

Transforming Growth Factor- β Signaling in Hepatocytes Promotes Hepatic Fibrosis and Carcinogenesis in Mice With Hepatocyte-Specific Deletion of TAK1

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BACKGROUND & AIMS: Transforming growth factor (TGF)- β -activated kinase 1 (TAK1) is activated in different cytokine signaling pathways. Deletion of *Tak1* from hepatocytes results in spontaneous development of hepatocellular carcinoma (HCC), liver inflammation, and fibrosis. TGF- β activates TAK1 and Smad signaling, which regulate cell death, proliferation, and carcinogenesis. However, it is not clear whether TGF- β signaling in hepatocytes, via TGF- β receptor-2 (*Tgfb2*), promotes HCC and liver fibrosis. **METHODS:** We generated mice with hepatocyte-specific deletion of *Tak1* (*Tak1ΔHep*), as well as *Tak1/Tgfb2ΔHep* and *Tak1/Smad4ΔHep* mice. *Tak1flox/flox*, *Tgfb2ΔHep*, and *Smad4ΔHep* mice were used as controls, respectively. We assessed development of liver injury, inflammation, fibrosis, and HCC. Primary hepatocytes isolated from these mice were used to assess TGF- β -mediated signaling. **RESULTS:** Levels of TGF- β , TGF- β R2, and phospho-Smad2/3 were increased in HCCs from *Tak1ΔHep* mice, which developed liver fibrosis and inflammation by 1 month and HCC by 9 months. However, *Tak1/Tgfb2ΔHep* mice did not have this phenotype, and their hepatocytes did not undergo spontaneous cell death or compensatory proliferation. Hepatocytes from *Tak1ΔHep* mice incubated with TGF- β did not activate p38, c-Jun N-terminal kinase, or nuclear factor- κ B; conversely, TGF- β -mediated cell death and phosphorylation of Smad2/3 were increased, compared with control hepatocytes. Blocking the Smad pathway inhibited TGF- β -mediated death of *Tak1*–/– hepatocytes. Accordingly, disruption of *Smad4* reduced the spontaneous liver injury, inflammation, fibrosis, and HCC that develops in *Tak1ΔHep* mice. Levels of the anti-apoptotic protein Bcl-xL, β -catenin, connective tissue growth factor, and vascular endothelial growth factor were increased in HCC from *Tak1ΔHep* mice, but not in HCCs from *Tak1/Tgfb2ΔHep* mice. Injection of N-nitrosodiethylamine induced HCC formation in wild-type mice, but less in *Tgfb2ΔHep* mice. **CONCLUSIONS:** TGF- β promotes development of HCC in *Tak1ΔHep* mice by inducing hepatocyte apoptosis and compensatory proliferation during early phases of tumorigenesis, and inducing expression of anti-apoptotic, pro-oncogenic, and angiogenic factors during tumor progression.

Keywords: Liver Cancer; Mouse Model; Signal Transduction; Oncogene.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths in the United States.¹ The increasing incidence of HCC is a result of the increased prevalence of hepatitis C virus infection, alcoholic cirrhosis, and nonalcoholic steatohepatitis. In humans, development of HCC is associated with chronic liver inflammation, fibrosis, and its subsequent irreversible cirrhosis.¹ In contrast, a mouse model for liver fibrosis induced by carbon tetrachloride does not induce HCC, and a mouse model for carcinogen-induced HCC is not accompanied by fibrosis. The current lack of an ideal animal model for liver fibrosis-associated HCC limits the mechanistic study of HCC.² We and others have recently established a mouse model that spontaneously develops HCC accompanied by liver fibrosis by deleting *Tak1* in hepatocytes, indicating that TAK1 is a tumor suppressor in the liver.^{3,4}

Transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1) is activated in various cytokine signaling, such as tumor necrosis factor (TNF), interleukin (IL)-1, Toll-like receptors, and transforming growth factor (TGF)- β . TAK1 then activates downstream kinases such as I κ B kinase (IKK), c-Jun-N-terminal kinase (JNK), and p38 that regulate cell survival, proliferation, and tumor growth.^{5,6} TNF receptor signaling activates both TAK1-IKK-nuclear factor- κ B (NF- κ B) pathway and caspase-mediated cell death pathway.⁷ Inactivation of TAK1 increases the susceptibility of hepatocytes to TNF-induced cell death through overactivation of caspases caused by lack of the TAK1-IKK-NF- κ B activation.³ Notably, deletion of TNF receptor prevented hepatocyte death in hepatocyte-specific *Tak1*-deleted (*Tak1ΔHep*) mice, indicating that TNF receptor signaling

Abbreviations used in this paper: ALT, alanine aminotransferase; CTGF, connective tissue growth factor; DEN, N-nitrosodiethylamine; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; IKK, I κ B kinase; IL, interleukin; JNK, c-Jun-N-terminal kinase; NF- κ B, nuclear factor- κ B; TAK1, TGF- β -activated kinase 1; *Tak1ΔHep* mice, hepatocyte-specific *Tak1*-deleted mice; TGF, transforming growth factor; *Tgfb2*, TGF- β receptor-2; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; VEGF, vascular endothelial growth factor; WT, wild-type.

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is responsible for spontaneous liver injury and inflammation in *Tak1* $^{\Delta Hep}$ mice.³

TGF- β signaling activates Smad-dependent canonical and Smad-independent noncanonical TAK1-mediated signal pathways that activate JNK and p38.⁸⁻¹⁰ TGF- β signaling regulates cell apoptosis, proliferation, differentiation, and extracellular matrix production. The importance of TGF- β signaling in cancer development has been demonstrated in several types of human cancers, including colon cancer, prostate cancer, and breast cancer.¹¹ In HCC, the functions of TGF- β signaling are paradoxical. In human HCC, persistent up-regulation of TGF- β was observed, and several reports indicate TGF- β signaling functions as a tumor suppressor. Alternatively, TGF- β signaling promotes the growth and migration of HCC through the induction of connective tissue growth factor (CTGF).^{9,12,13} It is unclear whether TGF- β signaling promotes or suppresses HCC development in *Tak1* $^{\Delta Hep}$ mice. In the present study, we investigated the role of TGF- β signaling in hepatocyte death, liver inflammation, fibrosis, and HCC spontaneously developed in *Tak1* $^{\Delta Hep}$ mice. We found that TGF- β signaling promoted HCC development through induction of hepatocyte death and its subsequent compensatory proliferation in early phase. Importantly, in HCC of *Tak1* $^{\Delta Hep}$ mice, TGF- β signaling is associated with overexpression of anti-apoptotic Bcl-xL, procarcinogenic β -catenin, and CTGF, which prevent tumor apoptosis and promote cancer growth. In addition, TGF- β signaling also contributes to angiogenesis through vascular endothelial growth factor (VEGF) expression to promote HCC growth in *Tak1* $^{\Delta Hep}$ mice.

Materials and Methods

Mouse Colonies

Albumin-Cre recombinase transgenic mice (Alb-Cre Tg mice), TGF- β receptor-2 (*Tgfb2*) $^{flax/flax}$ mice, and *Smad4* $^{flax/flax}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice carrying the floxed allele of *Tak1* (*Tak1* $^{flax/flax}$ mice) have been described previously.³ These mouse lines were crossed to generate the following types of mice with genes specifically deleted in hepatocytes: Alb-cre/+*Tak1* $^{flax/flax}$ mice (*Tak1* $^{\Delta Hep}$), Alb-cre/+*Tgfb2* $^{flax/flax}$ mice (*Tgfb2* $^{\Delta Hep}$), Alb-cre/+*Tak1* $^{flax/flax}$ *Tgfb2* $^{flax/flax}$ (*Tak1/Tgfb2* $^{\Delta Hep}$), and Alb-cre/+*Tak1* $^{flax/flax}$ *Smad4* $^{flax/flax}$ (*Tak1/Smad4* $^{\Delta Hep}$) mice on C57BL6 background. Both males and females were used in the study and Cre-negative animals were used as WT controls. In our previous report, we demonstrated that *Tak1* $^{\Delta Hep}$ mice spontaneously develop liver injury, inflammation, fibrosis, and tumors without sex disparity.^{3,4} One-month-old mice were used for assessing spontaneous liver injury, inflammation, and fibrosis, and 9-month-old mice were used for examining spontaneous liver fibrosis and HCC. In some experiments (Figure 7E), HCC was induced chemically via intra-peritoneal injection of 25 mg/kg N-nitrosodiethylamine (DEN) (Sigma-Aldrich, St Louis, MO) into 14-day-old male *Tgfb2* $^{flax/flax}$ and *Tgfb2* $^{\Delta Hep}$ mice.¹⁴ Nine months after DEN injection, the chemically induced HCC was evaluated. All mice received hu-

mane care according to the National Institutes of Health recommendations outlined in their Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Human Liver and HCC Samples

Paraffin-embedded human liver tissues were acquired from liver biopsy samples of patients with chronic hepatitis C and from explanted or surgically resected livers of patients with HCC. All patients provided written informed consent, and the study was approved by the University of California San Diego Institutional Review Board.

Other Materials and Methods

Other materials and methods for mouse tissue processing, histologic examination, Western blot analysis, quantitative real-time polymerase chain reaction, cell isolation, and treatment are described in the Supplementary Materials and Methods.

Statistical Analysis

Differences between 2 groups were compared using the Mann-Whitney *U* test or 2-tailed unpaired Student *t* test. Differences between multiple groups were compared using 1-way analysis of variance using SPSS software (SPSS Inc, Chicago, IL). *P* values <.05 were considered significant.

