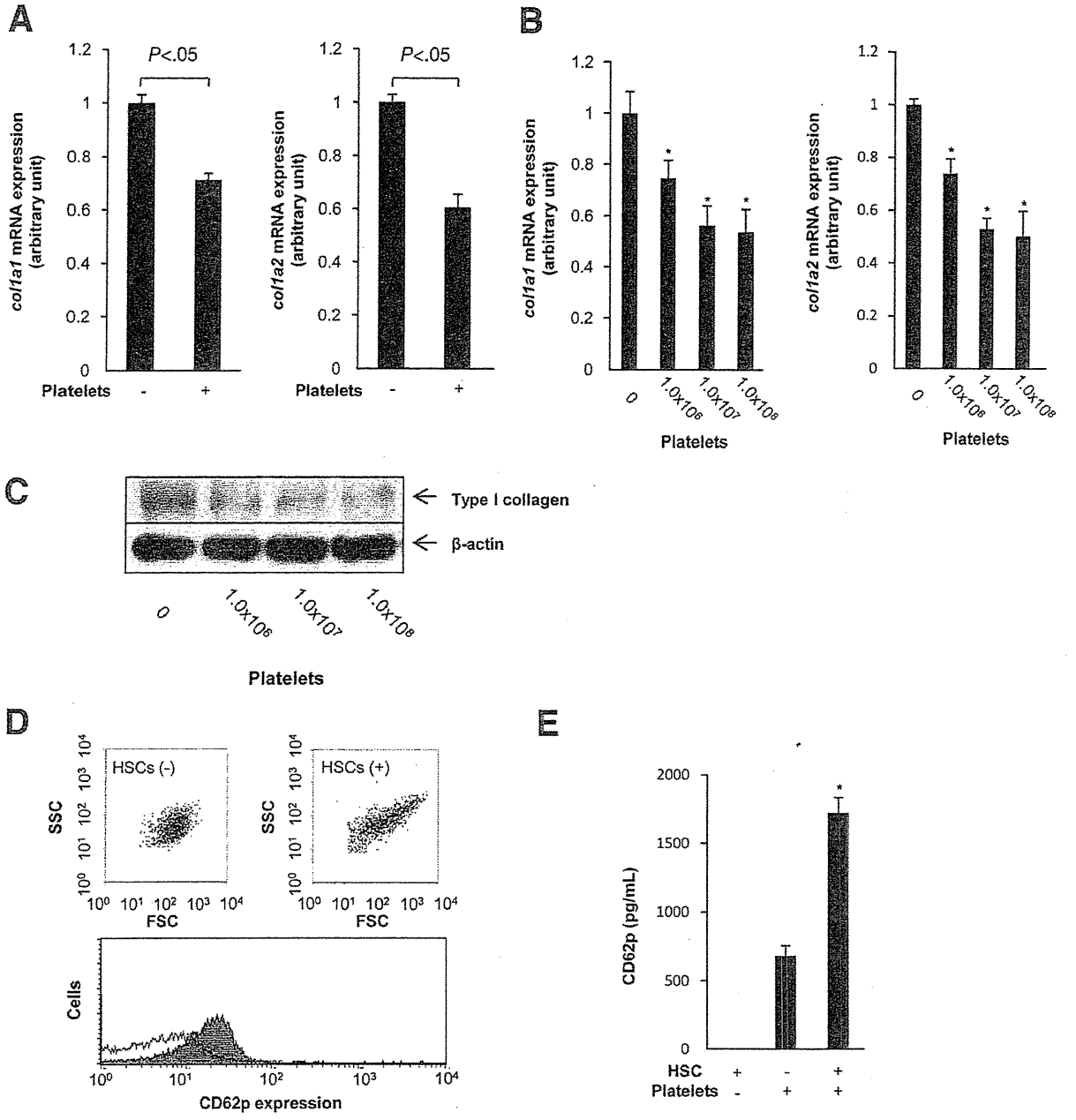


Figure 3. Platelets become activated and inhibit collagen synthesis in activated HSCs in vitro. (*A* and *B*) *col1a1* And *col1a2* mRNA levels in HSCs by real-time reverse-transcription polymerase chain reaction. Primary isolated HSCs were cocultured with 1.0×10^5 platelets for 6 hours (*A*), n = 3/group. HSCs (1.0×10^5) 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 (5.0×10^5) were cocultured with indicated dosages of platelets for 14 hours. (*D* and *E*) Activation of platelets on exposure to HSCs. Platelets (1.0×10^7) were cocultured with or without 1.0×10^5 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-

