

## ORIGINAL ARTICLE

# Jnk1 in murine hepatic stellate cells is a crucial mediator of liver fibrogenesis

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**ABSTRACT**

**Objective** The c-Jun N-terminal kinase-1 (Jnk1) gene has been shown to be involved in liver fibrosis. Here, we aimed to investigate the molecular mechanism and define the cell type involved in mediating the Jnk1-dependent effect on liver fibrogenesis.

**Design** Jnk1<sup>fl/fl</sup> wildtype (WT), Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> (hepatocyte-specific deletion of Jnk1) mice were subjected to (i) bile duct ligation (BDL) and (ii) CCl<sub>4</sub>-induced liver fibrosis. Additionally, we performed bone marrow transplants (BMT), isolated primary hepatic stellate cells (HSCs), studied their activation in vitro and investigated human diseased liver samples.

**Results** Phosphorylated Jnk was expressed in myofibroblasts, epithelial and inflammatory cells during the progression of fibrogenesis in humans and mice. In mice, liver transaminases, alkaline phosphatase, bilirubin and liver histology revealed reduced injury in Jnk1<sup>-/-</sup> compared with WT and Jnk1<sup>Δhepa</sup> mice correlating with lower hepatocyte cell death and proliferation. Consequently, parameters of liver fibrosis such as Sirius red staining and collagen IA1 and α-smooth muscle actin expression were downregulated in Jnk1<sup>-/-</sup> compared with WT and Jnk1<sup>Δhepa</sup> livers, 4 weeks after CCl<sub>4</sub> or BDL. BMT experiments excluded bone marrow-derived cells from having a major impact on the Jnk1-dependent effect on fibrogenesis, while primary HSCs from Jnk1<sup>-/-</sup> livers showed reduced transdifferentiation and extracellular matrix production. Moreover, Jnk1 ablation caused a reduced lifespan and poor differentiation of HSCs into matrix-producing myofibroblasts.

**Conclusions** Jnk1 in HSCs, but not in hepatocytes, significantly contribute to liver fibrosis development, identifying Jnk1 in HSCs as a profibrotic kinase and a promising cell-directed target for liver fibrosis.

**INTRODUCTION**

Hepatic fibrosis is a dynamic and reversible process and a major cause of morbidity and mortality worldwide.<sup>1–3</sup> After acute hepatocellular injury, parenchymal cells regenerate and replace the necrotic or apoptotic tissue, a process that is associated with an inflammatory response and a limited deposition of extracellular matrix (ECM). In contrast, chronic liver injury triggers a persistent wound-healing response and continuously increases ECM protein deposition.<sup>4</sup> Advanced fibrosis is characterised by accumulation of activated α-smooth muscle actin (αSMA)-positive periportal

**Significance of this study****What is already known about this subject?**

- The Jnk signalling pathway has been implicated in many pathological conditions, including cancer, stroke, heart disease and liver fibrosis.
- Jnk1 has been linked with the development of hepatocyte death, hepatic inflammation, steatohepatitis and fibrogenesis.
- Jnk contributes to α-smooth muscle actin expression during hepatic stellate cells (HSCs) activation and differentiation, which suggests a potential role for Jnk in this cell type.

**What are the new findings?**

- Jnk1 in hepatocytes has no major impact on the progression of liver fibrosis and injury; however, Jnk1 in non-parenchymal cells is of major relevance.
- Jnk1 in bone marrow-derived cells has no significant effect on liver fibrosis and injury.
- Jnk1 is an essential profibrogenic kinase in HSCs and portal myofibroblasts by mediating transactivation and inhibiting apoptosis of HSCs.

**How might it impact on clinical practice in the foreseeable future?**

- These results provide the molecular basis to consider cell-type-specific Jnk1 inhibition in HSCs as an attractive concept to treat liver fibrosis.

and perisinusoidal myofibroblasts that lead to scar formation composed of collagens (predominantly collagens I and III). The cell types and patterns of fibrosis are different in the various forms of liver diseases. In biliary diseases, portal myofibroblasts are the main cell types causing periportal fibrosis and portal–portal septa, while in hepatocytic damage, mainly activated hepatic stellate cells (HSCs) are involved in fibrosis.<sup>1–5</sup>

Liver injury activates Jnk—members of the mitogen-activated protein kinase family. Jnk1 and Jnk2 are ubiquitously found, including the liver, while Jnk3 is exclusively expressed in the central nervous system, testis and heart.<sup>6</sup> Several authors have demonstrated that Jnk contributes to αSMA

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expression during HSCs activation and differentiation, which suggests a potential role for Jnk in liver fibrogenesis.<sup>2-7</sup> However, the specific role of the Jnk genes, and specifically of Jnk1, during liver fibrogenesis is not yet defined.

Therefore, the present study aimed to characterise and define the cell-type-specific contribution of Jnk1 activation during liver fibrosis *in vivo*. We addressed this task by combining general and hepatocyte-specific Jnk1 knockout animals. By including bone marrow transplants (BMT) and isolation of primary HSCs, we show that Jnk1 in HSCs, but not in hepatocytes, plays a crucial role during liver fibrogenesis, thus identifying Jnk1 as a profibrotic kinase.