Results

Expression of TGF- β Signaling Components in Tumors From *Tak1* $^{\Delta Hep}$ Mice

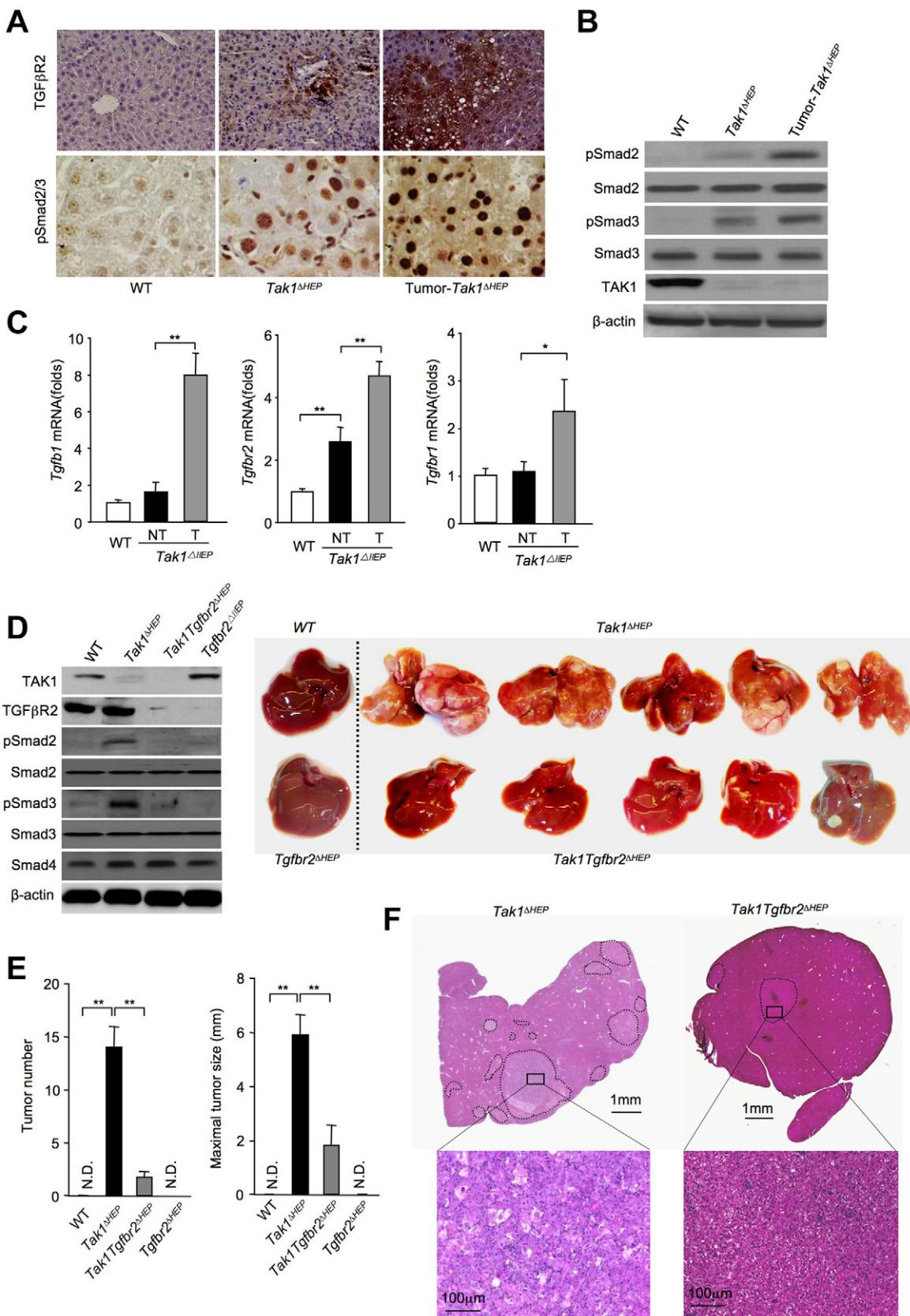
As reported previously, *Tak1* $^{\Delta Hep}$ mice develop spontaneous liver inflammation, fibrosis, and HCC without treatment with carcinogens.³ In contrast, their WT counterparts did not develop liver tumors and fibrosis, even at 12 months of age.³ We initially investigated the expressions of TGF- β signaling components in liver tumors of 9-month-old *Tak1* $^{\Delta Hep}$ mice. An immunohistochemical study demonstrated that expressions of TGF- β type 2 and phosphorylated Smad2/3 were modestly increased in nontumor livers of *Tak1* $^{\Delta Hep}$ mice and remarkably increased in HCC lesions of *Tak1* $^{\Delta Hep}$ mice in comparisons with WT normal livers (Figure 1A). Immunoblots showed dramatically increased phosphorylation and nuclear translocation of Smad2/3, as well as blunted expression of TAK1 protein in tumors of *Tak1* $^{\Delta Hep}$ mice (Figure 1B). Expressions of genes related to TGF- β signaling, including *Tgfb1*, *Tgfb2*, and *Tgfb1*, were also increased in tumors of *Tak1* $^{\Delta Hep}$ mice (Figure 1C). These results suggest that activation of TGF- β signaling is associated with spontaneous HCC development in *Tak1* $^{\Delta Hep}$ mice.

Ablation of *Tgfb2* Prevents Spontaneous HCC Development in *Tak1* $^{\Delta Hep}$ Mice

To investigate the contribution of TGF- β signaling in spontaneous hepatocarcinogenesis in *Tak1* $^{\Delta Hep}$ mice, we generated *Tak1/Tgfb2* $^{\Delta Hep}$ mice by crossing *Tak1* $^{\Delta Hep}$ mice with *Tgfb2* $^{\Delta Hep}$ mice. Deletion of hepatic TAK1 and/or TGF β R2 protein in *Tak1* $^{\Delta Hep}$, *Tgfb2* $^{\Delta Hep}$, or *Tak1/Tgfb2* $^{\Delta Hep}$ mice was confirmed by immunoblotting (Figure 1D). In

the liver from 1-month-old *Tak1^{ΔHep}* mice, Smad2 and Smad3 were strongly phosphorylated, however, such signaling was diminished by *Tgfb2* deletion (Figure 1D). This demonstrates that the deletion of *Tgfb2* blocks

TGF- β signaling in *Tak1^{ΔHep}* mice. *Tak1^{flox/flox}* mice and *Tgfb2^{ΔHep}* mice were used as corresponding WT controls, and these mice showed no appearances of spontaneous liver tumors, even at 12 months of age. *Tak1^{ΔHep}* mice



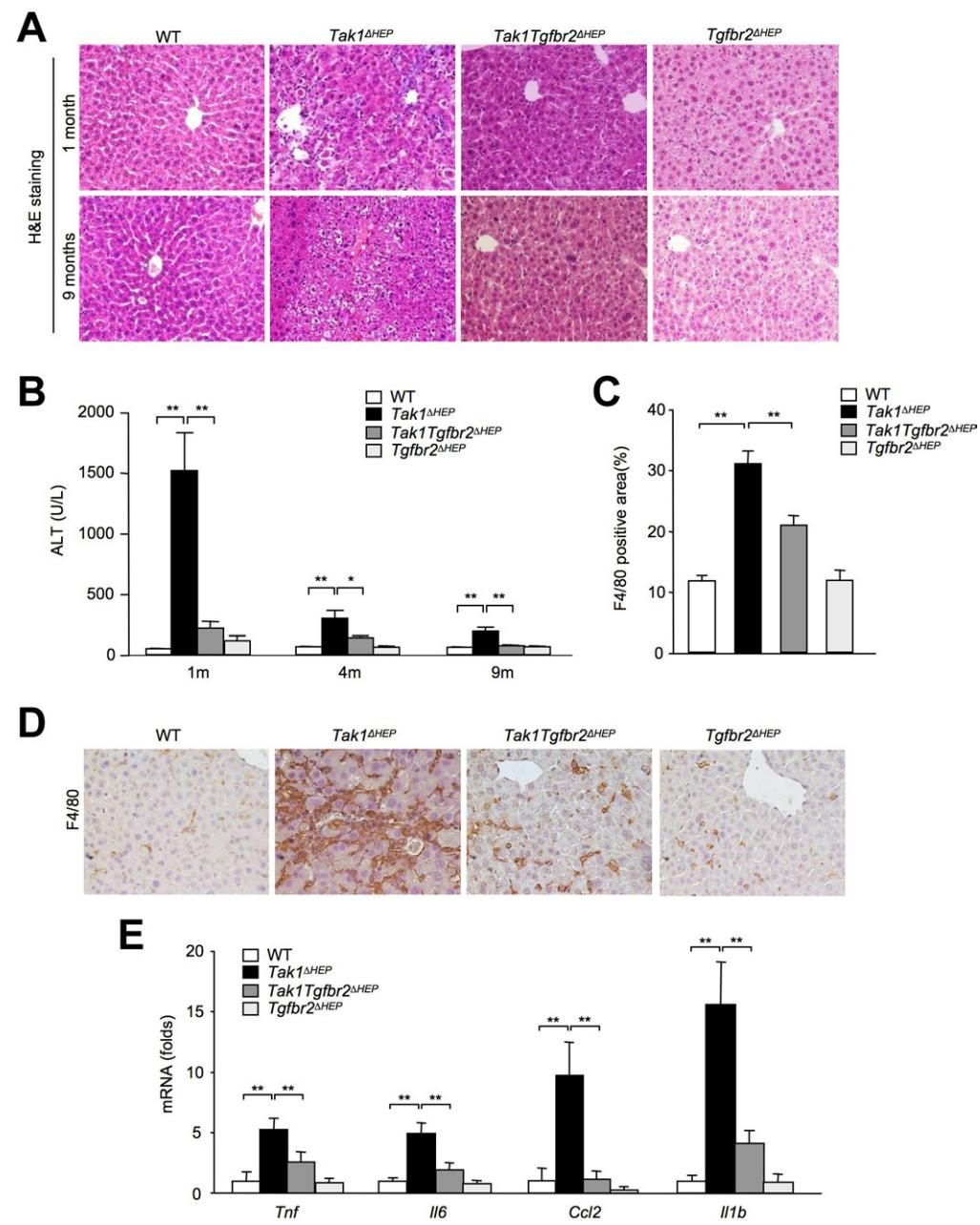


Figure 2. Ablation of *Tgfb2* in *Tak1 Δ Hep* mice reduces spontaneous liver inflammation. (A) H&E staining in the livers of 1- and 9-month-old WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice. Original magnification 200 \times . (B) Serum ALT levels were measured in WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice at age 1, 4, and 9 months ($n = 8$ at the each time point). (C) Immunohistochemistry for F4/80 in WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice at 1 month of age. Quantification (C) and representative pictures (D). Original magnification 200 \times . (E) Hepatic messenger RNA expression of inflammatory genes (*Tnf*, *Il6*, *Ccl2*, and *Il1b*) in mice at the age of 1 month was determined by quantitative real-time polymerase chain reaction. Data are represented as mean \pm standard error of mean. * $P < .05$; ** $P < .01$.

developed spontaneous HCC at 9 months of age; in comparisons, *Tak1/Tgfb2 Δ Hep* mice developed tumors significantly lower in multiplicity and maximal size of tumors (Figure 1D–F). These results indicate that TGF- β signal-

ing is responsible for spontaneous HCC development in *Tak1 Δ Hep* mice. Additionally, we confirmed no significant sex disparity in HCC formation in both *Tak1 Δ Hep* mice and *Tak1/Tgfb2 Δ Hep* mice (Supplementary Figure 1).

Figure 1. TGF- β signaling is required for spontaneous hepatocarcinogenesis in *Tak1 Δ Hep* mice. (A) Immunohistochemistry for TGF β R2 and phosphorylated Smad2/3 in liver tissues from 9-month-old WT, and nontumor livers and tumors from 9-month-old *Tak1 Δ Hep* mice is shown. Original magnification $\times 200$ for TGF β R2 staining and $\times 400$ for Smad2/3 staining. (B) Western blotting for phosphorylated Smad2 and Smad3 and TAK1 in liver tissues from 9-month-old WT, and nontumor liver tissues and tumors from 9-month-old *Tak1 Δ Hep* mice. β -actin was used as a loading control. (C) Expression of *Tgfb1*, *Tgfb2*, and *Tgfb2* messenger RNA in liver tissues from 9-month-old WT, and nontumor liver tissues and tumors from 9-month-old *Tak1 Δ Hep* mice was measured by quantitative real-time polymerase chain reaction. NT, nontumor liver, T, tumors. ($n = 6$, each samples) (D, left) Immunoblots for TAK1, TGF β R2, phospho-Smad2, phospho-Smad3, Smad4 in the livers of WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice at 1 month of age are shown. β -actin was used as a loading control. (D, right) Representative macroscopic pictures of livers of 9-month-old WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice. (E) The number of tumors per mouse was counted and the maximum diameter of individual tumor nodules was measured (WT, $n = 10$; *Tak1 Δ Hep*, $n = 29$; *Tak1/Tgfb2 Δ Hep*, $n = 27$; *Tgfb2 Δ Hep*, $n = 10$). (F) H&E staining. Data are represented as mean \pm standard error of mean.* $P < .05$; ** $P < .01$.