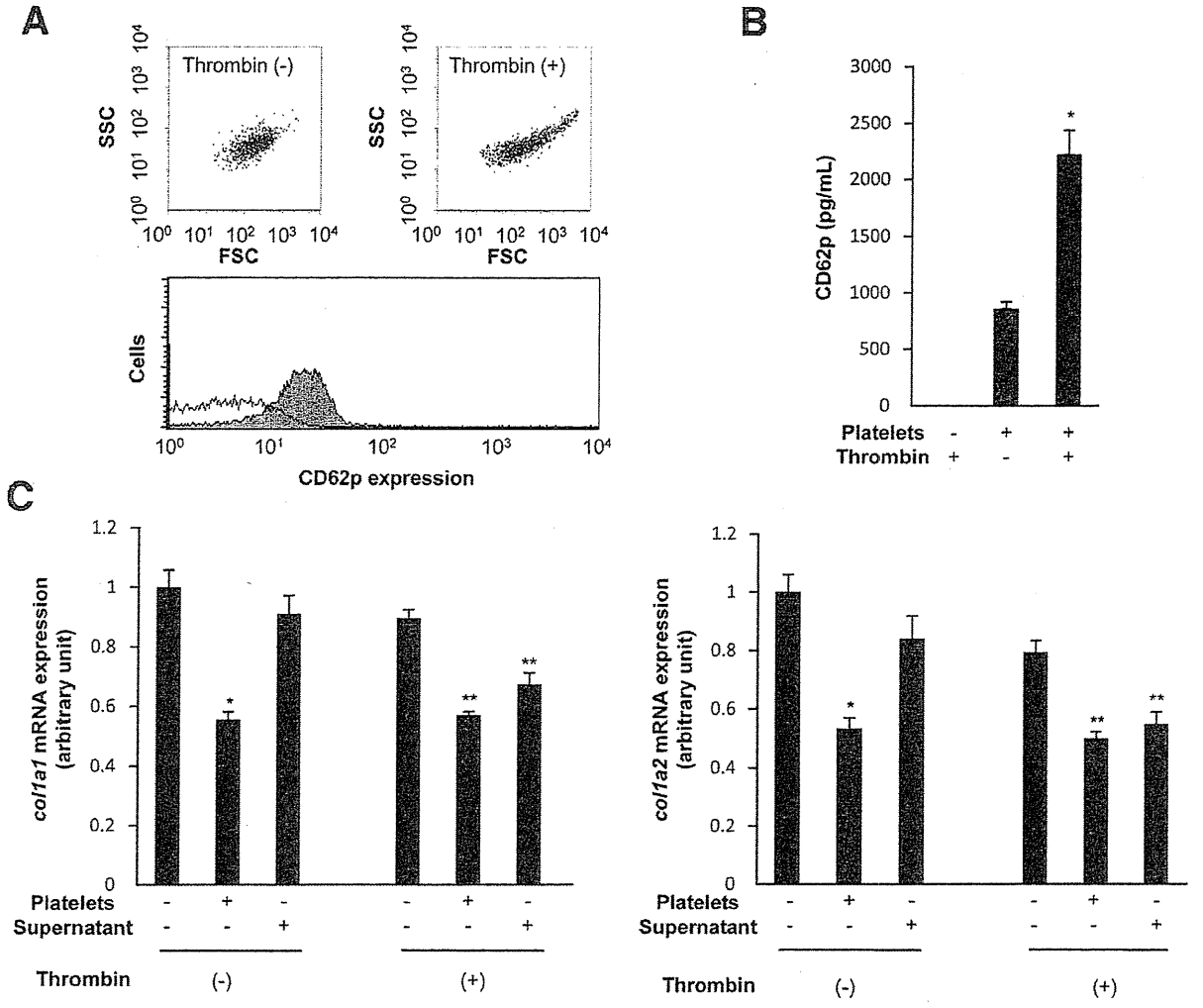
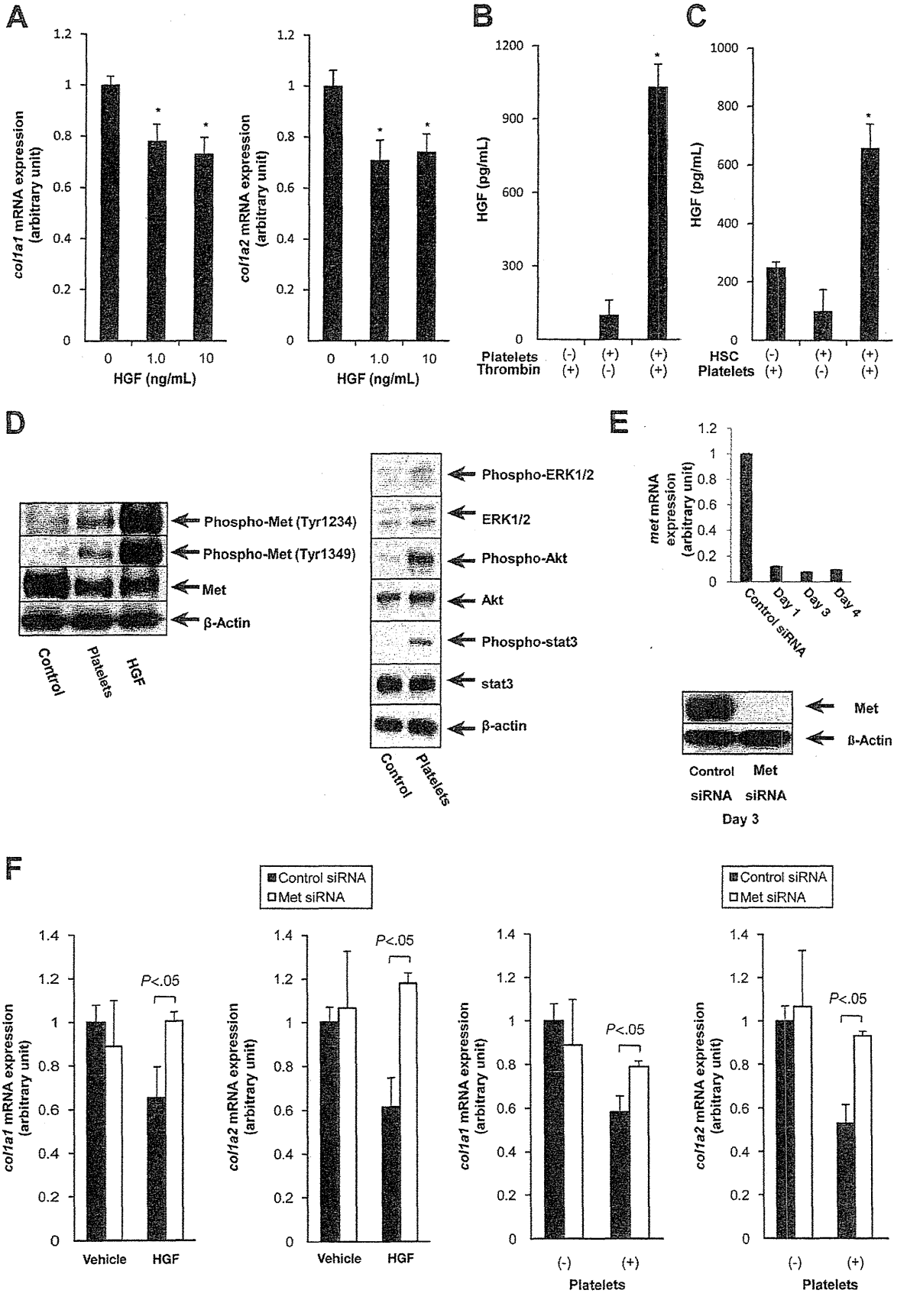