### METHODS

#### Jnk1 chimeric mice and experimental models of liver fibrosis

Animal husbandry and procedures were approved by the authority for environment conservation and consumer protection of the state of North Rhine-Westphalia (LANUV, Germany) and the University Hospital RWTH Aachen Animal Care Facility's guidelines. Jnk1-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Jnk1<sup>f/f</sup> controls (wildtype (WT)) were crossed with transgenic animals expressing the Cre transgene giving yield to the Jnk1<sup>Δhepa</sup> strain, mice with specific deletion of Jnk1 only in hepatocytes as previously described.<sup>8-10</sup> Induction of liver fibrosis was performed in 7–8-week-old age-matched male mice (n=9–10 per group) by ligating the common bile duct (BDL) or treatment with CCl<sub>4</sub> (0.6 mL/kg body weight, diluted in corn oil, injected intraperitoneally every 3 days) during 4 weeks. Control animals were opened and immediately closed (sham) or corn oil-injected, respectively.

#### Isolation of primary hepatocytes

Primary hepatocytes were isolated by perfusing the portal vein of the liver with Earle's balanced salt solution EBSS containing 100 mM ethylene glycol tetraacetic acid pH 8.3, followed by an EBSS solution containing 20 mg collagenase-D and 2 mg trypsin inhibitor. Hepatocytes ( $5 \times 10^5$ / well) were cultured in Dulbecco's modified medium with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics on six-well plates with pre-coated and phosphate-buffered saline (PBS) washed 2.5 mg/mL type I collagen.

### RESULTS

#### Jnk activation in chronic liver disease in humans and mice

Hyperactivation of the Jnk pathway in the liver has been associated with the development of inflammation, metabolic disorders (including obesity, steatosis and insulin resistance), fibrosis and hepatocellular carcinoma (HCC).<sup>11</sup> We thus analysed pJnk expression in different stages of human liver fibrosis and cirrhosis (see online supplementary figure S1A). We found strong upregulation of pJnk in myofibroblasts of human diseased liver (see online supplementary figure S1A, upper panel). pJnk expression was also evident in infiltrating cells (see online supplementary figure S1A, lower panel). To evaluate the activation of Jnk in human myofibroblasts during human liver disease, we performed costaining for pJnk and αSMA. We observed that activated myofibroblasts elicit Jnk activation during chronic liver disease (figure 1A). Additionally, coimmunostaining of pJnk and mouse monoclonal antibody to mature granulocytes (BM2), an antibody that recognises mature granulocytes, identified inflammatory cells as pJnk-positive in human diseased liver (figure 1B). Thus, Jnk is activated in the fibrotic compartment and in

inflammatory cells during the progression of liver disease in humans.

Previous studies described the presence of pJnk-positive hepatocytes during liver injury.<sup>12</sup> To differentiate between the different cell types in vivo, we generated hepatocyte-specific Jnk1 knockout mice (Jnk1<sup>Δhepa</sup>). Besides Jnk1<sup>Δhepa</sup> animals, constitutive Jnk1<sup>-/-</sup> and respective littermate controls (floxes) were included. Primary hepatocytes were isolated and Jnk1 protein expression was assessed, showing lack of Jnk1 expression in primary hepatocytes of Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> in contrast to WT mice (see online supplementary figure S1B). Additionally, PCR of tail DNA for Jnk1 confirmed the respective knockout strain (see online supplementary figure S1C).

We next investigated the phosphorylation status of Jnk in murine models of chronic liver injury. We performed coimmunostaining for F4/80 (orange) and pJnk (black) in WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> mice after repetitive injections of CCl<sub>4</sub> or BDL surgery for 28 days (figure 1C,D). After CCl<sub>4</sub> injection, we found periportal pJnk expression in WT and Jnk1<sup>Δhepa</sup> mice (figure 1C), while we also detected peribiliary pJnk expression after BDL in WT and Jnk1<sup>Δhepa</sup> mice (figure 1D). In contrast, Jnk1<sup>-/-</sup> mice displayed no significant pJnk expression (figure 1C,D).

#### Jnk1 in hepatocytes has no impact on acute and chronic hepatic injury during experimental liver fibrosis

We next evaluated the relevance of Jnk1 for acute liver injury after BDL or CCl<sub>4</sub> treatment (figure 2A,B). After 48 h, BDL-treated mice of all strains included showed no differences in serum aspartate aminotransferase (AST), while liver injury was significantly reduced in Jnk1<sup>-/-</sup> mice 48 h after CCl<sub>4</sub> compared with WT and Jnk1<sup>Δhepa</sup> animals (figure 2A,B).