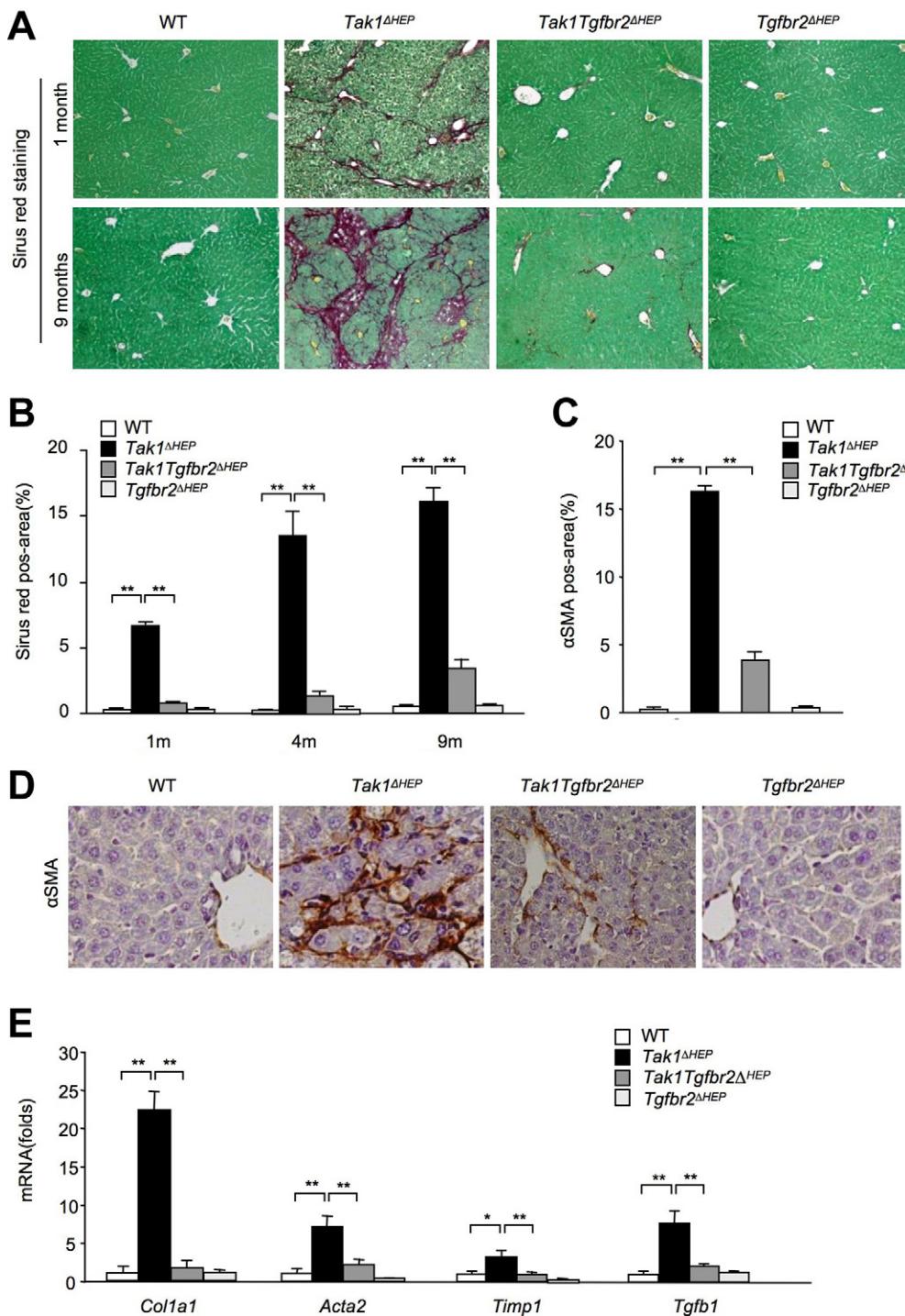


Figure 3. Loss of *Tgfb2* in *Tak1 Δ Hep* mice suppresses spontaneous liver fibrosis. (A, B) WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice at the age of 1, 4, and 9 months ($n = 7$ at the each time point) were used for analysis. Fibrillar collagen deposition was determined by Sirius red staining (A) and its quantification are shown in (B). Original magnification 100 \times . (C, D) Expression of α -smooth muscle actin in 9-month-old mice was determined by immunohistochemistry. Quantification (C) and representative pictures (D). Original magnification 320 \times . (E) Hepatic messenger RNA expressions of fibrogenic markers, including *Col1a1*, *Acta2*, *Timp1*, and *Tgfb1*, were determined by quantitative real-time polymerase chain reaction in 1-month-old WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice. Data are reported as mean \pm standard error of mean. * $P < .05$; ** $P < .01$.

TGF- β Signaling Is Required for Spontaneous Liver Injury and Inflammation in *Tak1 Δ Hep* Mice

Next, we investigated whether TGF- β signaling contributes to spontaneous liver injury and inflammation in *Tak1 Δ Hep* mice. Histologic analysis showed evident hepatocyte death and inflammatory cell infiltration to have occurred in both 1- and 9-month-old *Tak1 Δ Hep* mice (Figure 2A). These phenotypes were abolished in the livers of *Tak1/Tgfb2 Δ Hep* mice (Figure 2A). Elevation of serum alanine aminotransferase (ALT) levels in *Tak1 Δ Hep* mice were

also suppressed by *Tgfb2* deletion (Figure 2B). Kupffer cells/macrophages are the major source of inflammatory cytokines produced in response to hepatocyte damage.^{15,16} According to the immunohistochemical staining of F4/80 in 1-month-old mice, the number of infiltrated hepatic macrophages in *Tak1 Δ Hep* mice was significantly lowered in *Tak1/Tgfb2 Δ Hep* mice (Figure 2C and D). Accordingly, *Tak1/Tgfb2 Δ Hep* livers had attenuated expression of inflammatory genes, including TNF α , IL-6, CCL2, and IL-1 β compared with *Tak1 Δ Hep* livers (Figure 2E). These results indicate that spontaneous liver injury and

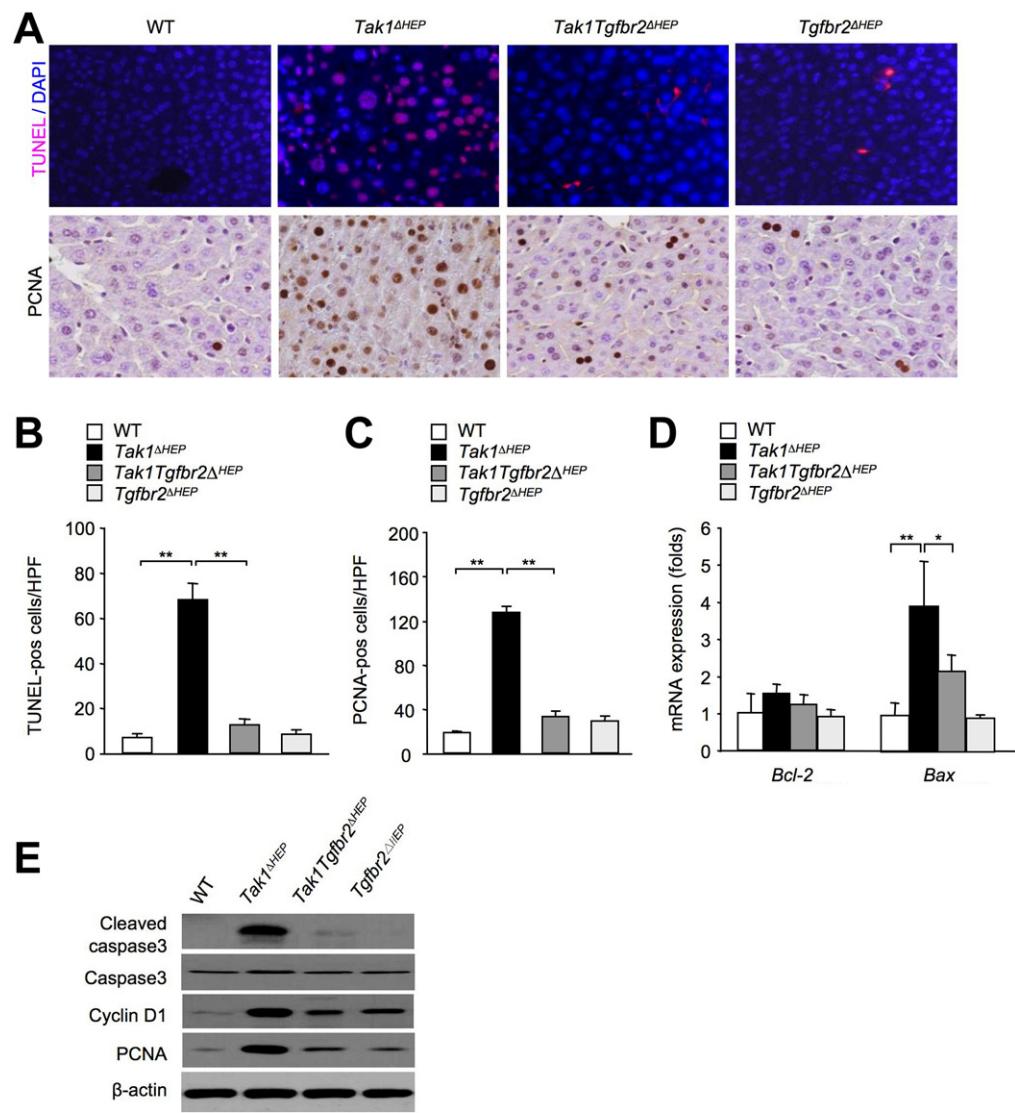


Figure 4. Additional deletion of *Tgfb2* in hepatocytes inhibits spontaneous apoptosis and compensatory regeneration in the livers of *Tak1 Δ Hep* mice. (A) Apoptotic hepatocytes were evaluated by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining (A, upper) and proliferating hepatocytes were evaluated by immunohistochemistry for proliferation cell nuclear antigen (PCNA) (A, lower) in the livers of WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice at the 1 month of age ($n = 7$). Original magnification 320 \times . (B, C) Quantification for TUNEL staining (B) and staining for PCNA (C). (D) Hepatic messenger RNA expression of apoptotic genes (*Bcl-2*, *Bax*) in the livers of WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice at 1 month of age was determined by quantitative real-time polymerase chain reaction. (E) Immunoblots for caspase 3, cleaved caspase 3, PCNA, and cyclin D1 in the livers of WT, *Tak1 Δ Hep*, *Tak1/Tgfb2 Δ Hep*, and *Tgfb2 Δ Hep* mice at 1 month of age. Data are reported as mean \pm standard error of mean. * $P < .05$; ** $P < .01$.

inflammation in *Tak1 Δ Hep* mice are associated with TGF- β -mediated hepatocyte damage, which drives Kupffer cell/macrophage activation and production of inflammatory cytokines.