Figure 4. Soluble factors released from activated platelets are involved in the inhibition of collagen synthesis in HSCs. (*A* and *B*) Activation of platelets stimulated with thrombin. Platelets (1.0×10^7) were stimulated with or without thrombin (1 U/mL) for 15 minutes. Shape change and P-selectin 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/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 1.0×10^7 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/group. $^*P < .05$ vs HSC with control group and HSC with platelet supernatant group. $^{**}P < .05$ vs HSC with thrombin group.

topenic mice (Figure 6*A*). 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 6*B*). 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 6*C*). Finally, to investigate whether attenuation 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,[20-22] 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 6*D*). 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 6*E*).


A
col1a1 mRNA expression (arbitrary unit)
HGF (ng/mL)
col1a2 mRNA expression (arbitrary unit)
HGF (ng/mL)
B
HGF (pg/mL)
Platelets
Thrombin
C
HGF (pg/mL)
HSC
Platelets
D
Phospho-Met (Tyr1234)
Phospho-Met (Tyr1349)
Met
β-Actin
Control
Platelets
HGF
Phospho-ERK1/2
ERK1/2
Phospho-Akt
Akt
Phospho-stat3
stat3
β-actin
E
met mRNA expression (arbitrary unit)
Control siRNA
Day 1
Day 3
Day 4
Met
ß-Actin
Control siRNA
Met siRNA
Day 3
F
Control siRNA
Met siRNA
col1a1 mRNA expression (arbitrary unit)
P<.05
Vehicle
HGF
col1a2 mRNA expression (arbitrary unit)
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Platelets
col1a2 mRNA expression (arbitrary unit)
P<.05
Platelets

Discussion

Platelets are circulating blood cells with the daily job of handling hemostasis and thrombosis.[14] On the other hand, they are also involved in inflammation,[23] angiogenesis, and tissue repair. Platelets have been shown to accumulate in the liver under some pathologic conditions such as acute viral hepatitis[24] and cholestasis.[25] 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.[26] Moreover, it has been reported that TPO-induced thrombocytosis attenuates progression of liver fibrosis and accelerates liver fibrolysis.[27,28] However, the mechanisms remain obscure, and the extrathrombocytotic 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.[5] However, traditional knockout of both alleles of the *bcl-x* gene leads to embryonic lethality.[6] 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[6,7] and transgenic mice expressing the Cre-recombinase under regulation of the promoter of the Pf4 gene.[8] The expression of Pf4 promoter is reported to be specific to thrombocytes,[8] 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.[23-25] 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.[29] Recent research has shown that platelets mediate liver regeneration after partial hepatectomy.[26] 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,[29] 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.[1,2] 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 thromobocytopenic mice compared with control mice (Supplementary Figure 5*A*). 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*) *col1a1* And *col1a2* mRNA levels in HSCs stimulated with murine HGF for 6 hours by real-time reverse-transcription polymerase chain reaction, n = 3/group. *$P < .05$ vs control. (*B*) Secretion of HGF from activated platelets. Platelets (1.0×10^7) 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/group. *$P < .05$ vs other 2 groups. (*C*) Production of HGF in platelet/HSC coculture. HSCs (1.0×10^5) were cocultured with 5.0×10^7 of platelets for 3 hours, and the levels of HGF in the culture medium were determined by ELISA, n = 3/group. *$P < .05$ vs the other 2 groups. (*D*) Activation of Met and its downstream pathways in platelet/HSC coculture. HSCs (5.0×10^5) were cocultured with or without 5.0×10^7 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*) *col1a1* And *col1a2* 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 1.0×10^7 platelets (*right*), n = 3/group.