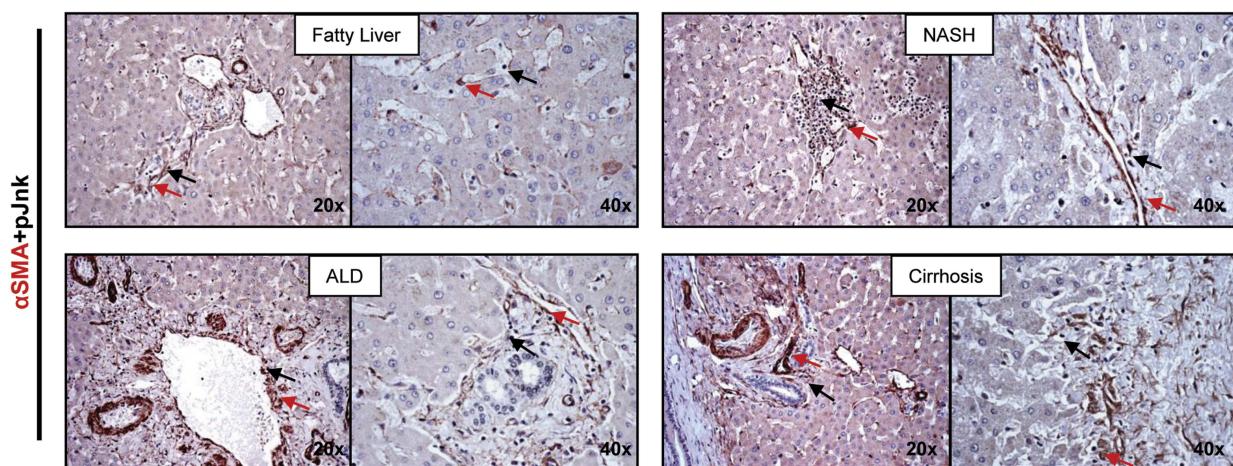
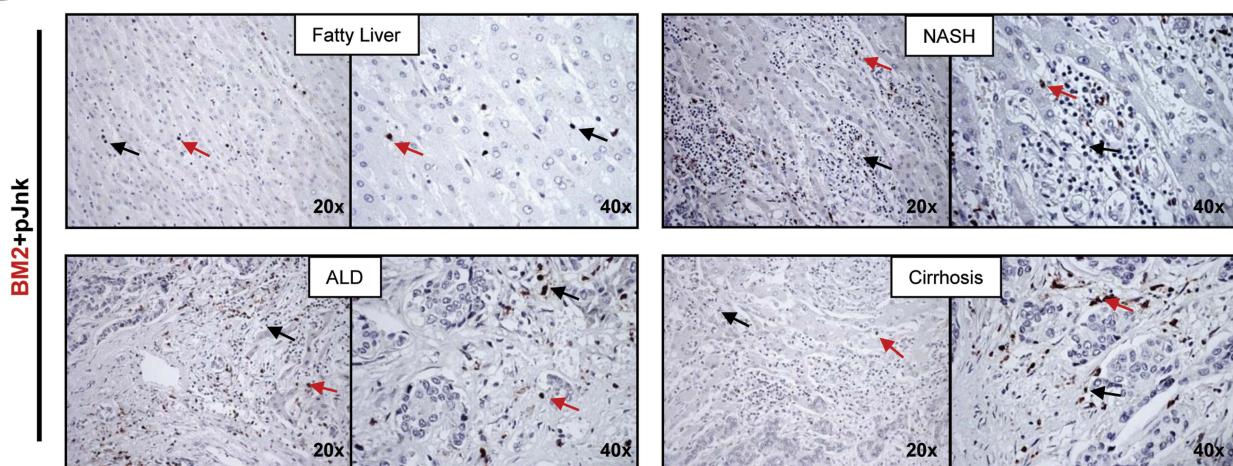
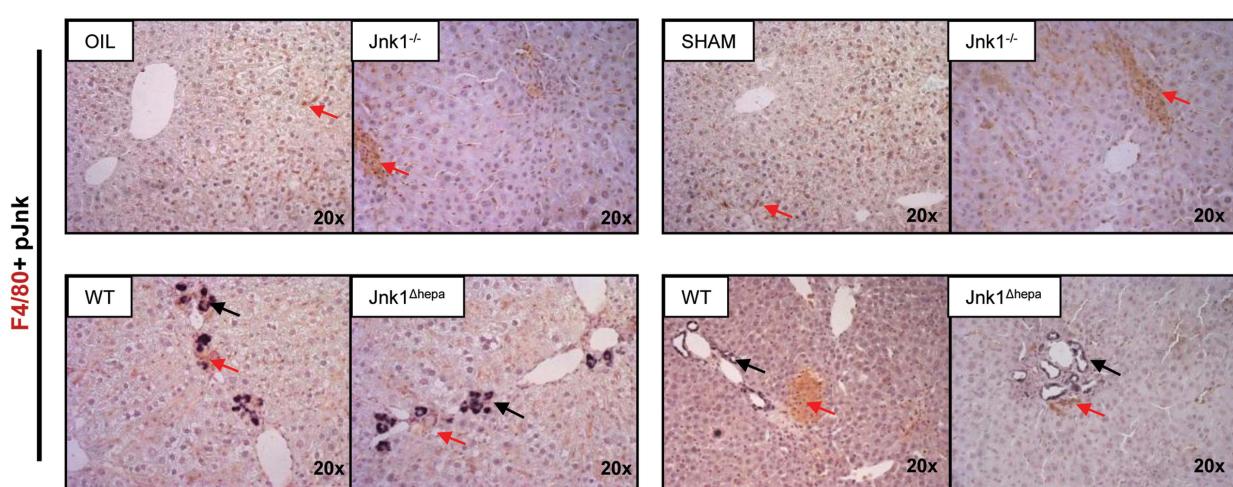
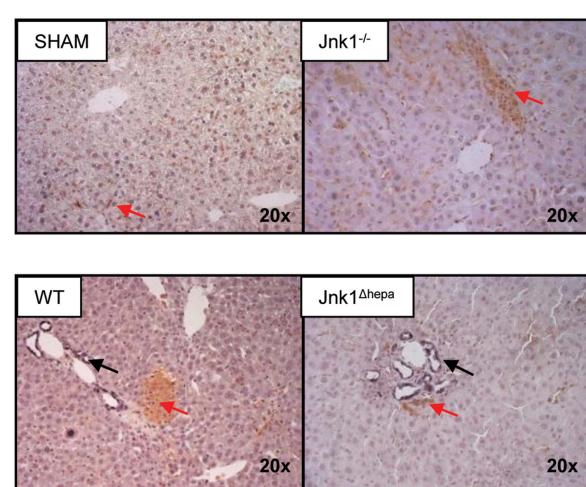
Since BDL triggers liver damage through biliary fibrosis, we analysed biochemical markers at 14 and 28 days after BDL. Plasma AST and alanine aminotransferase (ALT) levels increased approximately 10-fold in Jnk1<sup>Δhepa</sup> and WT but only 7-fold in Jnk1<sup>-/-</sup> mice compared with respective sham-operated animals at both time points (figure 2C, see online supplementary figure S1D). Given that changes in transaminase levels between the animal strains were similar at both time points and a longer time point is more suitable to study liver fibrogenesis, we selected 28 days after BDL for further analysis.