TGF- β Signaling in Hepatocytes Drives Spontaneous Liver Fibrosis in *Tak1 Δ Hep* Mice

Subsequently, we investigated the role of hepatocyte's TGF- β signaling in the development of liver fibrosis in *Tak1 Δ Hep* mice. Spontaneous liver fibrosis in *Tak1 Δ Hep* mice was significantly inhibited by the additional deletion of *Tgfb2* (Figure 3A and B). The number of activated hepatic stellate cells that express α -smooth muscle actin was also decreased in *Tak1/Tgfb2 Δ Hep* mice (Figure 3C and D). Expressions of fibrogenic genes, such as *Col1a1*, *Acta2*, *Timpl*, and *Tgfb1* were significantly reduced in *Tak1/Tgfb2 Δ Hep* mice compared with the *Tak1 Δ Hep* mice (Figure 3E). These findings demonstrate that TGF- β signaling in hepatocytes is required for the development of spontaneous liver fibrosis in *Tak1 Δ Hep* mice.

TGF- β Signaling Is Associated With Spontaneous Hepatocyte Apoptosis and Compensatory Proliferation in *Tak1 Δ Hep* Mice

Hepatocyte death and compensatory proliferation are key drivers for the initiation of HCC after exposure to carcinogen or chronic liver inflammation.^{17,18} According to the elevated serum ALT levels caused by the deletion of hepatocyte's *Tak1* (Figure 2B), spontaneous hepatocyte apoptosis was remarkably increased in *Tak1 Δ Hep* mice, which was suppressed in *Tak1/Tgfb2 Δ Hep* mice (Figure 4A and B). Decreased hepatocyte apoptosis in *Tak1/Tgfb2 Δ Hep* mice was also demonstrated by the lack of cleaved caspase 3 induction (Figure 4E). The increased pro-apoptotic gene, *Bax*, in *Tak1 Δ Hep* livers was decreased in *Tak1/Tgfb2 Δ Hep* livers, as measured by quantitative real-time polymerase chain reaction (Figure 4D). Because the compensatory hepatocyte proliferation in response to massive liver cell death is associated with spontaneous hepatocarcinogenesis in *Tak1 Δ Hep* mice,³ we investigated the regenerative responses of *Tak1/Tgfb2 Δ Hep* mice. Cyclin D1 ex-

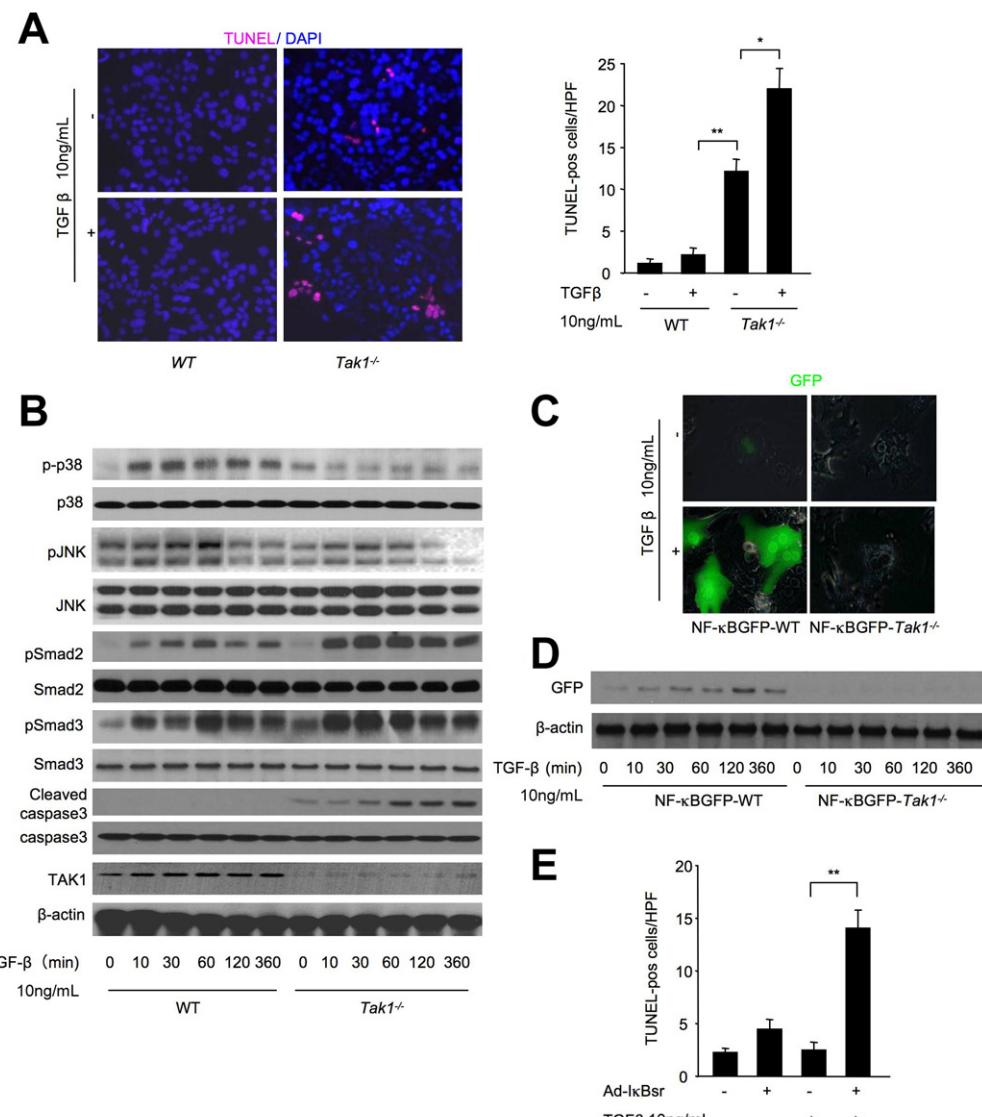


Figure 5. Ablation of *Tak1* in hepatocytes abolishes NF- κ B activation and increases susceptibility to TGF- β -mediated apoptosis. (A) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining (left) and its quantification (right) in the primary hepatocytes from WT and *Tak1* $^{\Delta Hep}$ mice after incubation with TGF- β 1 (10 ng/mL) for 24 h. Original magnification $\times 320$. (B) Phospho-p38, phospho-JNK, phospho-Smad2, phospho-Smad3, caspase 3, cleaved caspase 3, TAK1, and β -actin were determined by immunoblot analysis after primary hepatocytes from WT and *Tak1* $^{\Delta Hep}$ mice were incubated with TGF- β 1 (10 ng/mL) for the indicated time periods. (C, D) When stimulated with TGF- β (10 ng/mL) for 24 h, NF- κ B was activated in the primary hepatocytes from the NF- κ B-GFP reporter mice with *Tak1* sufficiency and deficiency, and their GFP expression was determined by microscopy (C) and Western blotting (D). Original magnification $\times 400$. (E) Adenoviral-IkB super repressor inhibited NF- κ B activation, which, as a result, caused apoptosis in primary hepatocytes from WT when stimulated with TGF- β (10 ng/mL) for 24 h. Apoptosis was determined with TUNEL staining. Data are reported as mean \pm standard error of mean. * P $<$.05; ** P $<$.01.

pression and proliferation cell nuclear antigen-positive cells were increased in *Tak1* $^{\Delta Hep}$ mice, but not in *Tak1*/*Tgfb2* $^{\Delta Hep}$ mice (Figure 4A, C, and E). These findings demonstrated that the loss of TGF- β receptor signaling in hepatocytes inhibits spontaneous hepatocyte death and its subsequent proliferation in *Tak1* $^{\Delta Hep}$ mice.

TAK1-NF- κ B Axis Protects Hepatocytes From Apoptosis Induced by TGF- β

We investigated whether TGF- β signaling participates in hepatocyte death under *Tak1*-deficient condition using primary culture hepatocytes. WT hepat-

cytes did not undergo apoptosis with or without TGF- β treatment. *Tak1* deficiency induced modest hepatocyte apoptosis, and TGF- β challenge dramatically increased apoptosis in *Tak1* $^{-/-}$ hepatocytes (Figure 5A). Consistently, TGF- β treatment induced cleavage of caspase 3 in *Tak1* $^{-/-}$ hepatocytes, but not in WT hepatocytes (Figure 5B). WT hepatocytes displayed phosphorylation of JNK and p38 induced by TGF- β treatment, but *Tak1* $^{-/-}$ hepatocytes did not. Interestingly, the signal intensity of phosphorylation of Smad2 and Smad3 was stronger in *Tak1* $^{-/-}$ hepatocytes than in WT hepatocytes (Figure 5B), suggesting that the TAK1-dependent pathway negatively regulates Smad2/3

