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Hepatocyte Mitogen-Activated Protein Kinase Kinase 7 Contributes to Restoration of the Liver Parenchyma Following Injury in Mice

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BACKGROUND AND AIMS: Mitogen-activated protein kinase kinase (MKK) 7 and MKK4 are upstream activators of c-Jun NH_2-terminal kinases (JNKs) and have been shown to be required for the early development of the liver. Although it has been suggested that MKK7 might be involved in the regulation of hepatocyte proliferation, the functional role of MKK7 in the liver has remained unclear.

APPROACH AND RESULTS: Here, we examined phenotypic alterations in liver-specific or hepatocyte/hematopoietic cell–specific MKK7 knockout (KO) mice, which were generated by crossing $MKK7^{LoxP/LoxP}$ with albumin–cyclization recombination (Alb-Cre) or myxovirus resistance protein 1–Cre mice, respectively. The livers of Alb-$Cre^{-/+}$ $MKK7^{LoxP/LoxP}$ mice developed without discernible tissue disorganization. MKK7 KO mice responded normally to liver injuries incurred by partial hepatectomy or injection of CCl_4. However, tissue repair following CCl_4-induced injury was delayed in MKK7 KO mice compared with that of control mice. Furthermore, after repeated injections of CCl_4 for 8 weeks, the liver in MKK7 KO mice showed intense fibrosis with increased protractive hepatocyte proliferation, suggesting that MKK7 deficiency might affect regenerative responses of hepatocytes in the altered tissue microenvironment. MKK7 KO hepatocytes demonstrated normal proliferative activity when cultured in monolayers. However, MKK7 KO significantly suppressed branching morphogenesis of hepatocyte aggregates within a collagen gel matrix. Microarray analyses revealed that suppression of branching morphogenesis in MKK7 KO hepatocytes was associated with a reduction in mRNA expression of transgelin, glioma pathogenesis related 2, and plasminogen activator urokinase-type (*Plau*); and forced expression of these genes in MKK7 KO hepatocytes partially recovered the attenuated morphogenesis. Furthermore, hepatocyte-specific overexpression of *Plau* rescued the impaired tissue repair of MKK7 KO mice following CCl_4-induced injury.

CONCLUSIONS: MKK7 is dispensable for the regenerative proliferation of hepatocytes but plays important roles in repair processes following parenchymal destruction, possibly through modulation of hepatocyte–extracellular matrix interactions. (HEPATOLOGY 2021;73:2510-2526).

The c-Jun NH_2-terminal kinases (JNKs) are known to be activated in response to environmental stresses and to phosphorylate their substrate c-Jun.[1] There are three members of the JNK family, JNK1, JNK2, and JNK3, which are encoded

Abbreviations: AAV, adeno-associated virus; AcGFP, green fluorescent protein derived from Aequorea coerulescens; Alb, albumin; ALT, alanine aminotransferase; CAG, cytomegalovirus enhancer/chicken β-actin promoter; cDNA, complementary DNA; Cre, cyclization recombination; E, embryonic day; EGF, epidermal growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; Glipr2, glioma pathogenesis related 2; Gluc, Gaussia princeps luciferase; HE, hematoxylin and eosin; HGF, hepatocyte growth factor; HH, hepatocyte/hematopoietic cell–specific; IRES, internal ribosome entry site; JNK, c-Jun NH2-terminal kinase; KO, knockout; Krt19, keratin 19; L, liver-specific; Mki67, marker of proliferation Ki-67; MKK, mitogen-activated protein kinase kinase; Mx1, myxovirus resistance 1; PH, partial hepatectomy; Plat, tPA gene; Plau, uPA gene; α-SMA, α-smooth muscle actin; TAA, thioacetamide; Tagln, transgelin; TGF, transforming growth factor; TNF, tumor necrosis factor; tPA, plasminogen activator tissue-type; uPA, plasminogen activator urokinase-type; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element.

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by distinct genes; JNK1 and JNK2 are expressed in many types of cells, including hepatocytes and bile duct cells, while the expression of JNK3 is restricted to the brain, heart, and testis.[1] Activation of JNK requires dual phosphorylation of Tyr and Thr residues, which is accomplished by the dual-specificity kinases mitogen-activated protein kinase kinase 7 (MKK7) and MKK4.[2] Studies have shown that MKK7 preferentially phosphorylates the Thr residues of JNKs, whereas MKK4 phosphorylates the Tyr residues.[3] Homozygous null mutations of either MKK4 or MKK7 in mice have been demonstrated to produce embryonic lethality on embryonic day 10.5 (E10.5) or E11.5, respectively; the lethal phenotype was associated with severely disorganized livers with reduced numbers of hepatoblasts, indicating that both MKK4 and MKK7 are indispensable for the early development of the liver bud.[4-7]

The physiological and pathological roles of MKK4 and MKK7 in the adult liver have remained obscure. Wuestefeld et al. used an *in vivo* RNA interference screening method and reported that MKK4 knockdown in adult hepatocytes promotes liver regeneration following various liver injuries and that the effect is mediated by activation of MKK7.[8] Although their findings raised an intriguing possibility that MKK7 could be a critical regulator of hepatocyte proliferation, the exact roles of MKK7 and its apparent reciprocal interactions with MKK4 in the adult liver have yet to be elucidated.

In this study, to investigate the roles of hepatocyte MKK7, we examined the effect of hepatocyte/hematopoietic cell–specific MKK7 knockout (KO) (hereafter referred to as MKK7 [HH] KO) or liver (hepatocytes and intrahepatic bile ducts)–specific MKK7 KO (hereafter referred to as MKK7 [L] KO) on compensatory liver regeneration following partial hepatectomy (PH) and regeneration and repair processes following CCl_4-induced tissue destruction. We found that MKK7 KO did not affect hepatocyte proliferation, although the lack of MKK7 led to partial suppression of the JNK–c-Jun pathway. However, MKK7 KO delayed the repair process in the liver following CCl_4 injury and enhanced liver fibrosis after repeated injuries. Furthermore, we found that hepatocytes from MKK7 KO mice demonstrated diminished branching morphogenesis within a collagen gel matrix, and this phenotype was associated with decreased mRNA expression of the genes encoding transgelin (*Tagln*), glioma pathogenesis related-2 (*Glipr2*), and plasminogen activator urokinase-type (uPA; *Plau*), which are known to be involved in cell migration and cell–extracellular matrix interactions. Our results indicate that MKK7 is not required for hepatocyte proliferation but plays important roles in tissue repair and remodeling following parenchymal destruction.

Materials and Methods

ANIMALS

Mice with the MKK7 flox allele were generated as described.[9] Myxovirus resistance 1 (Mx1)–cyclization

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recombination (Cre) mice and albumin (Alb)–Cre mice on the C57BL/6J background were obtained from Jackson Laboratories (Bar Harbor, ME). The protocols used for animal experimentation were approved by the Animal Research Committee of Asahikawa Medical University. All animal experiments adhered to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*. MKK7 (HH) KO or MKK7 (L) KO were generated by crossing $MKK7^{LoxP/LoxP}$ mice with Mx1-Cre mice and Alb-Cre mice, respectively. To induce Cre in the Mx1-Cre system, the mice were injected intraperitoneally with 250 μg polyinosinic:polycytidylic acid 3 times at 2-day intervals at the age of 6-8 weeks. We confirmed that hepatocytes are the only liver cells that are subjected to Mx1-Cre/loxP recombination.[10] Mice with heterologous KO ([$MKK7^{LoxP/+}$; Mx1-Cre] and [$MKK7^{LoxP/+}$; Alb-Cre]) were used as controls. In the experiments using MKK7 (HH) KO mice, $MKK7^{LoxP/LoxP}$ mice were also used as controls. All data shown here were obtained from experiments using 8-week-old to 12-week-old male mice.

To induce regenerative proliferation of hepatocytes, a two-thirds PH or acute CCl_4 liver injury (1 mL/kg body weight, subcutaneous injection) was used. To induce chronic liver injury, CCl_4 (1 mL/kg body weight) was subcutaneously injected into mice 3 times per week for 8 weeks. CCl_4 was also administered for 25 weeks to induce liver tumors. As another chronic injury–associated hepatocarcinogenesis model, mice were given thioacetamide (TAA; Sigma-Aldrich, St. Louis, MO) in drinking water (0.03% TAA in tap water) for 30 weeks.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

Livers were perfused with phosphate-buffered saline and fixed in 4% paraformaldehyde overnight. Paraffin sections (4 μm) were deparaffinized and stained with hematoxylin and eosin (HE). Immunohistochemical analyses were performed by the Envision (peroxidase; Dako, Carpinteria, CA) method using anti-Ki-67 (Nichirei, Tokyo, Japan), anti–phospho-c-Jun (Ser73; Cell Signaling Technology, Danvers, MA), antifibrinogen (Abcam, Cambridge, UK), anti–α-smooth muscle actin (α-SMA; Dako), anti–cleaved caspase 3 (Cell Signaling Technology), anti–green fluorescent protein (GFP; ThermoFisher Scientific, Waltham, MA), anti-transgelin (GeneTex, Irvine, CA), anti-uPA (Abcam), and anti–plasminogen activator tissue-type (tPA; Abcam) antibodies according to an antigen retrieval procedure using Target Retrieval Solution (Dako). To evaluate the extent of fibrosis, the sections were stained with sirius red F3B (Waldeck, Munster, Germany).