BDL-Jnk1<sup>Δhepa</sup> and WT mice exhibited significantly more cholestasis as characterised by hyperbilirubinemia with yellowish skin and alkaline phosphatase (AP) compared with Jnk1<sup>-/-</sup> mice (figure 2D, see online supplementary figure S1E). H&E staining of WT and Jnk1<sup>Δhepa</sup> livers showed severe cholestatic hepatitis with widespread bile infarcts—coincident with necrotic foci—along with bile ductular proliferation, portal oedema and hepatocellular damage 28 days after BDL (figure 2E). In contrast, Jnk1<sup>-/-</sup> mice exhibited signs of ductular proliferation but no necrotic foci (figure 2E). Moreover, biliary infarcts in Jnk1<sup>-/-</sup> mice compared with WT and Jnk1<sup>Δhepa</sup> 4 weeks after BDL were dramatically reduced (see online supplementary figure S1F).

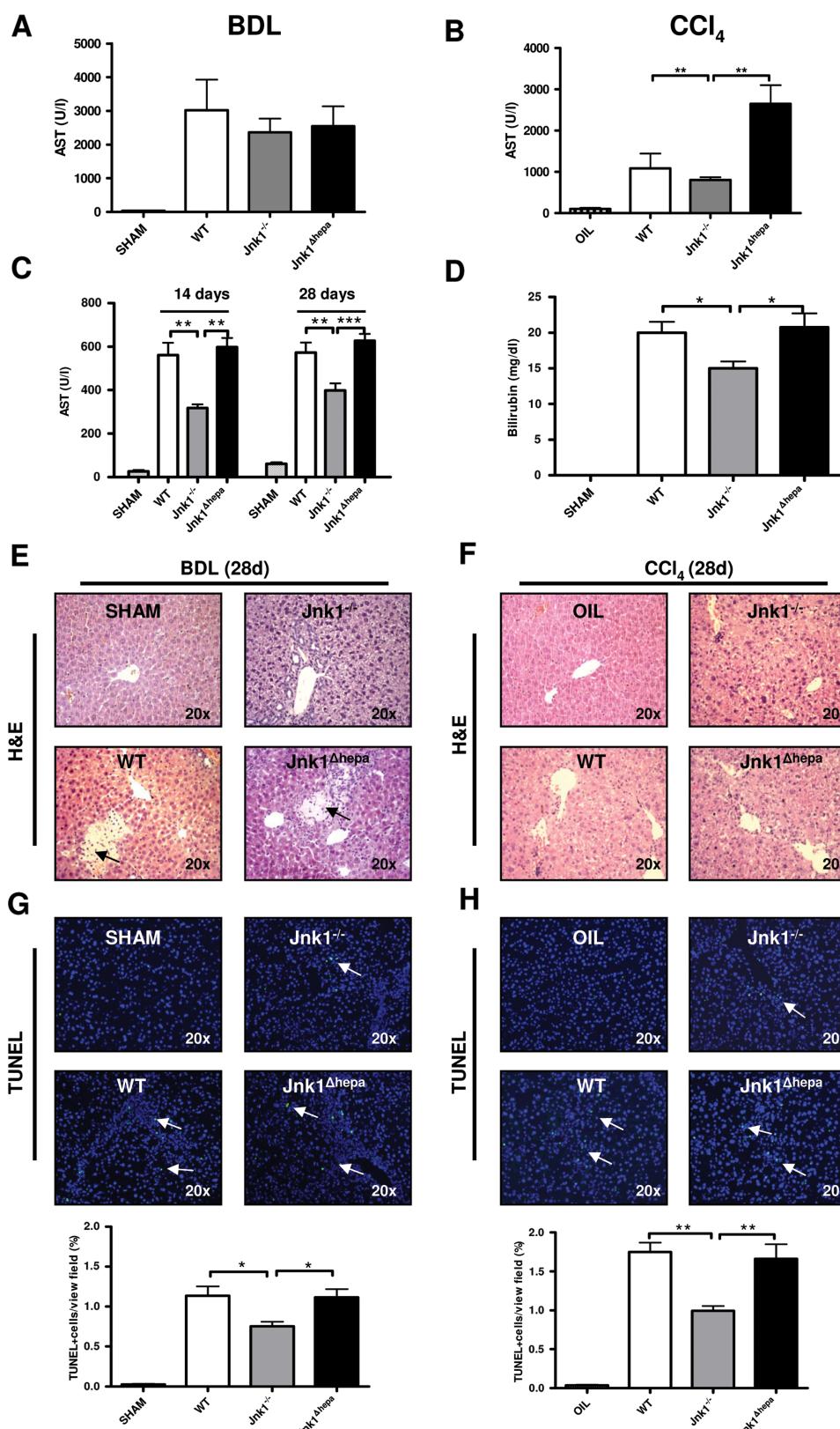
Next, WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> mice were treated with CCl<sub>4</sub> for 4 weeks. However, H&E staining of Jnk1<sup>Δhepa</sup> and WT livers revealed more cellular damage and centrilobular congestion of inflammatory cells compared with Jnk1<sup>-/-</sup> mice (figure 2F). Indeed, macroscopic examination of these 28-day-CCl<sub>4</sub>-subjected liver specimens revealed the presence of necrotic foci in distinct hepatic lobes with yellowish colour in WT and Jnk1<sup>Δhepa</sup> mice (see online supplementary figure 1G).