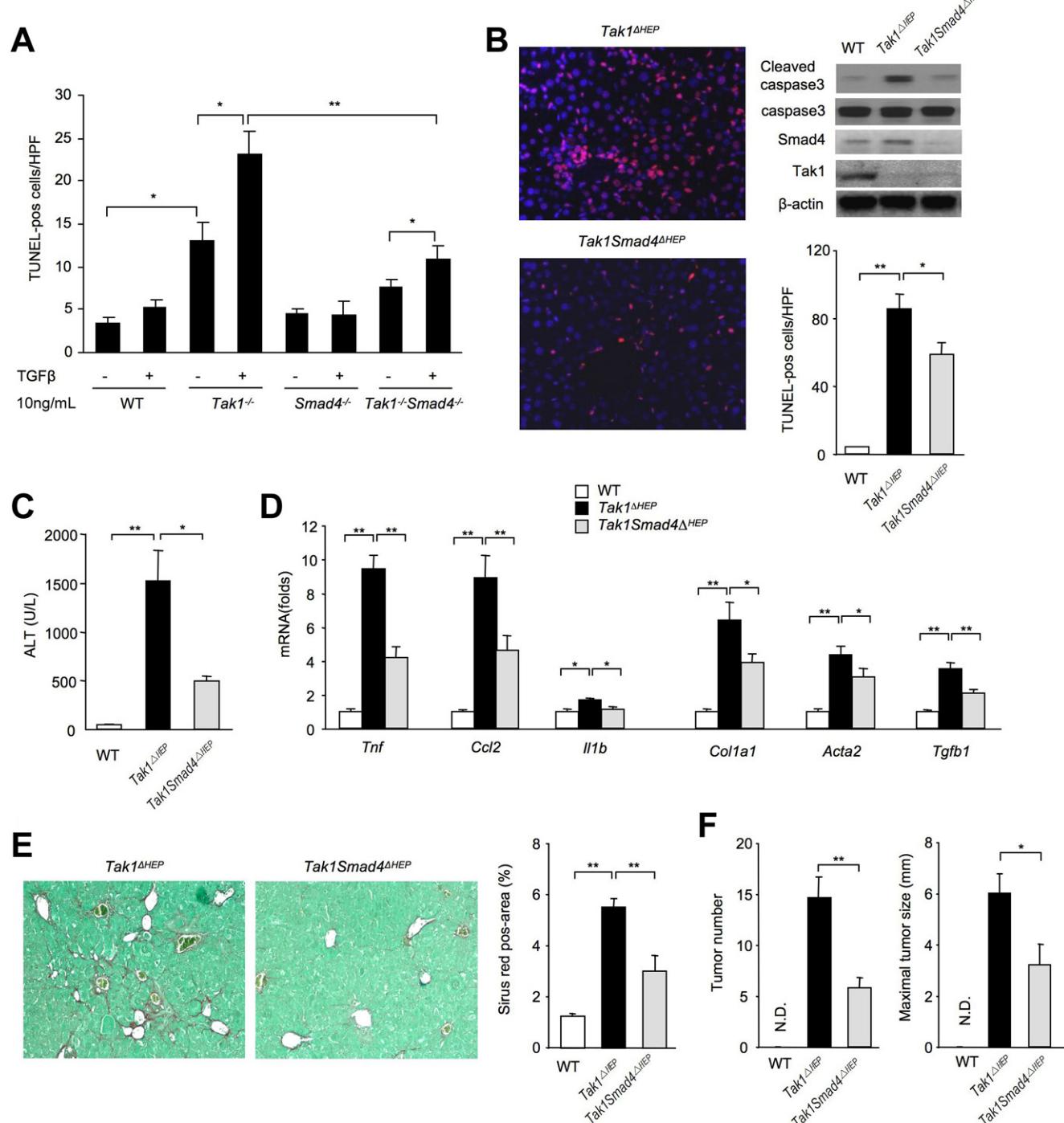


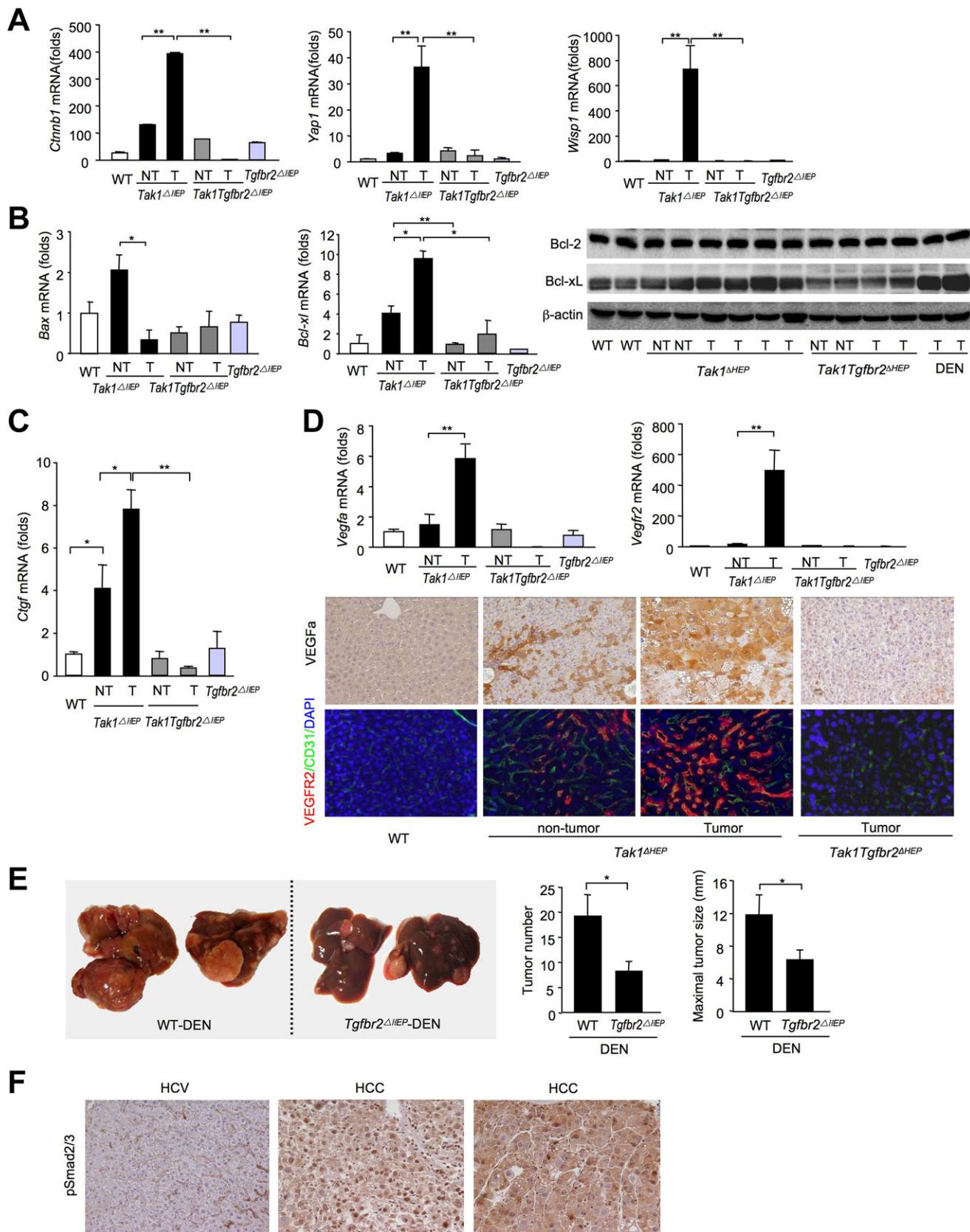
Figure 6. Inactivation of Smad molecules reduces spontaneous liver injury, inflammation, fibrosis, and HCC in *Tak1 Δ Hep* mice. (A) Apoptosis in the primary hepatocytes from WT, *Tak1 Δ Hep*, *Smad4 Δ Hep*, and *Tak1/Smad4 Δ Hep* mice after being treated with TGF- β (10 ng/mL) for 24 h were analyzed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining. The number of TUNEL-positive cells was counted. (B-E) WT, *Tak1 Δ Hep*, and *Tak1/Smad4 Δ Hep* mice at the age of 1 month ($n = 8$) were analyzed. (B) Apoptotic hepatocytes evaluated by TUNEL staining (left) and its quantification (lower right). Original magnification 200 \times . Immunoblots for cleaved caspase 3, caspase 3, Smad4, TAK1, and β -actin are shown (upper right). (C) Serum ALT levels. (D) Hepatic messenger RNA expression of inflammatory genes (*Tnf*, *Il1b*, and *Ccl2*) and fibrogenic genes (*Col1a1*, *Acta2*, and *Tgfb1*) determined by quantitative real-time polymerase chain reaction. (E) Fibrillar collagen deposition was determined by Sirius red staining (left) and its quantification (right). Original magnification $\times 100$. (F) The number of tumors per mouse was counted and the maximum diameter of individual tumor nodules was measured. (WT, $n = 10$; *Tak1 Δ Hep*, $n = 42$; *Tak1/Smad4 Δ Hep*, $n = 15$). Data are represented as mean \pm standard error of mean. * $P < .05$; ** $P < .01$.

activation and cell death signaling. To determine whether TGF- β signaling regulates NF- κ B activation, hepatocytes isolated from NF- κ B-green fluorescent protein (GFP) reporter mice were used. When the WT hepatocytes were

challenged with TGF- β , GFP reporter expression increased. In contrast, this was not observed in the hepatocytes isolated from *Tak1 $^{-/-}$* NF- κ B-GFP reporter mice (Figure 5C and D). To test whether NF- κ B-mediated sur-

vival signaling prevents TGF- β -mediated hepatocyte apoptosis, NF- κ B activation was blocked via infection with adenoviral vector expressing I κ B super-repressor, a potent inhibitor of NF- κ B. Upon inhibition of NF- κ B, TGF- β

treatment induced hepatocyte apoptosis (Figure 5E). Deletion of *Tak1* abolishes TGF- β -mediated NF- κ B activation and thereby increases susceptibility to TGF- β -mediated hepatocyte apoptosis.



Smad Signaling Is Required for TGF- β -Mediated Apoptosis in *Tak1*^{-/-} Hepatocytes

As shown in Figures 1D and 5B, Smad2 and Smad3 were overactivated in *Tak1*^{-/-} livers and in *Tak1*^{-/-} hepatocytes treated with TGF- β . This led us to investigate whether Smad activation contributes to TGF- β -mediated hepatocyte apoptosis. To inactivate Smad signaling in *Tak1*^{-/-} hepatocytes, we used primary hepatocytes isolated from *Tak1*/*Smad4*^{ΔHep} mice. Although TGF- β treatment increased the number of apoptosis in both *Tak1*^{-/-} and *Tak1*/*Smad4* double-knockout hepatocytes in comparison with the nontreatment group, apoptosis was suppressed in *Tak1*/*Smad4* double-knockout hepatocytes compared with *Tak1*^{-/-} hepatocytes (Figure 6A), indicating that Smad activation is required for the induction of apoptosis in *Tak1*^{-/-} hepatocytes mediated by TGF- β . We further confirmed the requirement of Smad pathways by silencing *Smad2* using small interfering RNA in primary hepatocytes; TGF- β -induced apoptosis in *Tak1*^{-/-} hepatocytes was suppressed in *Smad2*-silenced hepatocytes (Supplementary Figure 2).

We then investigated the in vivo roles of Smad pathway for the liver pathology in *Tak1*^{ΔHep} mice by using *Tak1*/*Smad4*^{ΔHep} mice. Spontaneous hepatocytes apoptosis was increased in 1-month-old *Tak1*^{ΔHep} mice, which was suppressed in *Tak1*/*Smad4*^{ΔHep} mice as assessed by counting the number of apoptotic terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive hepatocytes (Figure 6B). Consistent with the results from TUNEL staining, serum ALT levels in *Tak1*/*Smad4*^{ΔHep} mice were significantly lower than those in *Tak1*^{ΔHep} mice (Figure 6C). Expression of inflammatory and fibrogenic genes, as well as fibrillar collagen deposition, were significantly suppressed by *Smad4* deletion in 1-month-old *Tak1*^{ΔHep} mice (Figure 6D, E). Notably, HCC formation in *Tak1*/*Smad4*^{ΔHep} mice was significantly reduced compared with *Tak1*^{ΔHep} mice at 9 months of age (Figure 6F). These results indicate that Smad activation in hepatocytes is crucial for spontaneous liver injury, inflammation, fibrosis, and tumorigenesis in *Tak1*^{ΔHep} mice.