ISOLATION AND CULTURE OF MOUSE HEPATOCYTES

Hepatocytes were isolated from the livers of control ($MKK7^{LoxP/+}$; Alb-Cre) and MKK7 KO ($MKK7^{LoxP/LoxP}$; Alb-Cre) mice by the two-step collagenase perfusion method. Isolated hepatocytes were plated on collagen-coated plastic dishes or glass coverslips and cultured in serum-free Williams' E medium supplemented with epidermal growth factor (EGF; 10 ng/mL), insulin (10^{-7} M), and dexamethasone (10^{-6} M). Wound healing assays were also performed using monolayer cultures of hepatocytes in the absence or presence of hepatocyte growth factor (HGF; 10 ng/mL). To induce three-dimensional branching morphogenesis, hepatocytes were first plated on Primaria dishes (Becton-Dickinson, Franklin Lakes, NJ) in serum-free Williams' E medium supplemented with EGF (10 ng/mL) and insulin (10^{-7} M) for 5 days, and the formed spheroidal aggregates were then embedded within a collagen gel (Cellmatrix type I-A; Nitta Gelatin, Osaka, Japan) and cultured in Williams' E medium supplemented with 10% fetal bovine serum, EGF (10 ng/mL), and insulin (10^{-7} M) for 2 days, as described.[10] In some experiments, tumor necrosis factor (TNF)-α (10 ng/mL), transforming growth factor (TGF)-β (2 ng/mL), dexamethasone (10^{-6} M), or a specific JNK inhibitor (SP600125; 20 μM) was added to the medium. Quantification of branch lengths was performed using ImageJ 1.51n (National Institutes of Health, Bethesda, MD).

WESTERN BLOTTING

Protein samples (15 μg per lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and immunoblotted with anti-MKK7, anti-MKK4, anti-JNK, anti-phospho-JNK, anti-c-Jun, anti-phospho-c-Jun (Ser73), anti-phospho-histone H3 (Cell Signaling Technology), anti-glyceraldehyde

3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) and anti-β-actin (Sigma) antibodies. All blots were developed using an enhanced chemiluminescence detection system (GE Healthcare, Chalfont St. Giles, UK).

MICROARRAY ANALYSES

Total RNA was prepared from liver tissues (quiescent and 2 days after PH) and cultured hepatocytes using Sepasol (Nacalai Tesque Inc., Kyoto, Japan). Equal amounts of samples from four independent experiments were mixed, reverse-transcribed, and analyzed by one-color microarrays (3D-Gene Microarray; TORAY, Tokyo, Japan). To extract candidate genes involved in branching morphogenesis, the genes with expression levels that paralleled the branch lengths were extracted.

QUANTITATIVE RT-PCR

Total RNA was extracted and subjected to quantitative RT-PCR analyses. Quantitative RT-PCR was performed using the ΔΔCt method with FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany). Each reaction was conducted in duplicate, and the mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase. The primers used in the quantitative RT-PCR experiments are listed in Supporting Table S1.

TRANSDUCTION OF CLONED COMPLEMENTARY DNA IN HEPATOCYTES BY AN ADENO-ASSOCIATED VIRUS 8 VECTOR SYSTEM

The Gaussia princeps luciferase (*Gluc*) with NheI and MluI sites was amplified by PCR from phosphorylated cytomegalovirus–Gluc (New England Biolabs, Ipswich, MA) with the primers 5′-ATCTAGCTAGCT CGGATCCAGCCACCATG-3′ and 5′-ATCGACG CGTGCTCGAGCGGCCGCTTAGT-3′. The internal ribosome entry site 2–GFP derived from *Aequorea coerulescens* gene (*IRES2-AcGFP*) with MluI sites was amplified by PCR from the pIRES2-AcGFP plasmid (Takara Bio, Ohtsu, Japan) with the primers 5′-ATCG ACGCGTCGAGCTCAAGCTTCGAAT-3′ and 5′-ATCGACGCGTCACTTGTACAGCTCATCCAT G-3′. Then, the purified *Gluc* and *IRES2-AcGFP* gene segments were inserted into the NheI-MluI and MluI sites, respectively, of psubCytomegalovirus enhancer/chicken β-actin promoter (CAG)-Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (a gift from Dr. Kari Alitalo, Helsinki University, Finland) to obtain psubCAG-Gluc-IRES2-AcGFP-WPRE. Full-length complementary DNAs (cDNAs) of *Tagln*, *Plau*, *Glipr2*, and tPA (*Plat*) were amplified from cDNA from hepatocytes cultured for 3 days within a collagen gel matrix by PCR. These cDNAs were inserted at the NheI and XhoI sites of the psubCAG-WPRE-Gluc-IRES2-AcGFP vector after excision of the Gluc sequences. Packaging the plasmids into the recombinant adeno-associated virus 8 (AAV8) vector was performed as described.[11] Mouse hepatocytes were cultured on collagen-coated plastic dishes for 3 hours and infected with 4×10^{11} copies of AAV8. After 5 days, the hepatocytes were trypsinized, cultured on ultralow attachment surface plates (Corning, Kennebunk, ME) for 2 days, and embedded within the collagen gel matrix. For *in vivo* experiments, MKK7 (L) KO mice were injected with 1×10^{12} copies of AAV8 through the lateral tail vein and subjected to acute CCl_4 liver injury after 2 weeks.

STATISTICAL ANALYSES

All data are presented as the means ± SEM. Statistical analyses were performed using an unpaired two-tailed *t* test or Mann-Whitney U test.

Results

LIVER REGENERATION AFTER PH IS NOT AFFECTED BY MKK7 (HH) KO

In contrast to the embryonically lethal phenotype with aborted hepatogenesis in systemic MKK7 KO mice,[7] Alb-Cre-mediated or Mx1-Cre-mediated gene deletion did not induce discernible abnormalities in the liver (Alb-Cre: Supporting Fig. S1; Mx1-Cre: Fig. 1A, PH [–]). We then examined whether MKK7 KO affects compensatory regeneration of the liver following PH. The recovery of liver weight (Fig. 1B), the increase in hepatocyte mitoses (Fig. 1A, 2 days), and the increase in Ki-67-positive hepatocytes (Fig. 1C,D) in MKK7 (HH) KO mice were comparable to those in control mice. Consistent with this finding, there was an abrupt and transient increase in histone H3 phosphorylation at 2 days after PH in both