Both cholestatic injury and toxic challenge to the liver promote hepatocyte death. Thus, we examined the death of hepatic cells by TdT-mediated dUTP-biotin nick end labelling

## Human Liver Disease

**A****B****C****CCl<sub>4</sub> (28d)****D****BDL (28d)**

**Figure 1** Activation of Jnk is characteristic of patients with chronic liver disease and murine models of experimental liver injury. (A and B). Coexpression of pJnk (black) and α-smooth muscle actin (A) or pJnk (black) and mouse monoclonal antibody to mature granulocytes (BM2) (B). A specific marker of mature granulocytes (orange) was assessed in human liver samples with different stages of liver disease (C and D). Immunohistochemistry staining on paraffin sections for F4/80 (orange) and pJnk (black) was performed in wildtype, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers 28 days after CCl<sub>4</sub> treatment (C) or bile duct ligation (D), respectively. Arrows (→) indicate positive staining. Values are mean±SEM from at least 6–8 mice per group (\*\*p<0.01).



**Figure 2** Hepatic injury is mediated by non-parenchymally derived Jnk1 in experimental liver fibrosis. (A and B) Serum levels of aspartate aminotransferase (AST) were determined 48 h after bile duct ligation (BDL) (A) and CCl<sub>4</sub> (B) in wildtype (WT), Jnk $^{−/−}$  and Jnk $^{Δhepa}$  mice. (C and D) Levels of AST (C) and bilirubin (D) were determined in serum at 14 and 28 days after BDL in WT, Jnk $^{−/−}$  and Jnk $^{Δhepa}$  mice. (E) Representative H&E staining of liver sections from WT, Jnk $^{−/−}$  and Jnk $^{Δhepa}$  livers 28 days after BDL surgery. Sham mice were used as controls. (F) Representative H&E staining of liver sections of WT, Jnk $^{−/−}$  and Jnk $^{Δhepa}$  livers 28 days after repeated injections of CCl<sub>4</sub>. Corn-oil injections were used as controls. Arrows (→) indicate necrotic foci. (G and H) Representative TdT-mediated dUTP-biotin nick end labelling (TUNEL) staining performed on frozen liver sections after 4 weeks of BLD (G) or repeated CCl<sub>4</sub> injections (H) are shown. Quantification of TUNEL-positive cells was performed relative to total nuclei (DAPI). Arrows (→) indicate TUNEL-positive cells. Values are mean±SEM from at least 8–10 mice per group (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).

(TUNEL) staining in both experimental models of liver injury. After BDL, the percentage of TUNEL-positive cells in WT and *Jnk1*<sup>Δhepa</sup> livers was 1.8±0.3 and 1.6±0.4, 28 days after BDL, respectively. In contrast, *Jnk1*<sup>−/−</sup> livers revealed significantly less TUNEL-positive cells (1.0±0.1) (figure 2G). Similarly, *Jnk1*<sup>−/−</sup> animals showed significantly less TUNEL-positive cells than WT and *Jnk1*<sup>Δhepa</sup> mice 28 days after CCl<sub>4</sub> treatment (figure 2H).