Anti-Apoptotic and Pro-Oncogenic Gene Expression and Angiogenesis in HCC From *Tak1*^{ΔHep} Mice are TGF- β Signaling-Dependent

To explore additional TGF- β -dependent mechanisms that drive hepatocarcinogenesis, we further ana-

lyzed HCC tissues in 9-month-old *Tak1*^{ΔHep} mice. We investigated pro-oncogene and anti-apoptotic gene expression in HCC from *Tak1*^{ΔHep} mice. Although expressions of pro-oncogenes, such as *Ctnnb1*, *Yap1*, and *Wisp1*, were significantly increased in tumors from *Tak1*^{ΔHep} mice, these expressions were decreased in tumors from *Tak1*/*Tgfb2*^{ΔHep} mice (Figure 7A). Expression of anti-apoptotic *Bcl-2* was not affected in the tumors from *Tak1*^{ΔHep} or *Tak1*/*Tgfb2*^{ΔHep} mice. Notably, anti-apoptotic *Bcl-xL* increased significantly, and pro-apoptotic *Bax* decreased in the tumors from *Tak1*^{ΔHep} mice compared with WT normal livers and nontumor liver tissues from *Tak1*^{ΔHep} mice; however, these changes were not seen in *Tak1*/*Tgfb2*^{ΔHep} mice (Figure 7B). Because CTGF is known to promote growth and migration of HCC, *Ctgf* messenger RNA expression was assessed.^{9,12,13,19} Although tumors from *Tak1*^{ΔHep} mice exhibited increased *Ctgf* messenger RNA expression compared with nontumor tissues, tumors from *Tak1*/*Tgfb2*^{ΔHep} mice did not (Figure 7C). Due to the pivotal role of angiogenesis in the pathogenesis of HCC, we examined the expression of VEGFa, a potent proangiogenic factor, and its receptor, VEGFR2, in the tumors of *Tak1*^{ΔHep} mice and *Tak1*/*Tgfb2*^{ΔHep} mice. VEGFa was overexpressed in tumors from *Tak1*^{ΔHep} mice, but not from *Tak1*/*Tgfb2*^{ΔHep} mice (Figure 7D). VEGFR2-expressing endothelial cells were also increased in tumors from *Tak1*^{ΔHep} mice compared with tumors from *Tak1*/*Tgfb2*^{ΔHep} mice (Figure 7D). These results suggest that expression of *Bcl-xL*, CTGF, and VEGF-mediated angiogenesis are TGF- β -dependent in the development of HCC in *Tak1*^{ΔHep} mice.

TGF- β Signaling in a Mouse Model for Chemically Induced HCC and in Human HCC

To examine whether TGF- β signaling is a general promoter for HCC growth in vivo, we assessed DEN-induced HCC mouse model in WT mice and *Tgfb2*^{ΔHep} mice. Neonatal DEN treatment induced multiple, large-sized HCC in WT mice at 9 months after DEN injection. In contrast, inactivation of TGF- β signaling by deletion of *Tgfb2* reduced HCC formation induced by DEN (Figure 7E). Consistently, serum ALT levels were higher in WT mice than in *Tgfb2*^{ΔHep} mice after DEN treatment (Supplementary Figure 3). Analogous in pattern to HCC from *Tak1*^{ΔHep} and *Tgfb2*^{ΔHep} mice, HCC from DEN-treated WT mice had increased expressions of pro-oncogenes (*Ctnnb1*, *Yap1*, and *Wisp1*), *Bcl-xL*, and *Ctgf*,

Figure 7. Oncogenic gene expression in HCC from *Tak1*^{ΔHep} mice, and TGF- β signaling in DEN-induced murine HCC and in human HCC. (A–D) Liver tissues in 9-month-old WT and *Tgfb2*^{ΔHep} mice, and nontumor liver tissues and tumor tissues in 9-month-old *Tak1*^{ΔHep}, *Tak1*/*Tgfb2*^{ΔHep} were harvested. (n=7, each samples) (A) Hepatic messenger RNA expression of pro-oncogenes (*Ctnnb1*, *Myc*, *Yap1*, and *Wisp1*) was determined by quantitative real-time polymerase chain reaction (qPCR). (B) Hepatic messenger RNA expression of *Bax* and *Bcl-xL* was assessed by qPCR, and protein expression of *Bcl-2* and *Bcl-xL* are shown by immunoblotting. (C, D) Hepatic messenger RNA expressions of *Ctgf* (C), *Vegfa*, and *Vegfr2* (D, upper) were assessed by qPCR. (D, lower) Immunostaining for VEGFa (upper), VEGFR2, and CD31 (lower) are shown. Original magnification 320 \times . (E) Diethylnitrosamine was injected (25 mg/kg) in 14-day-old WT and *Tgfb2*^{ΔHep} mice and their livers were harvested at 9 months after DEN injections. Representative macroscopic pictures (left). The number of tumors per mouse was counted and the maximum diameter of individual tumor nodules was measured (right). (WT, n = 16; *Tgfb2*^{ΔHep}, n = 16). NT, nontumor liver; T, tumors. (F) Immunohistochemistry for phospho-Smad2/3 in liver biopsy samples from patients with chronic hepatitis C (n = 4) and in liver tissues from patients with HCC (n = 4). Data are represented as mean \pm standard error of mean. *P < .05; **P < .01.

and these expressions were suppressed in HCC from *Tgfb2^{ΔHep}* mice treated with DEN (Supplementary Figure 4A–D). VEGFa expression and angiogenesis were increased in DEN-induced HCC from WT mice, but not in HCC from *Tgfb2^{ΔHep}* mice (Supplementary Figure 4E). These results indicate that TGF-β is a promoter for DEN-induced HCC. Finally, we assessed activation of TGF-β signaling by comparing liver biopsy specimens of patients with chronic hepatitis C with hepatic fibrosis (hepatitis C virus–non-HCC controls) with patients with chronic hepatitis C infection and HCC (hepatitis C virus–HCC cases) with immunohistochemistry for phosphorylation of Smad2/3. In liver tissues from patients with chronic hepatitis C-induced fibrosis, phosphorylation of Smad2/3 was seen only in nonparenchymal cells and not in liver parenchymal cells. As we predicted, in patients with chronic hepatitis C and HCC, we found increased phosphorylation and nuclear translocation of Smad2/3 (Figure 7F), providing clinical relevance of activation of TGF-β signaling in human HCC.

Discussion

TAK1 is a mitogen-activated protein kinase kinase kinase that is activated in the signaling cascade of IL-1β, Toll-like receptors, TNF, and TGF-β. TAK1 requires binding to TAB2 and TAB3 for activation of the downstream kinases JNK, p38, and IKK.^{5,6} These kinases further activate transcription factors AP-1 and NF-κB. The IKK-NF-κB pathway is anti-apoptotic and anti-oncogenic, whereas the JNK signaling is pro-apoptotic and pro-carcinogenic.⁷ Because TAK1 regulates both IKK-NF-κB and JNK pathways, the functions of TAK1 in hepatocyte death and carcinogenesis were uncertain. We and others have developed a genetically engineered mouse model that specifically knocked out *Tak1* gene in the liver.^{3,4} These mice spontaneously develop HCC accompanied by liver inflammation and fibrosis, which best mimics the progression of human HCC; therefore, *Tak1^{ΔHep}* mice serve as an excellent mouse model to analyze the mechanisms of hepatocarcinogenesis, especially for the link between inflammation, fibrosis, and carcinogenesis.

TGF-β signaling regulates cell proliferation, apoptosis, migration, angiogenesis, immunity, fibrosis, and cancer development.^{8,10,20} TGF-β binds to the receptor subunit TGF-β receptor 2, and then activates TGF-β receptor 1. Intracellular domain of TGF-β receptor 1 phosphorylates Smad2 and Smad3, and forms a complex with Smad4 that translocates into the nucleus to regulate gene transcription. TGF-β signaling also activates the Smad-independent, TAK1-dependent pathway that activates JNK and p38.^{8,20} In *Tak1^{ΔHep}* mice, increased production of TGF-β and overactivation of Smad2 and Smad3 were observed, indicating that the TGF-β receptor signaling is activated in *Tak1^{ΔHep}* livers. Due to the lack of TAK1, TGF-β-induced JNK and p38 phosphorylation were impaired. We demonstrate that TGF-β stimulation induced NF-κB ac-

tivation in WT hepatocytes, and inhibition of NF-κB in TGF-β-stimulated hepatocytes caused hepatocyte apoptosis (Figure 5C and E). In contrast, induction in hepatic apoptosis was not seen with inhibition of JNK or p38 in TGF-β-stimulated hepatocytes (Supplementary Figure 5). Because *Tak1^{-/-}* hepatocytes are more susceptible to TGF-β-mediated cell death, the TAK1-NF-κB pathway is an essential survival signal in the TGF-β signaling. *Tak1^{-/-}* deficiency augmented TGF-β-mediated Smad2/3 phosphorylation. This suggests that the TAK1-NF-κB pathway can negatively regulate Smad2/3 activation, and overactivation of Smad2/3 is associated with TGF-β-mediated cell death in *Tak1^{-/-}* hepatocytes. In fact, inactivation of Smad pathway attenuated TGF-β-mediated cell death in *Tak1^{-/-}* hepatocytes (Figure 6A, Supplementary Figure 2). The importance of Smad signaling in TGF-β-mediated hepatocyte apoptosis was also confirmed by decreased hepatocyte apoptosis in *Tak1/Smad4^{ΔHep}* mice (Figure 6B).

To determine the role of TGF-β-Smad signaling in hepatocytes for the regulation of spontaneous liver inflammation, fibrosis, and carcinogenesis in *Tak1^{ΔHep}* mice, we have generated hepatocyte-specific *Tak1* and *Tgfb2* double-knockout (*Tak1/Tgfb2^{ΔHep}*) mice, and *Tak1/Smad4^{ΔHep}* mice. The additional deletion of hepatocyte's TGF-β-Smad signaling in *Tak1^{ΔHep}* mice resulted in decreased spontaneous carcinogenesis, fibrosis, inflammation, and hepatocyte apoptosis. This implicates that TGF-β-Smad signaling in hepatocytes promotes liver fibrosis and formation of liver tumors that spontaneously develop in the setting of TAK1 inactivation (Figures 1–4). Notably, the suppression of liver pathology in *Tak1/Smad4^{ΔHep}* mice was less than that in *Tak1/Tgfb2^{ΔHep}* mice. This suggests that, in addition to Smad, TGF-β-mediated unknown pathways, such as phosphatidylinositol 3 kinase or RhoA/ROCK pathway, are involved in TGF-β-mediated liver phenotype in *Tak1^{ΔHep}* mice.