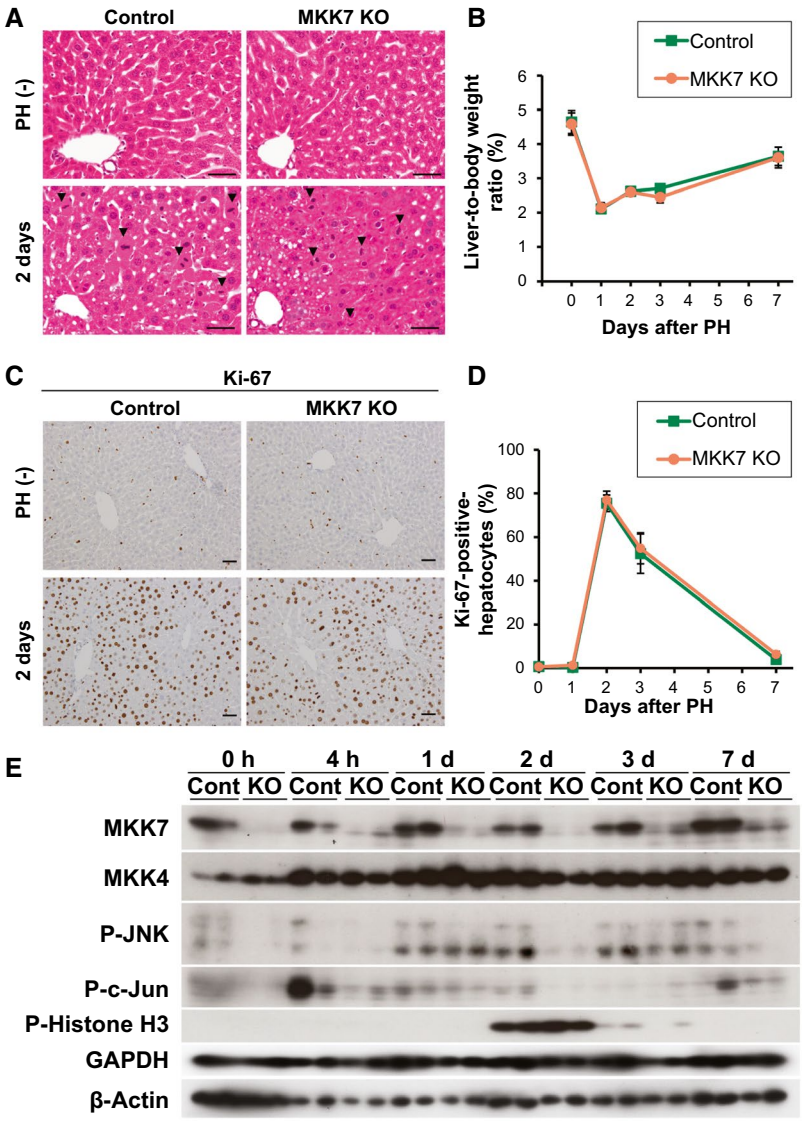

FIG. 1. MKK7 (HH) KO does not affect liver regeneration after PH. Control and MKK7 (HH) KO mice were subjected to two-thirds PH. (A) HE staining of tissue sections from quiescent (PH [–]) livers and regenerating livers at 2 days following PH. Mitotic cells are indicated by arrowheads. Scale bar, 50 μm. (B) Time course of liver-to-body weight ratios after PH (n = 5 for each). (C) Immunohistochemical analysis of Ki-67 in tissue sections from quiescent (PH [–]) livers and regenerating livers at 2 days following PH. Scale bar, 50 μm. (D) Time course of the proportion of Ki-67-labeled hepatocytes following PH (n = 3-5 for each). (E) Western blot analysis of MKK7, MKK4, phosphorylated JNK, phosphorylated c-Jun, phosphorylated histone H3, GAPDH, and β-actin in livers after PH.

control and MKK7 KO mice (Fig. 1E). The levels of MKK7 protein expression in the control mouse livers were almost unchanged following PH, whereas the expression of MKK4 was elevated 4 hours after PH, and the increased levels were maintained throughout the observation period (7 days) (Fig. 1E). MKK7 protein expression was greatly reduced in the livers of hepatocyte-specific MKK7 KO mice, but MKK4 protein expression was not affected in either quiescent or regenerating livers (Fig. 1E). There was slight phosphorylation of JNK and c-Jun following PH in the control livers, which was suppressed in the livers of MKK7 KO mice (Fig. 1E). We also found that the mRNA expression profiles of control and MKK7 KO

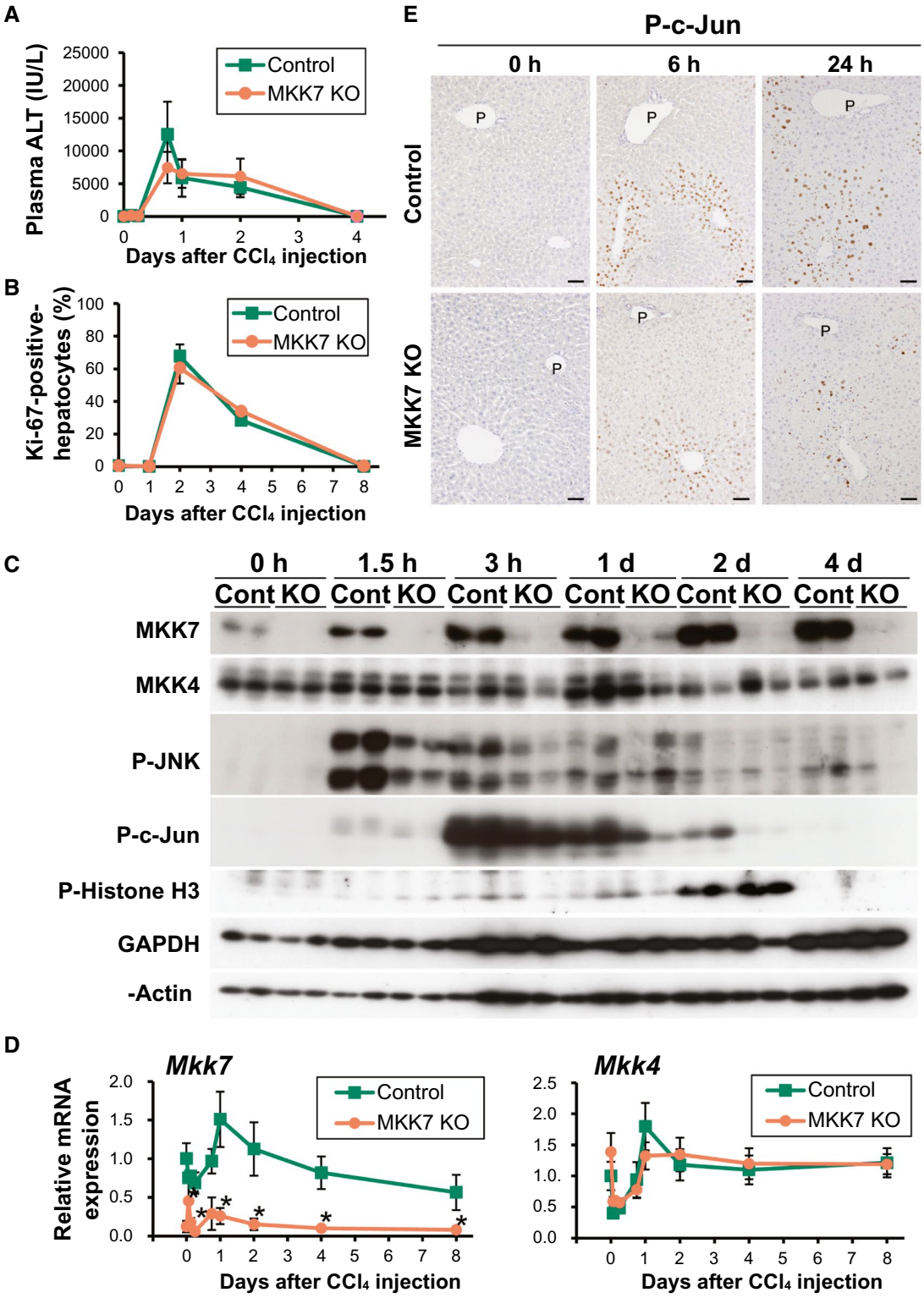
A
Plasma ALT (IU/L)
25000
20000
15000
10000
5000
0
Control
MKK7 KO
0 1 2 3 4
Days after CCl4 injection
B
Ki-67-positive-hepatocytes (%)
100 80 60 40 20 0
Control
MKK7 KO
0 1 2 3 4 5 6 7 8
Days after CCl4 injection
E
P-c-Jun
0 h
6 h
24 h
Control
MKK7 KO
P
C
0 h 1.5 h 3 h 1 d 2 d 4 d
Cont KO Cont KO Cont KO Cont KO Cont KO Cont KO
MKK7
MKK4
P-JNK
P-c-Jun
P-Histone H3
GAPDH
-Actin
D
Mkk7
Mkk4
Relative mRNA expression
2.0 1.5 1.0 0.5 0.0
2.5 2.0 1.5 1.0. 0.5 0.0
Control
MKK7 KO
0 1 2 3 4 5 6 7 8
Days after CCl4 injection

FIG. 2. MKK7 (HH) KO does not affect the proliferation of hepatocytes following acute CCl_4 injury. CCl_4 was injected subcutaneously into control and MKK7 (HH) KO mice. (A) Time course of plasma ALT following CCl_4 injection (n = 3 for each). (B) Time course of the proportion of Ki-67-labeled hepatocytes following CCl_4 injection (n = 3 for each). (C) Western blot analysis of MKK7, MKK4, phosphorylated JNK, phosphorylated c-Jun, phosphorylated histone H3, GAPDH, and β-actin in livers following CCl_4 injection. (D) Quantitative RT-PCR analysis of the mRNA expression of *Mkk7* and *Mkk4* following CCl_4 injection (n = 5 for each). Statistical analysis: unpaired two-tailed *t* test. **P* < 0.05 versus control. (E) Immunohistochemical analysis of phosphorylated c-Jun in the livers before (0 h) and at 6 hours (6 h) and 24 hours (24 h) following CCl_4 injection. Scale bar, 50 μm. Abbreviation: P, portal vein.

mouse liver tissues, either quiescent or regenerating (2 days after PH), were comparable (Supporting Fig. S2A,B). The numbers of differentially expressed genes between control and MKK7 KO livers 2 days after PH were 35 (increased) and 21 (decreased), corresponding to 0.37% and 0.22% of the total genes analyzed (Supporting Tables S4 and S5). A pathway analysis for the products of these differentially expressed genes did not demonstrate candidates for alternative proliferative pathways that might be activated in the regenerating livers of MKK7 KO mice (Supporting Fig. S2C).