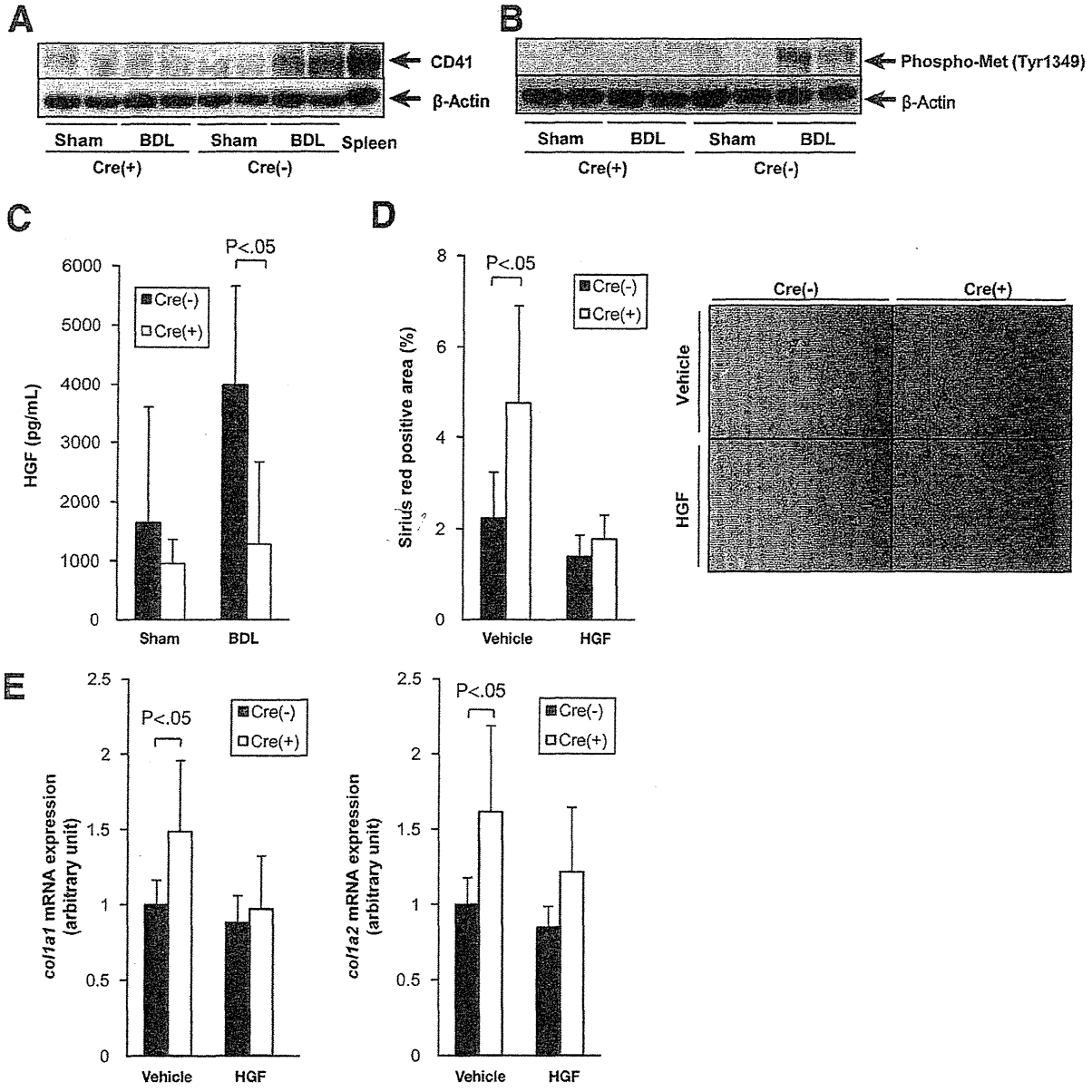

Figure 6. HGF administration prevents the exacerbation of liver fibrosis in the thrombocytopenic mice after BDL. (*A–C*) Platelet accumulation and Met activation in the liver following BDL. *bcl-x*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ mice were sham operated or subjected to BDL and analyzed 10 days later (4–7 mice per group). Cre(+) stands for *bcl-x*$^{flox/flox}$ *Pf4-Cre* mice and Cre(−) for *bcl-x*$^{flox/flox}$ 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*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ 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-β, and tumor necrosis factor-α, which are also known as cytokines involved in fibrosis, was not different between the 2 groups (Supplementary Figure 5*B*). 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 α-smooth muscle actin (SMA) and cause a parallel increase in collagen synthesis.[1,2] However, instead of increased collagen synthesis in thrombocytopenic mice, α-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 α complex protein.[30] Furthermore, it has recently been demonstrated that HGF suppresses *col1a2* promoter activation by inhibiting nuclear accumulation of Smad3 in activated HSCs.[31] We used activated HSCs for in vitro experiments and found that coculture with platelets did not affect mRNA expression of α-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.[10,11] 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 phosphorylation 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[16,17] and also from rat platelets.[18] HGF is known to be a multifunctional growth factor that shows mitogenic, motogenic, morphogenic, and antiapoptotic activities in a variety of cells.[16,17] Increasing evidence indicates that HGF has an antifibrotic effect in several experimental models, especially when administered exogenously.[20,21] Although platelets are known to contain HGF in their granules,[18] the functional role of platelet-derived HGF has remained unknown. Because human platelets contain a smaller amount of HGF than rodent platelets,[18] 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.[3,4] 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.[32,33] 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, and at doi: 10.1053/j.gastro.2010.02.054.