Altogether, these data indicate that *Jnk1* expression in hepatocytes has no impact on acute and chronic hepatocellular injury in two different models of chronic experimental liver injury.

### Non-hepatocytic *Jnk1* determines cell proliferation after BDL and CCl<sub>4</sub> treatment

Acute and chronic liver injury is followed by a phase of hepatocyte proliferation in order to restore liver mass.<sup>13</sup> Thus, we investigated changes in the expression of cell cycle markers. Proliferating cell nuclear antigen (PCNA), cyclin D and cyclin A levels were strongly upregulated in *Jnk1*<sup>Δhepa</sup> and WT livers 4 weeks after repetitive CCl<sub>4</sub> treatment or after BDL, respectively (figure 3A,B). In contrast, proliferation markers were significantly lower in *Jnk1*<sup>−/−</sup> mice, suggesting that *Jnk1* in non-parenchymal cells in both experimental models is required for compensatory proliferation (figure 3A,B). These results were further corroborated at the mRNA level. PCNA and cyclin D were highly expressed in *Jnk1*<sup>Δhepa</sup> and WT livers after chronic liver injury and significantly lower in *Jnk1*<sup>−/−</sup> mice (figure 3C, F). Moreover, quantification of cell proliferation with Ki-67 immunostaining demonstrated lower proliferation rates in *Jnk1*<sup>−/−</sup> compared with *Jnk1*<sup>Δhepa</sup> and WT livers treated with CCl<sub>4</sub> or BDL for 4 weeks (figure 3G,H).

### *Jnk1* in non-parenchymal cells promotes liver fibrogenesis

We now analysed fibrosis progression by measuring αSMA and collagen IA1 protein and mRNA levels after chronic BDL or CCl<sub>4</sub> treatment (figure 4A,F, see online supplementary figure 2A,B). *Jnk1*<sup>−/−</sup> livers show reduced αSMA and collagen IA1 expression compared with *Jnk1*<sup>Δhepa</sup> and WT animals in both experimental models. These findings were further validated by Sirius red staining. Indeed, repeated CCl<sub>4</sub> injections induced severe collagen deposition and septum formation in *Jnk1*<sup>Δhepa</sup> and WT livers, classified by two independent pathologists as bridging fibrosis (figure 4G). In contrast, quantification of Sirius red staining revealed significantly lower collagen fibre formation in *Jnk1*<sup>−/−</sup> mice (figure 4G). The essential role of *Jnk1* in non-hepatocytes for fibrosis progression was also found in the BDL model. *Jnk1*<sup>−/−</sup> livers showed reduced signs of fibrosis compared with *Jnk1*<sup>Δhepa</sup> and WT mice 4 weeks after BDL (figure 4H).

Since inflammatory mediators are key regulators in the development of liver fibrosis, we hypothesised that decreased inflammation after deletion of *Jnk1* could be linked to reduced levels of cytokines. Indeed, TNFα, TGFβ and IL-1β were decreased in *Jnk1*<sup>−/−</sup> compared with *Jnk1*<sup>Δhepa</sup> and WT livers 4 weeks after repeated CCl<sub>4</sub> injections or BDL (see online supplementary figure 3A,F). Conclusively, these findings support a role for *Jnk1* in non-parenchymal cells in the development of hepatic fibrosis.

### *Jnk1* expression in bone marrow-derived cells has no impact on the progression of liver fibrosis

Our results revealed that *Jnk1* is crucial for liver fibrogenesis, but other cells than hepatocytes mediate this effect. Hence, we investigated whether bone marrow-derived cells (BMDC) are essential in promoting liver fibrosis and thus performed BMT experiments. WT and *Jnk1*<sup>−/−</sup> mice were lethally irradiated and reconstituted with either *Jnk1*<sup>−/−</sup> or WT BM. Chimerism in the liver

was confirmed by PCR, demonstrating successful BM reconstitution (see online supplementary figure 4A,B and methods).

BDL-treated chimeric WT mice reconstituted with WT (WT→WT) or *Jnk1*<sup>−/−</sup> BM (*Jnk1*<sup>−/−</sup>→WT) showed macroscopically yellowish dots on their surface (figure 5A), which could be identified as bile infarcts by histological examination (figure 5B, see online supplementary figure S4C). After BDL, the quantification of bile infarcts revealed larger number of infarcts in WT recipients independent of the origin of the donor BM, whereas *Jnk1*<sup>−/−</sup> recipients showed significant protection (figure 5C). These results were strengthened measuring serum markers of injury such as liver transaminases, bilirubin and AP (figure 5D,F, see online supplementary figure S4D).