LIVER INJURY REPAIR IS DELAYED IN MKK7 KO MICE

We next examined the effects of MKK7 KO on the regenerative proliferation of hepatocytes following CCl_4-induced parenchymal destruction. A single necrotizing dose of CCl_4 caused massive death of hepatocytes in the centrilobular area in control and MKK7 (HH) KO mice. Both control and MKK7 KO mice demonstrated similar increases in plasma alanine aminotransferase (ALT) levels, which peaked at 18 hours after CCl_4 administration (Fig. 2A). The percentage of Ki-67-labeled nuclei in hepatocytes (Fig. 2B) and the phosphorylation of histone H3 in the livers (Fig. 2C) were also similar between control and MKK7 KO mice. Although the protein expression of MKK7 in the control livers was elevated soon after CCl_4 administration and remained at high levels thereafter (Fig. 2C), the mRNA levels of *Mkk7* were relatively constant (Fig. 2D). There were no significant changes in the expression of MKK4 protein or mRNA in either control or MKK7 KO livers (Fig. 2C,D). Phosphorylation of JNK and c-Jun, which peaked at 1.5 hours and 3 hours after CCl_4 injection, respectively, was suppressed in MKK7 KO livers (Fig. 2C). Immunohistochemically, phosphorylated c-Jun was detected in the nuclei of centrilobular hepatocytes in both control and MKK7 KO livers but was reduced in livers of MKK7 KO compared to control livers (Fig. 2E). The accumulation of F4/80-positive macrophages and the mRNA expression of cytokines (interleukin-6, *Hgf*, *Tnf*, and *Tgfb1*) in MKK7 KO mice were comparable to those in the control (Supporting Fig. S3).

We then compared the process of tissue repair in control and MKK7 KO mouse livers. CCl_4 administration induced similar levels of centrilobular necrosis after 2 days, and subsequent tissue repair was completed after 8 days in both control and MKK7 (HH) KO livers (Fig. 3A). However, the residual necrotic areas after 4 days were larger in MKK7 KO livers than in control livers (Fig. 3A). Although there were no differences in fibrinogen-positive necrotic areas between the control and MKK7 KO mouse livers at 2 days after CCl_4 administration, MKK7 KO livers contained larger fibrinogen-positive areas than control livers at 4 days (Fig. 3B,C). Similar results were obtained in MKK7 (L) KO mice (Supporting Fig. S4). These data suggest that hepatocyte MKK7 contributes to proper repair processes after parenchymal destruction.

LOSS OF MKK7 AUGMENTS FIBROSIS IN CHRONIC LIVER INJURY

We then examined whether MKK7 (HH) KO in mice affects liver pathology in chronic injury induced by repeated injections of CCl_4 for 8 weeks. In control mice, there was delicate bridging fibrosis between the injured centrilobular areas, which was highlighted by sirius red staining and associated with α-SMA-positive hepatic stellate cells; Ki-67-positive proliferating hepatocytes were scattered in the remodeled parenchyma (Fig. 4A). In MKK7 KO mouse livers, fibrosis was more extensive than in control mouse livers (Fig. 4A). Quantification of sirius red–positive areas confirmed that MKK7 KO augmented liver fibrosis (Fig. 4B). The mRNA expression of actin

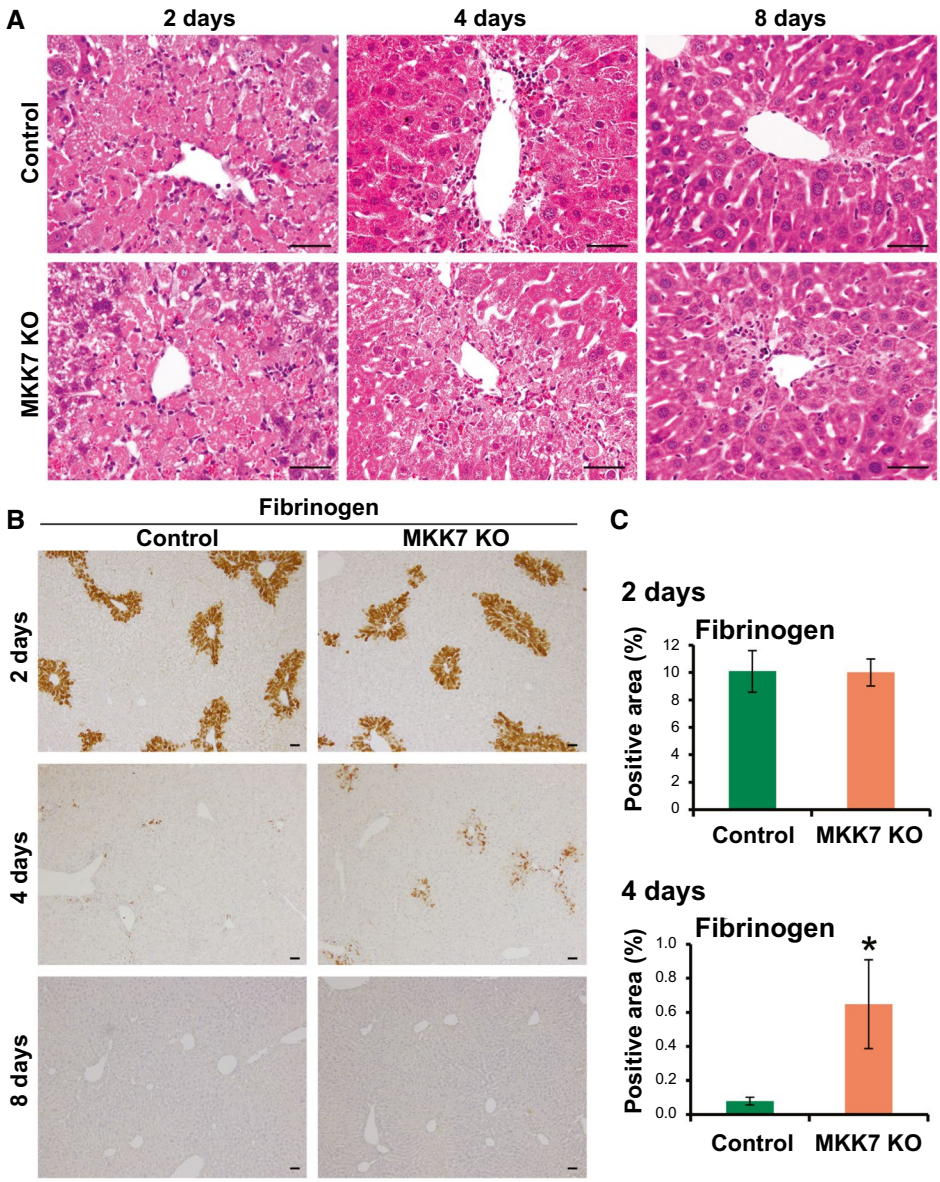

FIG. 3. Restoration processes following CCl_4-induced parenchymal destruction are delayed in MKK7 (HH) KO mice. CCl_4 was injected subcutaneously into control and MKK7 (HH) KO mice. (A) HE staining of tissue sections from control and MKK7 KO mouse livers at 2, 4, and 8 days after CCl_4 injection. Scale bar, 50 μm. (B) Immunohistochemical analysis of fibrinogen in tissue sections from control and MKK7 KO livers at 2, 4, and 8 days following CCl_4 injury. Scale bar, 50 μm. (C) Quantification of fibrinogen-positive area at 2 days (n = 5 for each) and 4 days (n = 8 for each) following CCl_4 injection. Statistical analysis: Mann-Whitney *U* test. **P* < 0.05 versus control.

alpha 2, smooth muscle and collagen type I alpha 1 chain in the livers of MKK7 KO mice was slightly higher than that of control mice, although there were no statistically significant differences (Supporting Fig. S5A,B). Quantification of α-SMA immunohistochemistry revealed that there was significantly more activation of hepatic stellate cells in MKK7 KO livers (Fig. 4C). In MKK7 KO mouse livers, mRNA expression of S100A4, which is known to be associated with liver fibrosis, was also significantly higher than that in control mouse livers (Fig. 4D). The mRNA expression of *Tnf*, but not *Tgfb1*, was elevated in the chronically injured livers; but there was no difference between control and MKK7 KO mice (Supporting