Because spontaneous hepatocyte death and carcinogenesis in *Tak1^{ΔHep}* mice were reduced in *Tak1/Tgfb2^{ΔHep}* and *Tak1/Smad4^{ΔHep}* mice, we concluded that TGF-β-Smad-mediated hepatocyte injury and compensatory proliferation of the surviving hepatocytes are the probable cause of spontaneous HCC development in *Tak1^{ΔHep}* mice. Particularly, HCC from 9-month-old *Tak1^{ΔHep}* mice overexpressed anti-apoptotic Bcl-xL, but not HCC from *Tak1/Tgfb2^{ΔHep}* mice. This suggests that hepatocytes lacking *Tak1* become resistance to TGF-β-mediated apoptosis when they transform to HCC. We also characterized the oncogenic properties of HCC developed in *Tak1^{ΔHep}* mice; the HCC of *Tak1^{ΔHep}* mice highly expressed pro-oncogene β-catenin (Figure 7). Yes-associated protein has been identified as an oncoprotein that is regulated by the Hippo signaling pathway.²¹ Yes-associated protein is overexpressed in lung, ovarian, colon, prostate, and liver cancer, and is associated with the prognosis for HCC patients.^{22–24} Our data demonstrated that Yes-associated protein 1 is highly expressed in HCC from *Tak1^{ΔHep}* mice (Figure 7). Wnt signaling downstream target, WISP1, is a

member of the CTGF family, and is associated with HCC development.²⁵ Although WISP1 expression was very high in tumors from *Tak1*^{ΔHep} mice, expression of this oncogene was significantly suppressed in tumors from *Tak1/Tgfbr2*^{ΔHep} mice, suggesting that TGF- β signaling regulates WISP1 expression in HCC. TGF- β signaling induces CTGF production in hepatocytes, which is associated with fibrosis, and HCC growth, and migration.^{19,26} Hepatocytes and HCC of *Tak1*^{ΔHep} mice express CTGF through TGF- β signaling. It is postulated that, in combination with TGF- β , CTGF promotes HCC growth and liver fibrosis in *Tak1*^{ΔHep} mice. Consistent with previous reports,^{27,28} TGF- β induced production of VEGF in hepatocytes that augmented angiogenesis in tumors and assisted tumor growth in *Tak1*^{ΔHep} mice.

There exist a number of studies demonstrating that TGF- β signaling is a tumor suppressor.⁹ Human gastric, colon, and pancreatic cancer express mutant forms of TGF- β signaling components that inhibit activity of TGF- β signaling.²⁹ Mice with inactivation of TGF β R2 by the ectopic expression of soluble TGF β R2, or haploinsufficiency for *Tgfbr2* or *elf*, a signaling molecule essential for TGF- β signaling, augmented HCC development.^{12,13,30} Tumor suppressive effect of TGF- β signaling can be mediated by increasing tumor-suppressing genes (eg, p15, p21) and inducing apoptosis of cancer cells.⁹ In contrast, sustained elevation of TGF- β is considered to promote malignancies and metastases in glioma cells and breast cancer.^{31–33} TGF- β signaling induces cell migration of HCC cells through induction of epithelial to mesenchymal transition, and expressions of anti-apoptotic genes and CTGF to promote HCC growth.^{19,34,35} Interestingly, additional deletion of *Tgfbr2* in *p53*^{ΔHep} mice inhibits spontaneous development of HCC.³⁶ A recent publication demonstrated that TGF- β signaling is required for liver progenitor cell-associated hepatocarcinogenesis, which corroborates our results.³⁷ These findings serve as additional evidence that TGF- β signaling promotes HCC formation in vivo. To address whether TGF- β signaling promotes HCC only in the specific genetic conditions, we provided additional evidence that *Tgfbr2* ablation in hepatocytes inhibits chemically induced HCC (Figure 7E). In addition to previous reports,^{38–41} our data demonstrated that TGF- β signaling is activated in human HCC as increased phosphorylation and nuclear translocation of Smad2/3 (Figure 7F), which provided evidence in the activation of TGF- β signaling in human HCC. It is postulated that function of TGF- β signaling as a tumor promoter or a tumor suppressor is dependent on cell types, tissues, organs, and stage of cancer. Our data clearly demonstrate that TGF- β signaling in hepatocytes contributes to tumor promotion.

Supplementary Materials

Note: To access the supplementary material accompanying this article, visit the online version of

Gastroenterology at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2013.01.056>.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Mouse Tissue Processing

Mouse tissues for RNA and protein preparations were snap-frozen with liquid nitrogen. Mouse tissues for histology were fixed in 10% or 4% neutral buffered formalin phosphate (Fisher Scientific, Pittsburgh, PA) and were subsequently embedded in paraffin or OCT compound, respectively, and sliced into 5- μ m sections for staining such as Sirius red staining, H&E, staining, and immunohistochemistry.

Histologic Examination

For immunostaining, liver sections were incubated with primary antibodies such as mouse anti- α -smooth muscle actin monoclonal antibody (DakoCytomation, Glostrup, Denmark), mouse anti-PCNA monoclonal antibody (Biologen, San Diego, CA), goat anti-mouse VEGFR2/Flk-1 affinity purified polyclonal antibody (R&D Systems, Minneapolis, MN), rabbit anti-VEGFa, rabbit anti-phospho-Smad2/3, rabbit anti-TGF β R2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and rat anti-mouse CD31 antibody (BD Bioscience Pharmingen, San Jose, CA). H&E, TUNEL, and Sirius red staining were performed as described previously.¹ TUNEL and PCNA-positive cells were counted on 10 fields of 100 \times and 200 \times magnification per slide, respectively. Sirius red-positive area was measured for 10 low-power (100 \times) fields per slide and quantified using National Institutes of Health imaging software.

Western Blot Analysis

Protein extracts were electrophoresed, blotted, and then incubated with antibodies for caspase 3, cleaved caspase 3, phospho-JNK, phospho-p38, TAK1, phospho-Smad2/Smad3, Smad2, Smad3, Smad4, Bcl-xL, Bcl-2 (Cell Signaling, Danvers, MA), β -actin (Sigma), cyclin D1, JNK,

and p38 (Santa Cruz Biotechnology) with appropriate secondary horseradish peroxidase-conjugated antibodies and developed.

Quantitative Real-Time Polymerase Chain Reaction Analysis

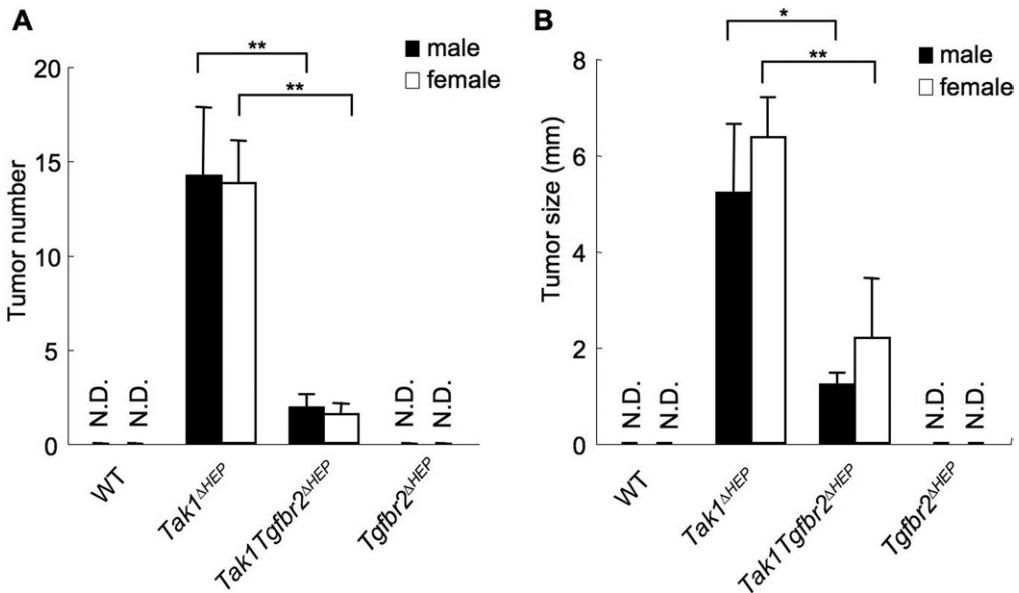
RNA extracted from liver was subjected to reverse transcription and subsequent polymerase chain reaction using a CFX96 real-time polymerase chain reaction system (Bio-Rad, Hercules, CA). Polymerase chain reaction primer sequences are listed in Supplementary Table 1. The expression of respective genes was normalized to 18S ribosomal RNA as an internal control.

Cell Isolation and Treatment

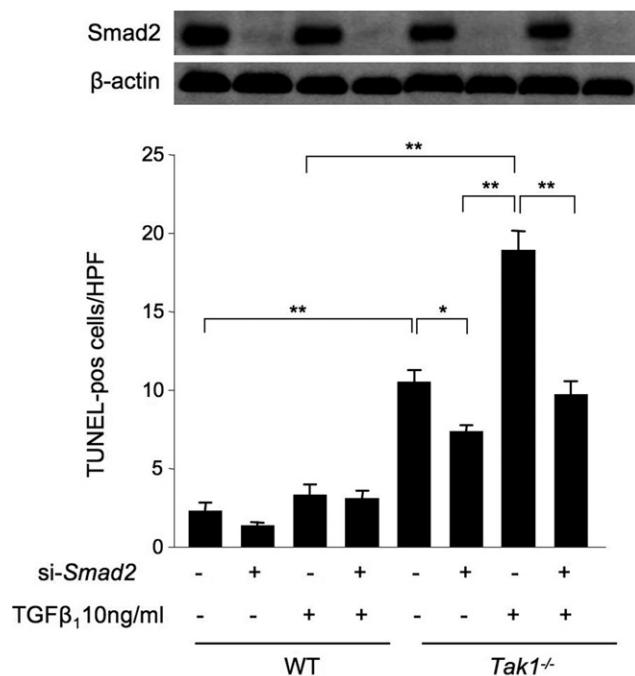
Hepatocytes were isolated from WT, *Tak1*^{ΔHEP}, *Smad4*^{ΔHEP}, and *Tak1/Smad4*^{ΔHep} mice. After cell attachment, hepatocytes were serum-starved for 14 h, and were then treated with 10 ng/mL murine TGF- β 1 (R&D Systems) for the indicated time periods. Apoptosis was examined using TUNEL staining. Recombinant adenovirus I κ Bsr and cells isolated from NF- κ B reporter GFP transgenic mice were used in some experiments.^{2,3} Small interfering RNA for mouse *Smad2* (sc-38375) was purchased from Santa Cruz Biotechnology.