References

1. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115: 209–218.
2. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008;134:1655–1669.
3. Afdhal N, McHutchison J, Brown R, et al. Thrombocytopenia associated with chronic liver disease. J Hepatol 2008;48:1000–1007.
4. Aster RH. Pooling of platelets in the spleen: role in the pathogenesis of "hypersplenic" thrombocytopenia. J Clin Invest 1966;45: 645–657.
5. Mason KD, Carpinelli MR, Fletcher JI, et al. Programmed anuclear cell death delimits platelet life span. Cell 2007;128:1173–1186.
6. 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.
7. 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.
8. 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.
9. Tsukamoto H, Matsuoka M, French SW. Experimental models of hepatic fibrosis: a review. Semin Liver Dis 1990;10:56–65.
10. Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil-interactions: linking hemostasis and inflammation. Blood Rev 2007;21:99–111.
11. Bennet JS. Structure and function of the platelet integrin αIIbβ3. J Clin Invest 2005;115:3363–3369.
12. 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.
13. 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.
14. Holmsen H. Physiological functions of platelets. Ann Med 1989; 21:23–30.
15. 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.
16. 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.
17. 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.
18. Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature 1989; 342:440–443.
19. Tulasne D, Foveau B. The shadow of death on the MET tyrosine kinase receptor. Cell Death Differ 2008;15:427–434.
20. Ueki T, Kaneda Y, Tsutsui H, et al. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nat Med 1999;5:226–230.
21. 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.
22. 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.
23. Iannacone M, Sitia G, Isogawa M, et al. Platelets mediate cytotoxic T lymphocyte-induced liver damage. Nat Med 2005;11: 1167–1169.
24. Lang PA, Contaldo C, Georgiev P, et al. Aggravation of viral hepatitis by platelet-derived serotonin. Nat Med 2008;14:756–761.
25. 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.
26. Lesurtel M, Graf R, Aleil B, et al. Platelet-derived serotonin mediates liver regeneration. Science 2006;312:104–107.
27. 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.
28. 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.
29. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology 2006;43:S45–S53.
30. Stefanovic B, Hellebrand C, Holcik M, et al. Posttranscriptional regulation of collagen α1(I) mRNA in hepatic stellate cells. Mol Cell Biol 1999;17:5201–5209.
31. 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.
32. Murata K, Ito K, Yoneda K, et al. Splenectomy improves liver function in patients with liver cirrhosis. Hepatogastroenterology 2008;55:1407–1411.
33. 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.

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

Conflicts of interest

The authors disclose no conflicts.

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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 picrosirius red. The percentage of oncotic necrosis or fibrotic area was calculated using image analysis software (winROOF 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,[1] 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.[2] To assess regenerative status, nuclear 5-bromo-2-deoxyuridine incorporation was evaluated as previously described.[3]

Determination of Liver Hydroxyproline Content

Hydroxyproline content was determined essentially as described previously.[4] 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.[5] Isolated HSCs were maintained at 37°C under 5% CO_2 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$^{flox/flox}$ Pf4-Cre* mice and *bcl-x$^{flox/flox}$* 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 μL/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.[6] 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 100*g* for 15 minutes at room temperature without braking. After incubation with 1 μmol/L prostaglandin E_1 (Sigma-Aldrich) and 1 U/mL apyrase (Sigma-Aldrich), the platelets were isolated by centrifugation at 200*g* at room temperature for 15 minutes.

Western Blot Analysis

Western blotting was performed as previously described.[2] 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.[2] 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

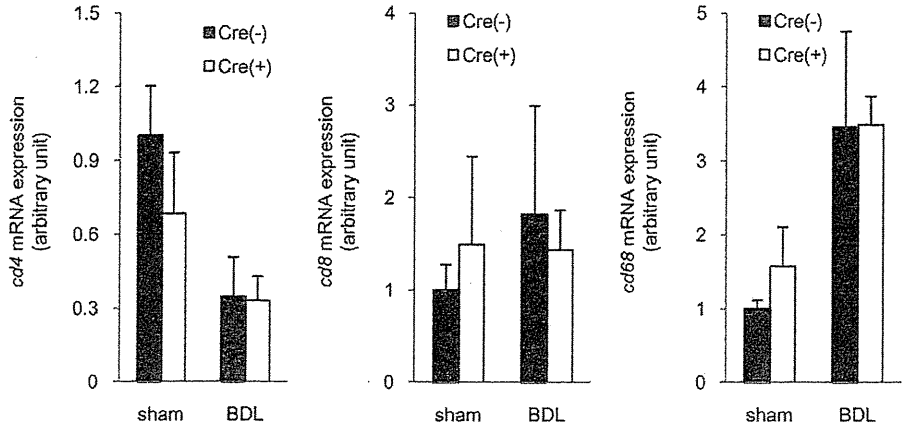
Mouse HGF and soluble CD62p (P-selectin) levels in plasma and culture supernatant were measured by using DuoSet enzyme-linked immunosorbent assay mouse hepatocyte growth factor (HGF) and CD62p (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's protocol.