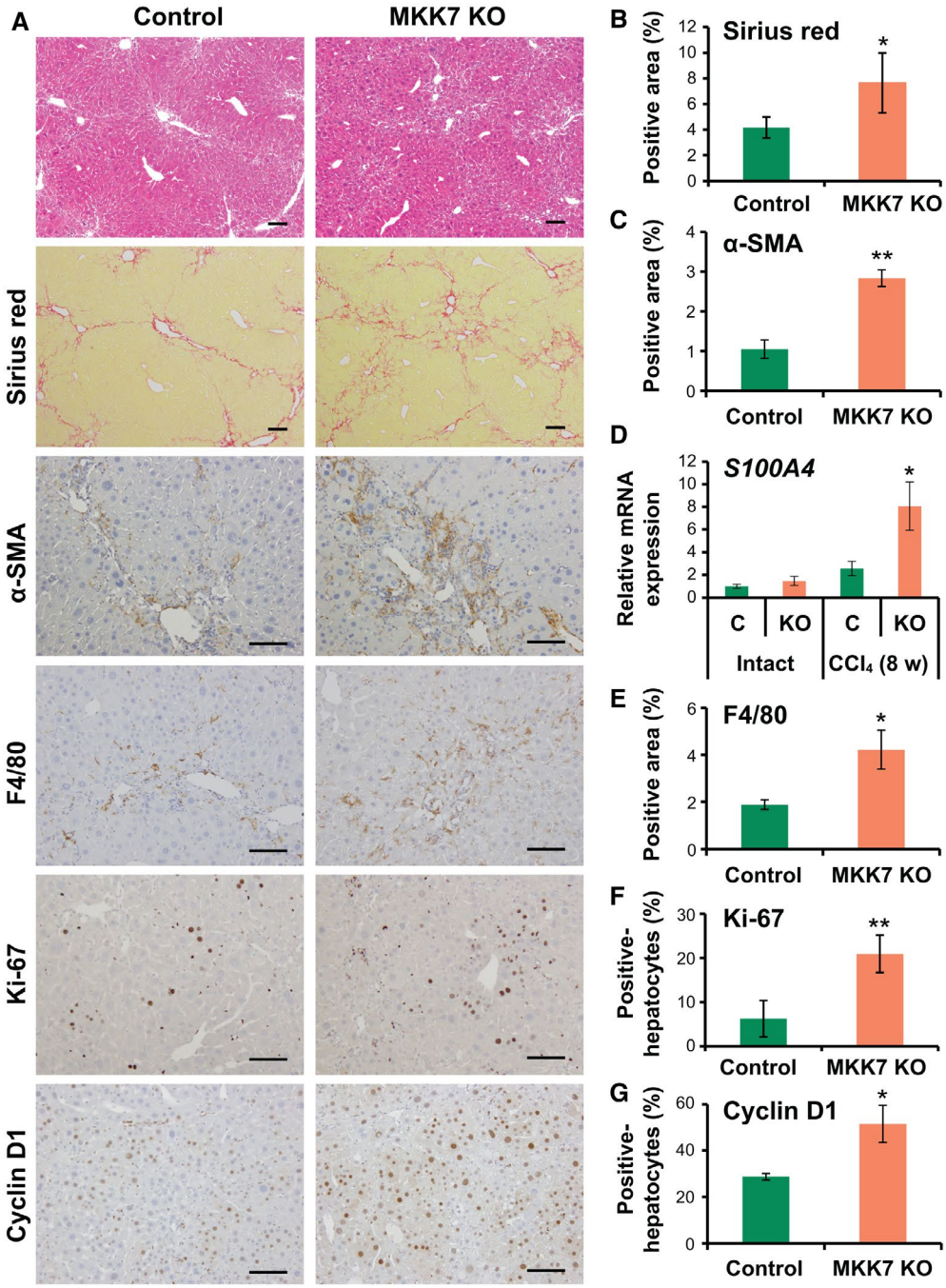

FIG. 4. MKK7 (HH) KO enhances liver fibrosis and regenerative proliferation of hepatocytes in chronic CCl_4 injury. CCl_4 was injected subcutaneously into control and MKK7 (HH) KO mice for 8 weeks. Mice were sacrificed 5 days after the last CCl_4 injection. (A) HE staining, sirius red staining, and immunohistochemistry for α-SMA, F4/80, Ki-67, and cyclin D1 in liver sections. Scale bar, 50 μm. (B) Quantification of sirius red–positive areas (n = 5 for each). (C) Quantification of α-SMA-positive areas (n = 4 for each). (D) Quantitative RT-PCR analysis of the mRNA expression of *S100A4* (intact, n = 3 for each; CCl_4 for 8 weeks, n = 5 for each). (E) Quantification of F4/80-positive areas (n = 4 for each). (F,G) The proportions of Ki-67-labeled (F) and cyclin D1–labeled (G) hepatocytes (n = 4 for each). Statistical analyses: unpaired two-tailed *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. Abbreviation: C, control.

Fig. S5C,D). However, the accumulation of F4/80-positive macrophages was more enhanced in MKK7 KO mouse livers (Fig. 4E). The number of Ki-67-positive hepatocytes, as well as cyclin D1-positive hepatocytes, was higher in MKK7 KO mice than in control mice (Fig. 4A,F,G). Cleaved caspase 3–positive apoptotic hepatocytes were scattered in the inflamed area of both control and MKK7 KO mouse livers (Supporting Fig. 5C). Similar results were obtained in MKK7 (L) KO mice (Supporting Figs. S6 and S7).

Repeated CCl_4 administration for more than 24 weeks induces multiple nodules of well-differentiated hepatocellular carcinoma in the context of liver cirrhosis.[12] We examined whether MKK7

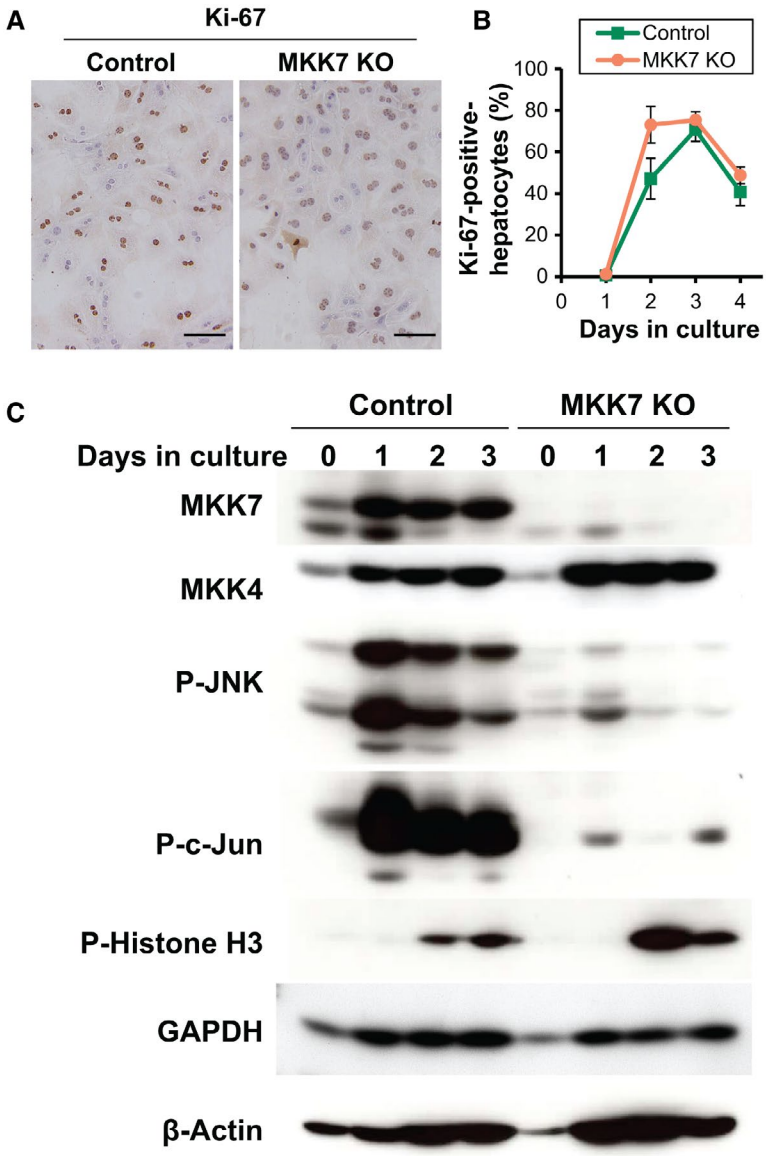

FIG. 5. MKK7 (L) KO does not significantly affect hepatocyte proliferation in monolayer cultures. Hepatocytes were isolated from control and MKK7 (L) KO mouse livers by two-step collagenase perfusion methods and cultured on collagen-coated dishes in the presence of EGF. (A) Immunohistochemical analysis of Ki-67 in hepatocytes that were cultured for 2 days. Scale bar, 100 μm. (B) Time course of the proportion of Ki-67-labeled hepatocytes (n = 5 for each). (C) Western blot analysis of MKK7, MKK4, phosphorylated JNK, phosphorylated c-Jun, phosphorylated histone H3, GAPDH, and β-actin.

(HH) KO or MKK7 (L) KO affected tumorigenesis in livers treated with CCl_4 for 25 weeks. Except for some increase in tumor number in MKK7 (HH) KO mice, there were no significant differences in tumorigenesis or liver weight between the control and MKK7 KO mice (Supporting Fig. S8A-C). We also treated control and MKK7 KO mice with TAA for 30 weeks and induced liver tumors and found that MKK7 KO did not affect tumorigenesis (Supporting Fig. S8D-F).

CELL CYCLE PROGRESSION IN PRIMARY CULTURED HEPATOCYTES OCCURS IN THE ABSENCE OF MKK7

To further confirm that hepatocyte proliferation was independent of MKK7, we performed *in vitro* experiments using primary mouse hepatocytes. Hepatocytes isolated and cultured on collagen-coated dishes in the presence of EGF underwent robust and synchronized cell cycle progression. Hepatocytes from MKK7 (L) KO mice demonstrated a proliferative response, which was slightly earlier but to the same extent as that observed in control hepatocytes (Fig. 5A-C). The protein expression of MKK7 and MKK4 in control hepatocytes was increased in culture (Fig. 5C). In MKK7 KO hepatocytes, there was a similar induction of MKK4 protein expression (Fig. 5C). Phosphorylation of JNK and c-Jun was strongly induced in the control hepatocytes, but it was significantly reduced in MKK7 KO hepatocytes (Fig. 5C).