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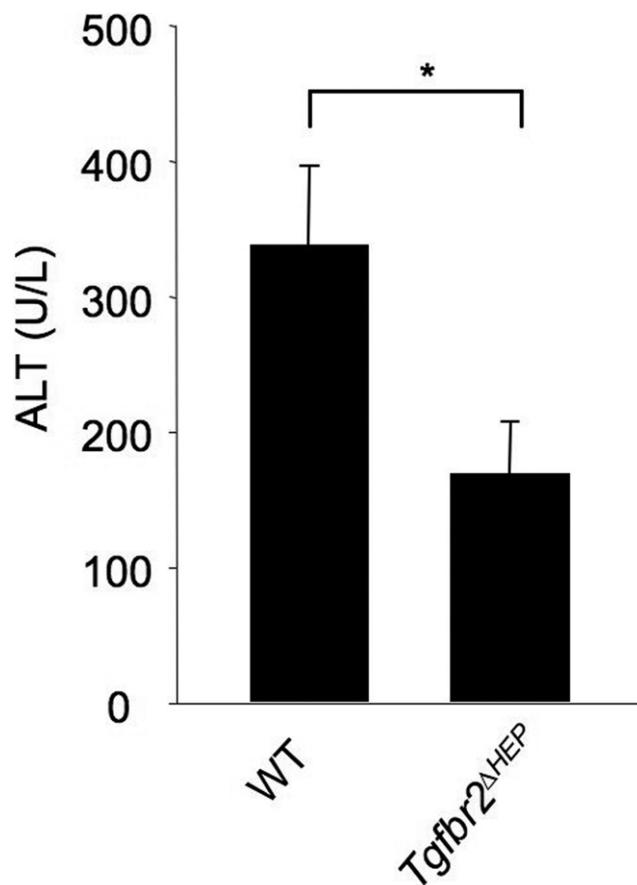
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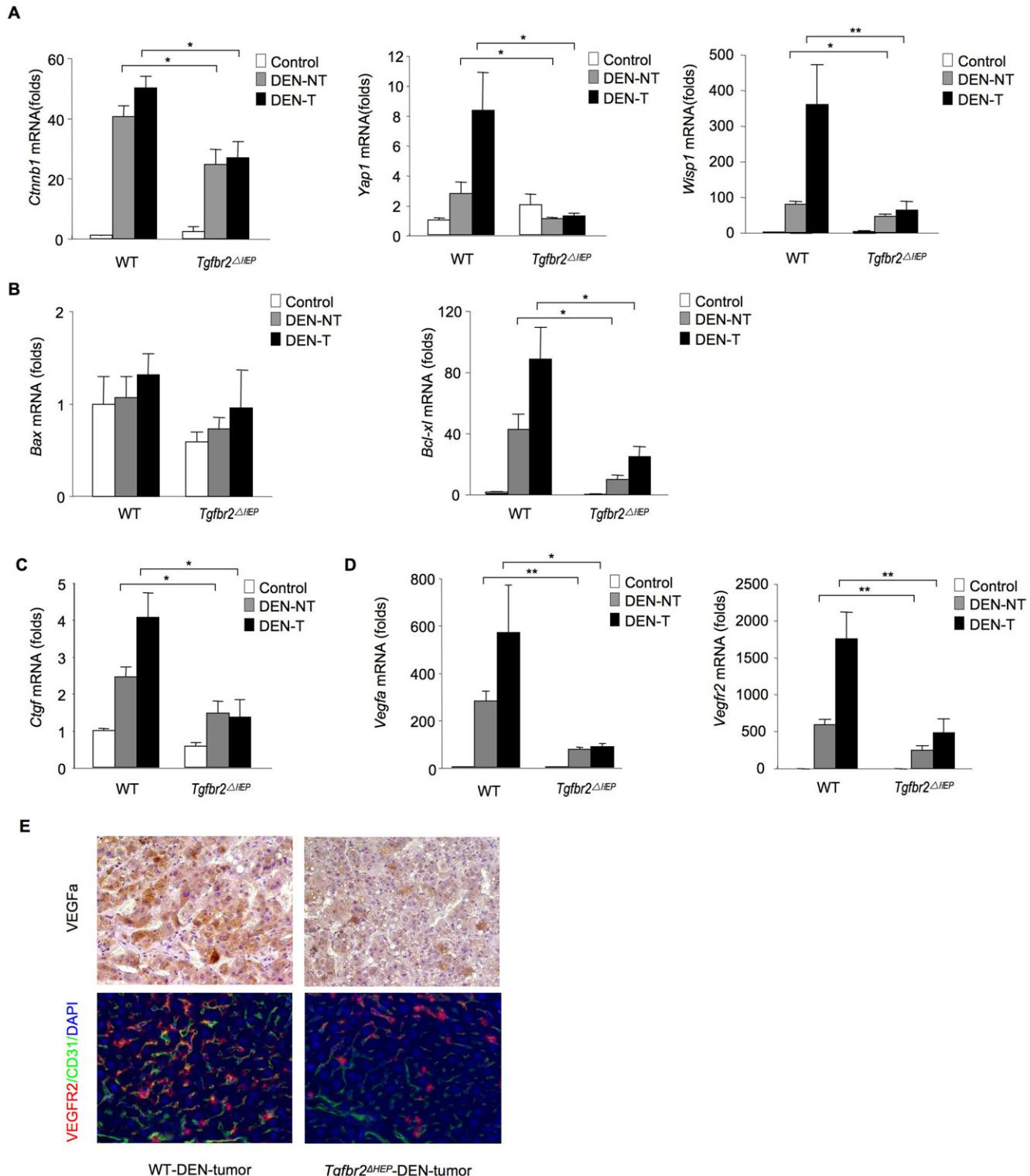
Supplementary Figure 1. No sex disparity in *Tak1^{ΔHep}* and *Tak1/Tgfb2^{ΔHep}* mice with respect to hepatocarcinogenesis. Male and female WT, *Tak1^{ΔHep}*, *Tak1/Tgfb2^{ΔHep}*, and *Tgfb2^{ΔHep}* mice WT and *Tak1^{ΔHep}* mice were analyzed at 9 months of age (male WT, n = 5; female WT, n = 5; male *Tak1^{ΔHep}*, n = 17; female *Tak1^{ΔHep}*, n = 25; male *Tak1/Tgfb2^{ΔHep}*, n = 14; female *Tak1/Tgfb2^{ΔHep}*, n = 21; male *Tgfb2^{ΔHep}*, n = 5; and female *Tgfb2^{ΔHep}*, n = 5). (A) The number of tumors per mouse was examined. (B) The maximum diameter of individual tumor nodules is presented. Data are presented as mean ± standard error of mean. *P < .05; **P < .01.



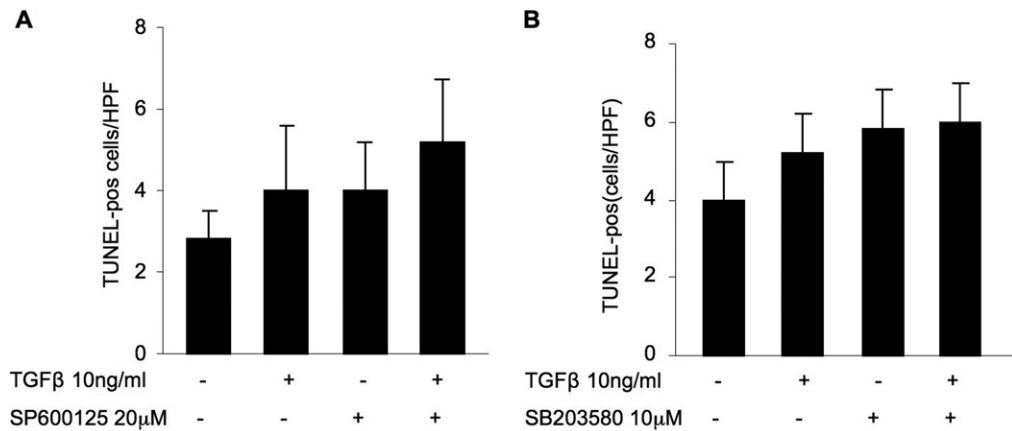
Supplementary Figure 2. Silencing *Smad2* reduces apoptosis in *Tak1^{-/-}* hepatocytes. *Smad2* and β-actin were determined by immunoblot analysis in WT and *Tak1^{-/-}* primary hepatocytes transfected with small interfering RNA (siRNA) for *Smad2*. Apoptosis were analyzed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining after transfection with siRNA for *Smad2* and then stimulation with TGF-β1 (10 ng/mL) for 24 h. Data are presented as mean ± standard error of mean. *P < .05; **P < .01.



Supplementary Figure 3. Liver damage is suppressed in *Tgfb2^{ΔHep}* mice at 9 months after DEN injection. Diethylnitrosamine was injected (25 mg/kg) in 14-day-old WT and *Tgfb2^{ΔHep}* mice and serum were sampled at 9 months after DEN injection. Serum ALT levels were measured in WT (n = 11), and *Tgfb2^{ΔHep}* mice (n = 10). Data are represented as mean ± standard error of mean. *P < .05.



Supplementary Figure 4. TGF- β signaling mediates pro-oncogene and anti-apoptotic gene expression and angiogenesis in DEN-induced HCC. (A–E) Diethylnitrosamine was injected (25 mg/kg) in 14-day-old WT and $Tgfb2^{\Delta HE}$ mice, and livers were harvested at 9 months after DEN injection. (WT, n = 11; $Tgfb2^{\Delta HE}$, n = 10). (A) Hepatic messenger RNA (mRNA) expression of pro-oncogenes (*Ctnnb1*, *Yap1*, and *Wisp1*) was determined by quantitative real-time polymerase chain reaction (qPCR). (B) Hepatic mRNA expression of *Bax* and *Bcl-xL* was assessed by qPCR. (C, D) Hepatic mRNA expressions of *Ctgf* (C), *Vegfa*, and *Vegr2* (D) were assessed by qPCR. (E) Immunostaining for VEGFa (upper), VEGFR2, and CD31 (lower) are shown. Original magnification used $\times 320$. NT, nontumor liver; T, tumors. Data are presented as mean \pm standard error of mean. *P < .05; **P < .01.



Supplementary Figure 5. No significant impact of cell death with inactivation of JNK and p38. (A) WT hepatocytes were pretreated with 20 μ M SP600125 (JNK inhibitor) or (B) 10 μ M SB203580 (p38 inhibitor). After stimulation with TGF- β (10 ng/mL) for 24 h, apoptotic hepatocytes were analyzed by counting cells positive for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining.

Supplementary Table 1. Sequence of Primers Used for Real-Time Quantitative Polymerase Chain Reaction

Gene	Forward	Reverse
18S	AGTCCCTGCCCTTGACACA	CGATCCGAGGGCTCACTA
Tnf	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTCTGCTAC
Il1b	GGTCAAAGGTTGGAAGCAG	TGTGAAATGCCACCTTTGA
Il6	ACCAGAGGAATTTCATAGGC	TGATGCACTTGAGAAAACA
Ccl2	ATTGGGATCATCTGCTGGT	CTGCTGTTACAGTGCC
Col1a1	TAGGCCATTGTTATGCAGC	ACATGTTCAGCTTGTGGACC
Acta2	GTTCAGTGGTGCCTCTGTCA	ACTGGGACGACATGGAAAAG
Tgfb1	GTGGAATCAACGGGATCAG	ACTTCCAACCCAGGTCTTC
Timp1	AGGTGGTCTCGTTGATTCT	GTAAGGCCTGTAGCTGTGCC
Tgfb1r1	AGACCATCTGTCACAGGTAAAA	CTCCTCATCGTGTGGTGG
Tgfb2r1	CTGGCCATGACATCACTGTT	GTCGGATGTGAAATGGAAG
Tgfb2r2		TTGCTGATGGCAACTTCAAC
Bax	GATCAGCTGGGACTTTAG	GTTGGATGGCCACCTATCTG
Bcl-xl	GCTGCATTGTCCTCGTAGAG	GAGCCGTCAGTGCAGGAG
Ctnnb1	CAGCTTGAGTAGCCATTGTCC	CCTGATGATGTACCACTGCC
Yap1	GCCATGTTGTTGTCGATCG	GTGGCAGTCCTGAGGGTG
Wisp1	GGCGTGTAGTCGTTCTCT	