HGF Treatment

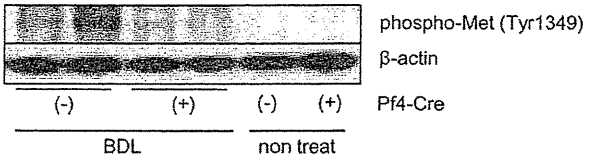
Wild-type (*bcl-x$^{fl/fl}$*) and knockout (*bcl-x$^{fl/fl}$ Pf4-Cre*) mice were subjected to bile duct ligation, followed by intraperitoneal injection of recombinant human HGF (500 μg/kg) or vehicle every 12 hours for 10 days and then killed to sample the liver tissues.

References

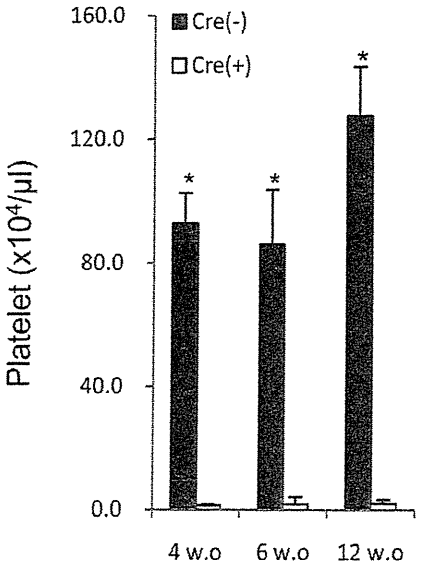
1. Gujral JS, Liu J, Farhood A, et al. Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. Hepatology 2004;40:998–1007.
2. Hikita H, Takehara T, Shimizu S, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing an adult murine 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-β 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.



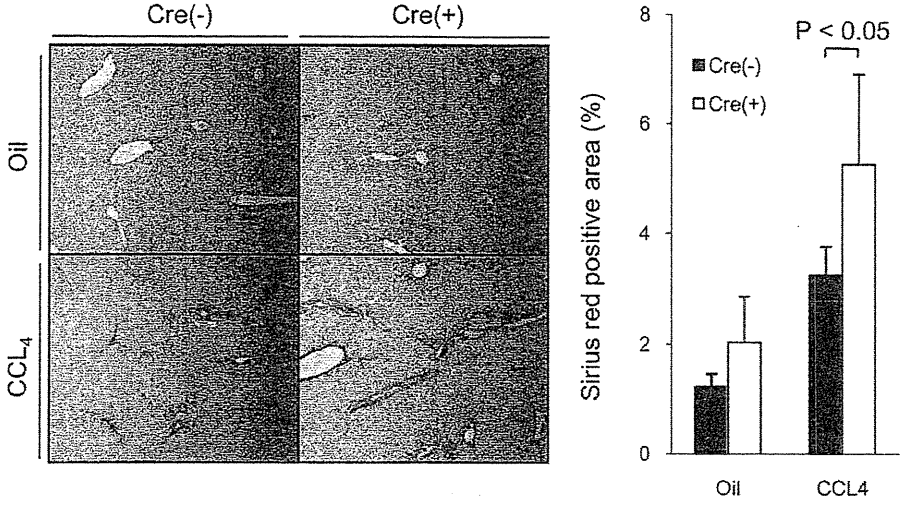
Supplementary Figure 1. Intrahepatic cell fractions upon bile duct ligation (BDL) treatment are not different between the thrombocytopenic mice and the control littermates. *bcl-x*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ mice were sham operated or subjected to BDL and analyzed 10 days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