MKK7 KO SUPPRESSES BRANCHING MORPHOGENESIS OF HEPATOCYTES WITHIN A COLLAGEN GEL MATRIX

MKK7 might have roles in the migratory behavior of regenerating hepatocytes through interactions with the extracellular matrix. We first performed wound healing assays using monolayer cultures of hepatocytes in the absence or presence of HGF, but there were no differences in wound closure between control and MKK7 KO hepatocytes (Supporting Fig. S9A,B). We then examined the effects of MKK7 KO on morphogenesis within a collagen gel matrix. As we demonstrated previously,[10] spheroidal aggregates of hepatocytes migrated into the gel and formed branching processes, which are associated with a decrease in albumin expression and the induction of bile duct–specific cytokeratin expression (ductular transdifferentiation). Branching morphogenesis was enhanced by inflammatory cytokines, such as TNF-α and TGF-β, but repressed by dexamethasone (Fig. 6A,B). The extent of branching morphogenesis was reduced in MKK7 KO hepatocytes compared with control hepatocytes (Fig. 6A). There were no statistically significant differences in the mRNA expression of marker of proliferation Ki-67 (*Mki67*), *Alb*, and keratin 19 (*Krt19*) between control and MKK7 KO hepatocytes (Fig. 6B). The mRNA expression of MET proto-oncogene and EGF receptor in MKK7 KO hepatocytes was also comparable to control hepatocytes (Supporting Fig. S9C). TGF-β-induced branching morphogenesis lacked *Mki67* and *Krt19* expression (Fig. 6B) and was followed by cell death after 1 week (data not shown), suggesting abortive ductular transdifferentiation (Fig. 6B). SP600125 significantly attenuated morphogenesis, which was consistent with the idea that the inhibitory effects of MKK7 KO might be mediated at least partly by the suppression of JNK signaling (Fig. 6C).

MKK7 KO SUPPRESSES GENE EXPRESSION OF *Tagln*, *Glipr2*, AND *Plau* IN CULTURED HEPATOCYTES AND uPA OVEREXPRESSION FACILITATES TISSUE REPAIR OF MKK7 KO MICE FOLLOWING CCl_4 INJURY

To gain insight into the mechanism by which MKK7 KO mediates suppression of branching morphogenesis, we performed an oligonucleotide microarray analysis of primary hepatocytes from either wild-type (control) or MKK7 KO mice cultured in collagen gels with or without TNF-α or TGF-β. The genes with expression patterns that corresponded with the changes in branch length within the collagen gel were screened (Fig. 7A). We focused on transgelin (encoded by *Tagln*), glioma pathogenesis-related protein 2 (encoded by *Glipr2*), and uPA (encoded by *Plau*) because these genes have been reported to be involved in cell migration and invasion.[13-15] Quantitative RT-PCR analyses confirmed that the mRNA expression of these genes was induced when hepatocyte spheroids were embedded within the collagen gel, but the extent

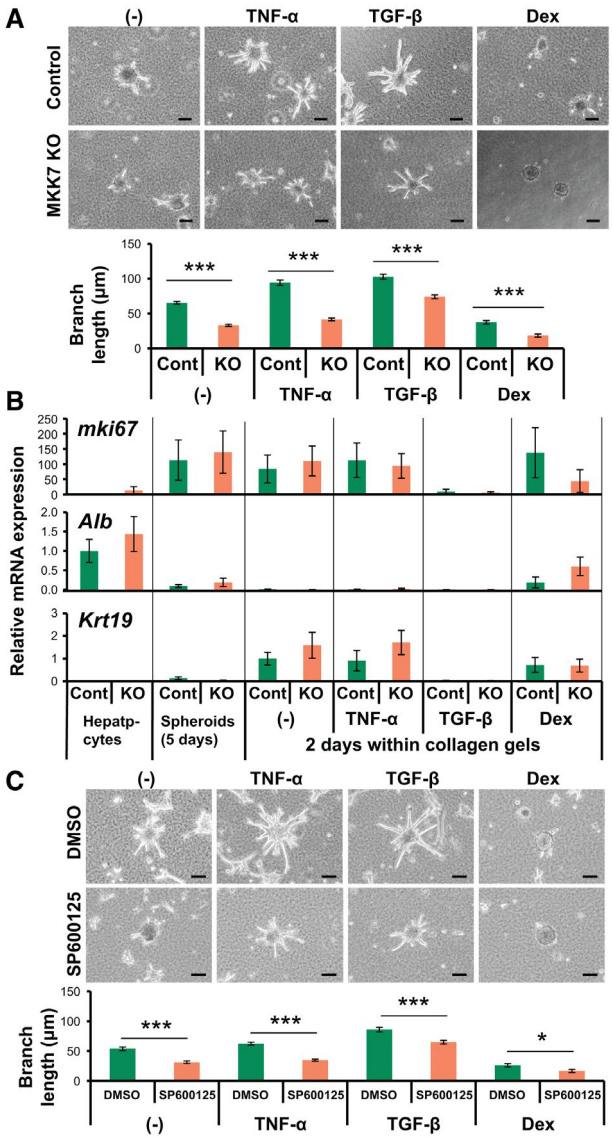

FIG. 6. MKK7 (L) KO suppresses branching morphogenesis of hepatocytes in collagen gel culture. Spheroidal aggregates of control and MKK7 (L) KO mouse hepatocytes were embedded within a collagen gel matrix and cultured for 2 days. (A) Branching morphogenesis in the absence or presence of TNF-α, TGF-β, or dexamethasone. Phase-contrast microscopic images and quantification of branch lengths. Branches (n = 111-114) from three independent experiments were measured. Scale bar, 100 μm. (B) Quantitative RT-PCR analyses of the mRNA expression of *mKi67*, *Alb*, and *Krt19* in freshly isolated hepatocytes and spheroidal aggregates (before and 2 days after being embedded and cultured within the gel) (n = 8 for each). (C) Branching morphogenesis in the absence or presence of a specific JNK inhibitor (SP600125). Phase-contrast microscopic images and quantification of branch lengths. Branches (n = 53-77) from three independent experiments were measured. Scale bar, 100 μm. Statistical analyses (A-C): unpaired two-tailed *t* test. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control. Abbreviations: Dex, dexamethasone; DMSO, dimethyl sulfoxide.

of induction was generally reduced in MKK7 KO hepatocytes (Fig. 7B). The reduced branching morphogenesis of MKK7 KO hepatocytes was partially reversed by overexpression of *Tagln*, *Glipr2*, or *Plau* (Fig. 7C), suggesting that these gene products might be candidate effectors for MKK7-induced migration of hepatocytes. We also found that the expression of tPA (encoded by *Plat*), which is functionally related to uPA, slightly enhanced branching morphogenesis of MKK7 KO hepatocytes (Supporting Fig. S10). The mRNA expression of *Tagln* and *Plau*, but not *Glipr2* and *Plat*, was strongly suppressed by SP600125 (Fig. 7D; Supporting Fig. S10).

Finally, we studied whether overexpression of transgelin, uPA, or tPA *in vivo* might rescue the impaired tissue reparation following CCl_4 injury in MKK7 (L) KO mice. In the control livers (Gluc), transgelin was detected in hepatic arteries and some endothelial cells; uPA was detected in sinusoids and some endothelial cells; and tPA was detected in bile ducts, sinusoids, and some endothelial cells (Supporting Fig. S11A). Although they were barely expressed in MKK7 (L) KO hepatocytes (Supporting Fig. S11A), infection of AAV8 vectors harboring these genes induced the expression of their products preferentially in the centrilobular area (Supporting Fig. S11A,B). MKK7 (L) KO mice were infected with AAV8 vectors and treated with CCl_4 after 2 weeks. Compatible with previous reports,[16,17] overexpression of uPA induced hepatocyte injury (Supporting Fig. S11B), but the extent of CCl_4-induced injury was comparable to Gluc-expressing, transgelin-expressing, or tPA-expressing livers (Fig. 8A,C). Interestingly, overexpression of uPA, but not *Tagln* or tPA, significantly facilitated tissue reparation (Fig. 8B,D). Although MKK7 KO did not affect the levels of *Plau* mRNA expression in the whole liver in either acute or chronic CCl_4 injury (Supporting Fig. S12), our results suggest that diminished hepatocyte uPA expression could be a key determinant for the delayed tissue repair in MKK7 KO mice.