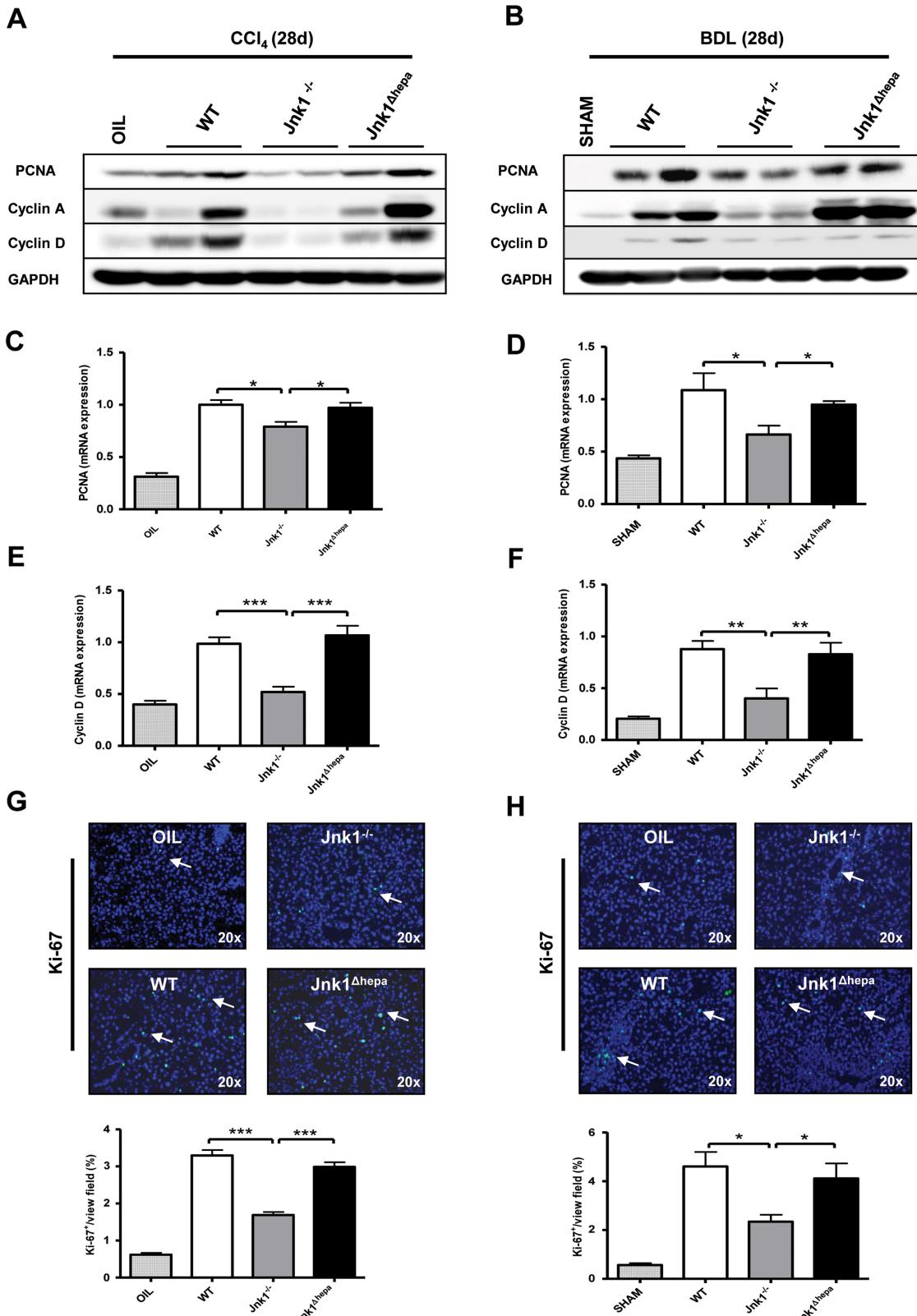
Next, we evaluated the impact on liver fibrosis after CCl<sub>4</sub> or BDL and BMT by Sirius red staining (see online supplementary figure S5A,B). Quantification of Sirius red-positive areas revealed that *Jnk1*<sup>−/−</sup> compared with WT recipients showed significantly less liver fibrosis in both models of experimental liver fibrosis (see online supplementary figure 5A,B). These results were further confirmed after analysing collagen deposition by collagen IA1 immunofluorescence (see online supplementary figure 5C,D).