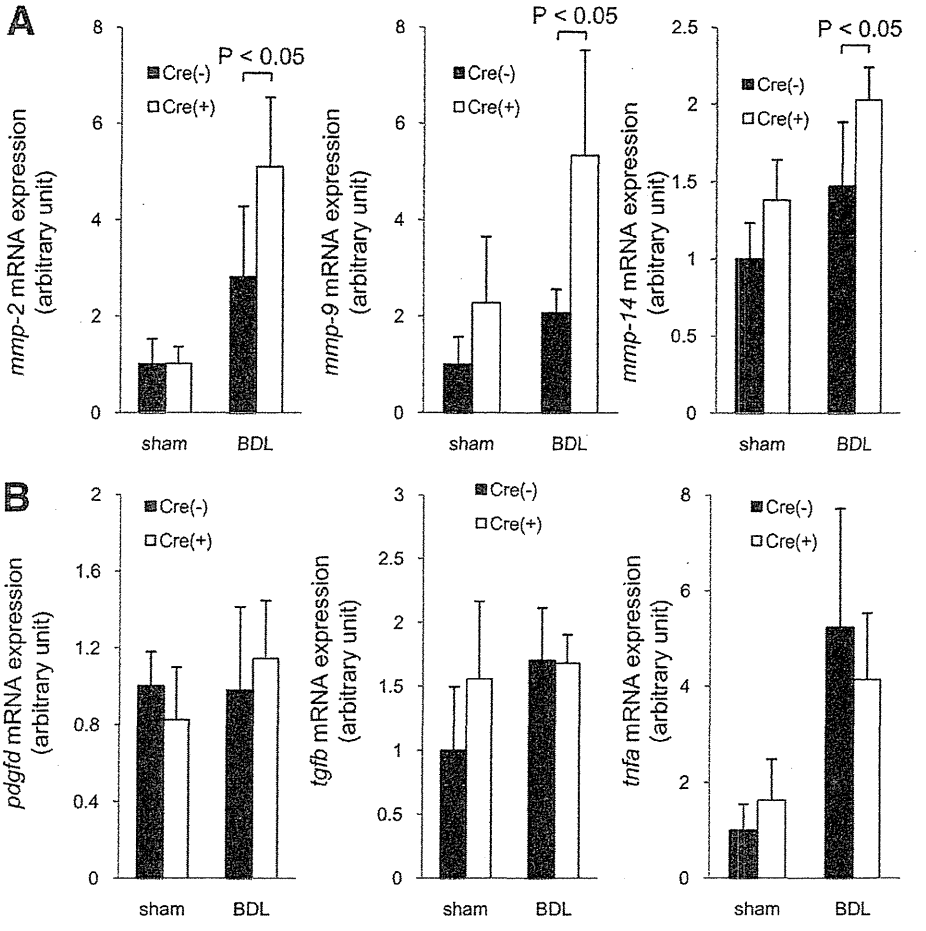
Supplementary Figure 2. Phosphorylation of Met protein in the liver is stronger in the control littermates than in the thrombocytopenic mice at 3 days after bile duct ligation (BDL) treatment. *bcl-x*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ mice were subjected to BDL and analyzed 3 days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



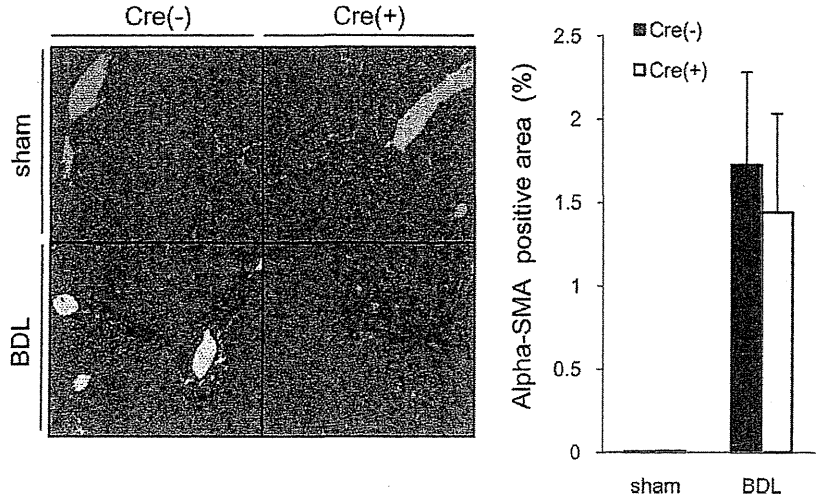
Supplementary Figure 3. *bcl-x*$^{flox/flox}$ *Pf4-Cre* mice exhibit severe thrombocytopenia at as early as 4 weeks of age, and it persists for a longer time. Circulating platelet counts of *bcl-x*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ mice at the age of 4, 6, and 12 weeks. Cre(+) and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



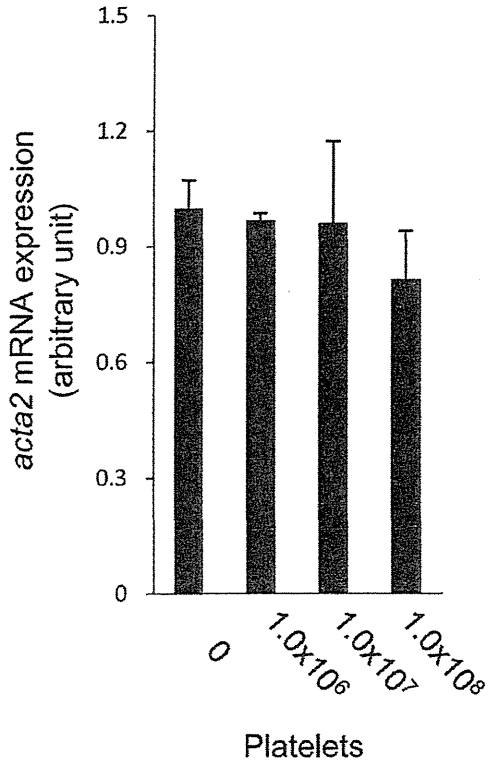
Supplementary Figure 4. Thrombocytopenia exacerbates liver fibrosis induced by chronic CCl_4 administration. *bcl-x*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ mice were administered intraperitoneal injection of CCl_4 (0.3 mL/kg) or oil 2 times per week and examined 6 weeks later (7 mice per group). Cre(+) and Cre(−) stand for *bcl-x*$^{flox/flox}$ *Pf4-Cre* and *bcl-x*$^{flox/flox}$, respectively. Liver fibrosis was evaluated by picrosirius 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*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ 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*$^{flox/flox}$ *Pf4-Cre* and *bcl-x*$^{flox/flox}$, 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*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ 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*$^{flox/flox}$ *Pf4-Cre* and *bcl-x*$^{flox/flox}$, 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*$^{flox/flox}$ *Pf4-Cre* mice and *bcl-x*$^{flox/flox}$ 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*$^{flox/flox}$ *Pf4-Cre* and *bcl-x*$^{flox/flox}$, respectively. To assess HSC activation, liver sections were stained with monoclonal anti-α-smooth muscle actin (α-*SMA*) (Dako, Glostrup, Denmark).