Discussion

In contrast to systemic homologous disruption of MKK7, which induces embryonic lethality with severely hampered liver development,[7] MKK7 KO either in the developing liver ($MKK7^{LoxP/LoxP}$;

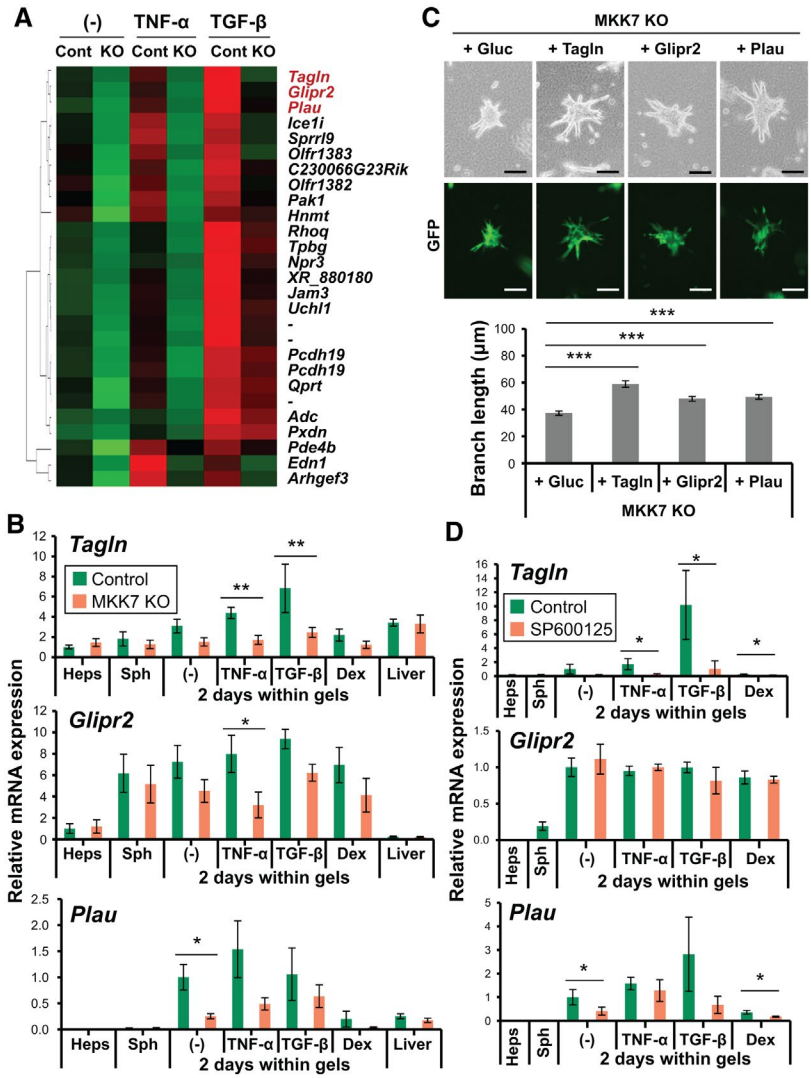

FIG. 7. MKK7 (L) KO suppresses the mRNA expression of *Tagln*, *Glipr2*, and *Plau* in collagen gel culture. (A) Oligonucleotide microarray screening and hierarchical clustering of the genes with expression patterns that corresponded with the changes in branch length within the collagen gel. Spheroidal aggregates of control and MKK7 (L) KO mouse hepatocytes were embedded within a collagen gel matrix and cultured for 2 days. The criteria for gene selection: higher expression levels (>1.2-fold) in control hepatocytes than in MKK7 KO hepatocytes, higher expression levels (>1.2-fold) in TNF-α-treated or TGF-β-treated control hepatocytes than in untreated control hepatocytes and maximum normalized expression levels >25. (B) Quantitative RT-PCR analyses of the mRNA expression of *Tagln*, *Glipr2*, and *Plau* in control and MKK7 (L) KO mouse hepatocytes (freshly isolated, spheroidal aggregates of hepatocytes before and after being embedded and cultured within the gel in the presence or absence of various soluble factors) and intact liver tissues (n = 8 for each). (C) Branching morphogenesis of spheroidal aggregates of MKK7 (L) KO hepatocytes with virally transduced *Gluc* (control), *Tagln*, *Glipr2*, and *Plau*. The spheroidal aggregates were cultured within the gel for 2 days. Phase-contrast and fluorescence microscopic images and quantification of the branch lengths (n = 73-82). Shown are representatives of three independent experiments with equivalent results. Expression of the transduced genes was monitored by coexpressing GFP. Scale bar, 100 μm. (D) Quantitative RT-PCR analyses of the mRNA expression of *Tagln*, *Glipr2*, and *Plau* in freshly isolated wild-type hepatocytes and spheroidal aggregates of wild-type hepatocytes before and after being embedded and cultured within the gel in the presence or absence of various soluble factors. The JNK inhibitor SP600125 was added to the spheroids (n = 4 for each). Statistical analyses: unpaired two-tailed *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (B,D) or Gluc (C). Abbreviations: Dex, dexamethasone; Heps, hepatocytes; Sph, spheroidal aggregates.

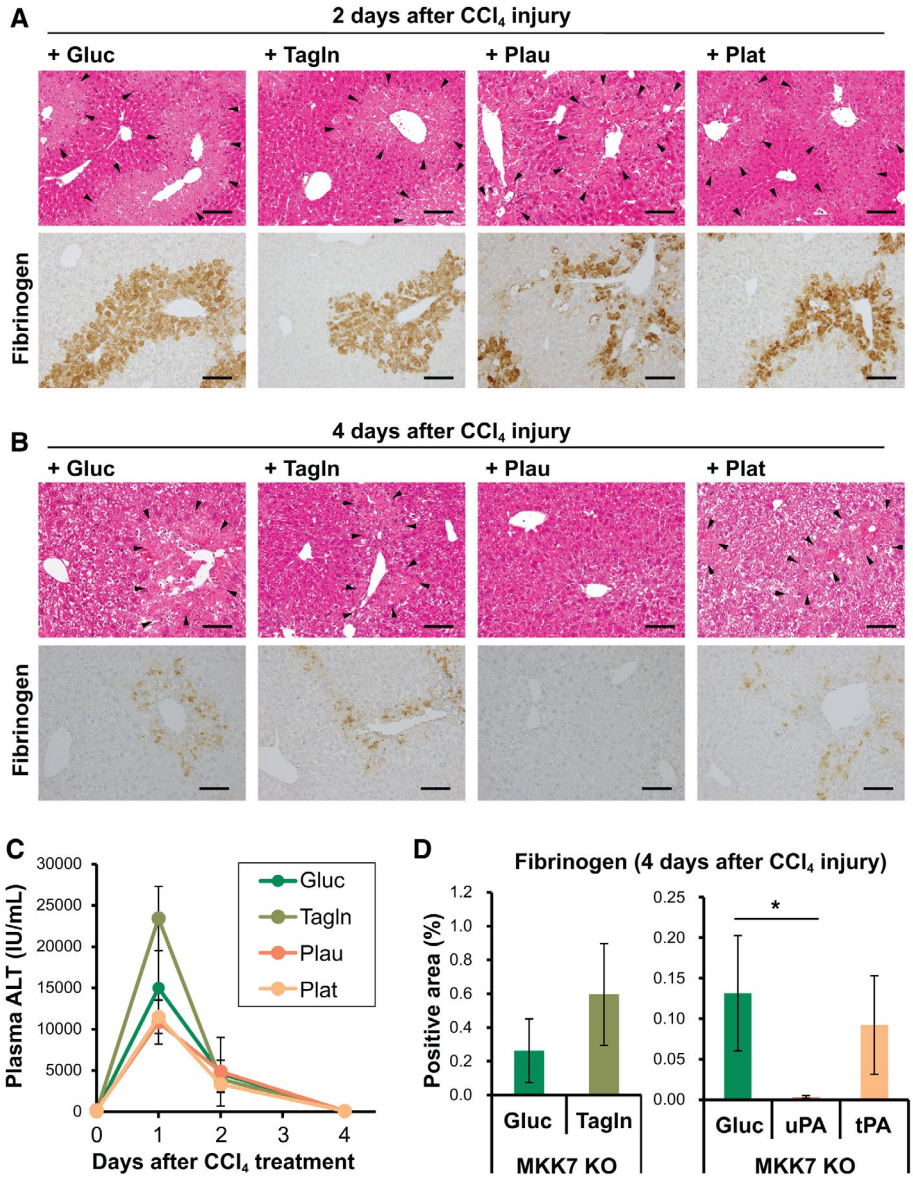

FIG. 8. Hepatocyte-specific uPA overexpression rescues retarded tissue reparation of MKK7 (L) KO mice following CCl_4 injury. MKK7 (L) KO mice were infected with AAV8 vectors harbouring the *Gluc* (control), *Tagln*, *Plau*, or *Plat* gene and treated with CCl_4 after 2 weeks. (A,B) HE staining and immunohistochemistry for fibrinogen at 2 days (A) and 4 days (B) following CCl_4 injection. Necrotic areas are demarcated by arrowheads. Scale bar, 50 μm. (C) Time course of plasma ALT following CCl_4 injection (n = 3 for each). (D) Quantification of fibrinogen-positive area at 4 days (n = 6 for Gluc, uPA, and tPA; n = 5 for Tagln) following CCl_4 injection. Statistical analysis: Mann-Whitney U test. $^*P < 0.05$ versus control.

Alb-Cre) or in the adult liver ($MKK7^{LoxP/LoxP}$; Mx1-Cre) did not show discernible abnormal gross or microscopic features in the liver. In germline MKK7 KO mice, the liver anlage at E8.5 to E9.5 has been shown to be apparently normal; but subsequent liver development fails, and the animals die at E11.5, suggesting that MKK7 is required for the earliest hepatoblastic differentiation.[7] Because Alb mRNA is first detected at E10.5 in hepatic primordia[18,19] and gene recombination takes at least several days to complete,[20] MKK7 KO occurs in cells that are already committed to becoming hepatoblasts in the Alb-Cre/LoxP system. MKK7 might not be required for the proliferation and differentiation of hepatoblasts.

Similarly, although deficient in both the JNK1 and JNK2 genes in mice that are embryonically lethal,[21] no obvious abnormalities are detected in the livers of hepatic JNK double KO mice (Alb-Cre$^{-/+}$ Jnk1$^{LoxP/LoxP}$ Jnk2$^{-/-}$ or Mx1-Cre$^{-/+}$ Jnk1$^{LoxP/LoxP}$ Jnk2$^{-/-}$).[22] Although it is possible that the activities of MKK7 or JNKs in nonparenchymal cells or very immature hepatoblasts are critical in normal liver development, our results indicate that the later development of the liver is not dependent on MKK7–JNK–c-Jun signaling in hepatoblasts or hepatocytes.

MKK7 KO did not affect the regenerative proliferation of hepatocytes following PH or CCl_4 injury. Furthermore, EGF-stimulated cell cycle progression in hepatocytes *in vitro* also occurred normally in the absence of the *MKK7* gene. Wuestefeld et al.[8] proposed that MKK7 is crucial in hepatocyte proliferation and liver regeneration because their experimental findings suggested that *in vivo* knockdown of MKK4 facilitated liver regeneration through the induction of MKK7 expression. However, our results demonstrated that MKK4 protein expression is increased in regenerating livers following PH as well as in cultured hepatocytes and that hepatocytes robustly proliferate regardless of hepatocyte MKK7 expression, indicating that MKK4 suppression and subsequent MKK7 activation might not be required for hepatocyte proliferation. It is interesting to note that the increase in MKK7 protein expression was only modest in compensatory regeneration (PH), while there was a dramatic increase in MKK7 protein expression in reparatory regeneration following CCl_4-induced tissue destruction, suggesting important roles of hepatocyte MKK7 in response to parenchymal injury. In contrast, MKK4 protein expression was slightly increased in compensatory regeneration, but its levels were almost constant in reparatory regeneration, suggesting differential roles of MKK4 and MKK7. MKK7 KO did not affect the extent of liver damage following acute CCl_4 injury. This is consistent with a previous report showing that increased activity of MKK4, but not MKK7, is associated with the extent of acetaminophen-induced or tumor necrosis factor/galactosamine-induced liver injuries.[23]

The activity of JNK1 and JNK2 is low in quiescent hepatocytes, but liver injuries, such as PH[24] or CCl_4 injury,[25] have been shown to rapidly induce their activity. Our results demonstrated that MKK7 KO significantly attenuated the phosphorylation of JNKs and c-Jun following PH or CCl_4 injury, suggesting that activation of the JNK–c-Jun pathway might not be critical for hepatocyte proliferation. In fact, in mice with liver-specific disruption of JNK1 and JNK2 (Alb-Cre$^{-/+}$ Jnk1$^{LoxP/LoxP}$ Jnk2$^{-/-}$), liver regeneration has been demonstrated to be accomplished eventually, although there was transient suppression of hepatocyte proliferation at 2 days.[26] While it has been reported that mice with genetic deletion of JNK1 demonstrate decreased hepatocyte proliferation following PH,[27]this might be explained by the lack of bone marrow–derived cells with JNK1 activity, which mediate liver regeneration.[28]

Tissue repair processes following CCl_4 injury were delayed in MKK7 KO mice which had increased areas of residual fibrinogen-positive necrotic tissues after 4 days. This delay in tissue repair could induce protracted wound repair reactions in chronic CCl_4 injury, causing more intense liver fibrosis, as well as more enhanced regenerative proliferation of hepatocytes, in MKK7 KO mice than in control mice. This phenotypic change might be due to attenuated JNK signaling because liver-specific JNK KO (Alb-Cre$^{-/+}$ Jnk1$^{LoxP/LoxP}$ Jnk2$^{-/-}$) has been reported to promote liver injury, fibrosis, and inflammation in CCl_4-induced chronic liver injury.[29] Because there is a well-established relationship between chronic liver injury and tumorigenesis, it is important to examine the role of MKK7 in hepatocarcinogenesis. Our study demonstrated that the effect of MKK7 KO on hepatocarcinogenesis induced by chronic CCl_4 or TAA injury was marginal and at least not suppressive, suggesting that JNK–c-Jun signaling does not play important roles in the neoplastic growth of hepatocytes. Our data are consistent with those in a previous study showing that compound deficiency of JNK1 and JNK2 in hepatocytes does not prevent diethylnitrosamine-induced hepatocarcinogenesis.[26] However, because JNK1 has been shown to be required in chemically induced mouse hepatocarcinogenesis in studies using conventional JNK1 KO mice,[26,27] it is possible that JNK1 expression in nonparenchymal cells might be involved in tumorigenesis by providing an environment rich in inflammatory cytokines.

We observed that MKK7 KO hepatocytes demonstrated decreased branching morphogenesis in a three-dimensional collagen matrix. These results suggest that MKK7 is involved in the migration of hepatocytes within the extracellular matrix. Interestingly,

previous reports demonstrated that MKK7 deletion in the nervous system (Nestin-Cre$^{-/+}$ MKK7$^{LoxP/LoxP}$) delays radial migration of neurons in the cortex.[30] Furthermore, a similar phenotype has been documented in mice overexpressing a dominant-negative form of JNK[31] with deletion of the *MKK4* gene in the central nervous system.[32]

The JNK–c-Jun pathway is activated by inflammatory cytokines, such as TNF-α and TGF-β.[24,33] We reported that branching morphogenesis of spheroidal aggregates of rat and mouse hepatocytes within a three-dimensional collagen gel matrix is augmented by TNF-α but suppressed by SP600125.[10,34] A recent work also demonstrated that TNF-α-induced activation of the JNK–c-Jun pathway is important in the proliferation and tumorigenesis of bile duct cells, as well as cells transdifferentiated from hepatocytes.[35] Although TGF-β signaling has also been shown to be involved in ductular transdifferentiation of hepatocytes,[36] our study demonstrated that excessive TGF-β signaling might abort transdifferentiation. We hypothesize that the three-dimensional branching morphogenesis of hepatocytes includes complex cell–cell and cell–matrix interactions induced by inflammatory cytokines and therefore mimics tissue remodeling following injury *in vivo*.

We found that the mRNA expression of *Tagln*, *Glipr2*, and *Plau* was suppressed in MKK7 KO hepatocytes and that forced expression of these genes in MKK7 KO hepatocytes partially recovered the diminished branching morphogenesis within the collagen gel matrix. The mRNA expression of *Tagln* and *Plau* in cultured mouse hepatocytes was suppressed by SP600125, suggesting that their expression is regulated by the JNK signaling pathway, which is compatible with previous reports.[37,38] Tagln is an actin-binding protein that has multiple biological functions, including cell migration and the regulation of matrix metalloproteinase-9.[39] Of note, Glipr2 has been demonstrated to augment cell migration following epithelial–mesenchymal transition.[15] Importantly, plasminogen has been demonstrated to play critical roles in the recovery and remodeling from acute CCl_4-induced injury through the removal of fibrin-rich matrix and necrotic cells within injured areas.[17,40] Mice with plasminogen deficiency have been reported to exhibit more prominent liver fibrosis following chronic CCl_4 injury than control mice.[41] Furthermore, it has been shown that plasminogen activators, especially uPA, are required for proper reorganization of the damaged parenchyma.[42] Despite the toxic effect of uPA on hepatocytes,[16,17] our *in vivo* study demonstrated that hepatocyte-specific transduction of uPA rescued the delayed tissue repair following CCl_4 injury in MKK7 KO mice, suggesting its participation in tissue remodeling after parenchymal destruction.

In conclusion, our study demonstrates that MKK7 is not required for the regenerative proliferation of adult hepatocytes but plays an important role in repair processes and tissue remodeling following parenchymal destruction. The actions of MKK7 appear to be mediated by activation of the JNK–c-Jun pathway, which in turn activates multiple effectors, especially uPA, that are involved in cell migration and cell–extracellular matrix interactions.

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Author Contributions: T.O. designed and performed experiments, analyzed data, and wrote the paper; M.Y., K.F., B.X., K.W., M.G., and Y.O. performed experiments and analyzed data; A.S., J.M.P., and H.N. provided essential experimental materials and critically reviewed and edited the manuscript; Y.N. supervised the whole project, designed and performed experiments, analyzed data, and wrote the paper. All the authors approved the version to be published.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31565/suppinfo.