Supplementary Figure 7. Coculture with platelets does not affect messenger RNA expression of α-SMA in activated HSCs. HSCs (1.0×10^5) 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-α2B/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 β-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
col1a1	Mm00801666_g1
col1a2	Mm01165187_m1
met	Mm01156980_m1
mmp-2	Mm00439506_m1
mmp-9	Mm00600164_g1
mmp-14	Mm01318969_g1
acta2	Mm01546133_m1
actb	Mm02619580_g1
cd4	Mm01182108_m1
cd8	Mm00442754_m1
cd68	Mm03047343_m1
Tnfa	Mm01178820_m1
tgfb	Mm00546829_m1
pdgfd	Mm01135193_m1

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Fatal exacerbation of type B chronic hepatitis triggered by changes in relaxed circular viral DNA synthesis and virion secretion

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ABSTRACT

Virological features of fulminant liver disease-causing hepatitis B virus (HBV) have not been fully elucidated. We studied longitudinally the viruses obtained before and after fulminant liver disease in a patient with chronic HBV infection showing fatal exacerbation. HBV strains were obtained before and after exacerbation (designated as FEP1 and FEP2). Their virological features were investigated by *in vitro* transfection. FEP1 and FEP2 possessed higher activity of overall HBV DNA synthesis than the wild-type. FEP1 lacked competence for relaxed circular (RC) HBV DNA synthesis and RC HBV DNA-containing virion secretion, but FEP2 maintained it. Chimeric analysis revealed that the preS/S gene, where FEP1 had a considerable number of mutations and deletions but FEP2 did not, was responsible for impaired RC HBV DNA synthesis and virion secretion. Furthermore, incompetence of FEP1 strain was transcomplemented by the preS/S protein of wild-type strain. In conclusion, the viral strain after exacerbation showed resurgent RC HBV DNA synthesis and virion secretion, which was caused by conversion of the preS/S gene from a hypermutated to hypomutated state. This may have been responsible for disease deterioration in the patient. This is a novel type of HBV genomic variation associated with the development of fulminant liver disease.

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1. Introduction

Type B fulminant hepatitis develops in approximately 1% of patients with acute hepatitis B virus (HBV) infection and results in a high rate of mortality [1]. Serious disease exacerbation like fulminant hepatitis can also occur during chronic HBV infection. The virological characteristics of fulminant hepatitis-causing HBV strains have been widely studied. An A1896 mutation in the precore gene, and T1762/A1764 and V1753/V1754 mutations (V = not T) in the core promoter have been shown to be detected more frequently in fulminant hepatitis-related strains than in non-fulminant hepatitis-related ones [2–4], although these viral mutations do not completely account for the pathogenesis of fulminant hepatitis. A few investigators have conducted detailed studies on the strain-specific virological feature of an individual fulminant hepatitis-causing HBV strain in comparison with the representative wild-type HBV strain [5–8]. Baumert et al. [5,6] reported that a fulminant hepatitis-causing HBV strain with rare types of mutations in the core promoter showed a robust increase of viral encapsidation and strong induction of cellular apoptosis. Pult et al. [7] also revealed that the strain isolated from a patient with heart transplantation-associated fulminant hepatitis had the 11-bp insertion in the core promoter and revealed the elevated viral transcription via generation of a novel binding site of hepatocyte nuclear factor 1. In addition, Kalinina et al. [8] reported that the strain derived from a fulminant hepatitis patient after liver transplantation was secretion-defective due to several mutations in the surface (S) gene. According to these observations, in fulminant hepatitis-causing HBV strains, both frequent mutations and strain-specific viral genomic variations may contribute to the development of the disease. However, there have been no longitudinal virological studies of HBV strains obtained before and after the onset of fulminant liver disease in chronic HBV carriers showing serious disease exacerbation such as fulminant hepatitis. Such investigations may lead to better understanding of the role of viral genomic changes on the pathogenesis of HBV-related fulminant liver disease.

HBV is a double-stranded circular DNA virus approximately 3.2 kb long and has four open reading frames, preS/S, precore/core,

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