### Genes involved in matrix formation are downregulated in *Jnk1*<sup>−/−</sup> mice

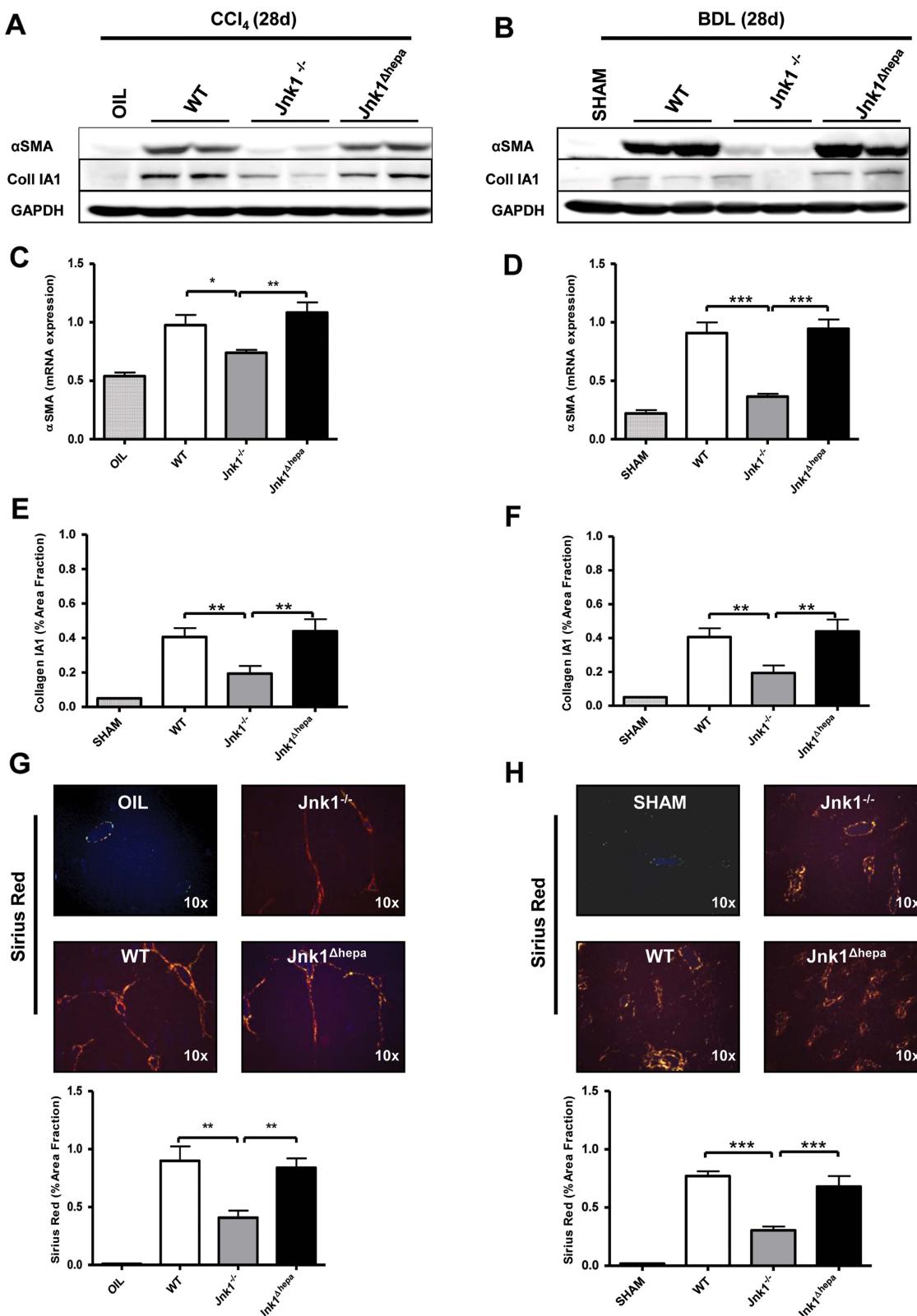
Our results excluded the possibility that BM-derived cells or hepatocytes are involved in mediating the profibrogenic effects of *Jnk1*. We thus performed microarray analysis to potentially link the gene profile to a specific liver cell type. Our experimental approach allowed the detection of changes in mRNA expression of genes associated with liver fibrosis in WT, *Jnk1*<sup>−/−</sup> and *Jnk1*<sup>Δhepa</sup> mice 28 days after BDL (figure 6A). The results as depicted in figure 6A show that genes involved in HSCs activation, hepatic fibrogenesis and liver damage exhibit major changes between the knockout strains. HSCs marker genes such as *raet1d* or genes associated with hepatic fibrosis, for example, *mmd2*, *gas2*, *rps11* and *tead-2*, were differentially regulated (fold change) in *Jnk1*<sup>−/−</sup> compared with WT and *Jnk1*<sup>Δhepa</sup> livers. Hence, our microarray data suggested that genes directly involved in matrix formation showed specific differences in *Jnk1*<sup>−/−</sup> compared with *Jnk1*<sup>Δhepa</sup> and WT livers 28 days after BDL.

We thus analysed αSMA expression in liver paraffin sections to study HSCs activation during liver injury (figure 6B,C). We found that lobular-activated αSMA HSCs in *Jnk1*<sup>−/−</sup> mice were less in number compared with WT and *Jnk1*<sup>Δhepa</sup> mice and that the septa are more slender, 28 days after CCl<sub>4</sub> treatment (figure 6B). Indeed, αSMA-positive-activated HSCs were observed in a perisinusoidal pattern and formed fine portal-central septa (figure 6B). Moreover, 28 days after BDL, αSMA-positive cells were mainly portal myofibroblasts, surrounding ductular proliferation areas (figure 6C). Periportal fibrosis and portal–portal septa were formed in WT and *Jnk1*<sup>Δhepa</sup> mice and were strongly reduced in *Jnk1*<sup>−/−</sup> livers (figure 6C), suggesting that HSCs are directly involved in mediating the *Jnk1*-dependent profibrogenic effect on liver fibrogenesis.

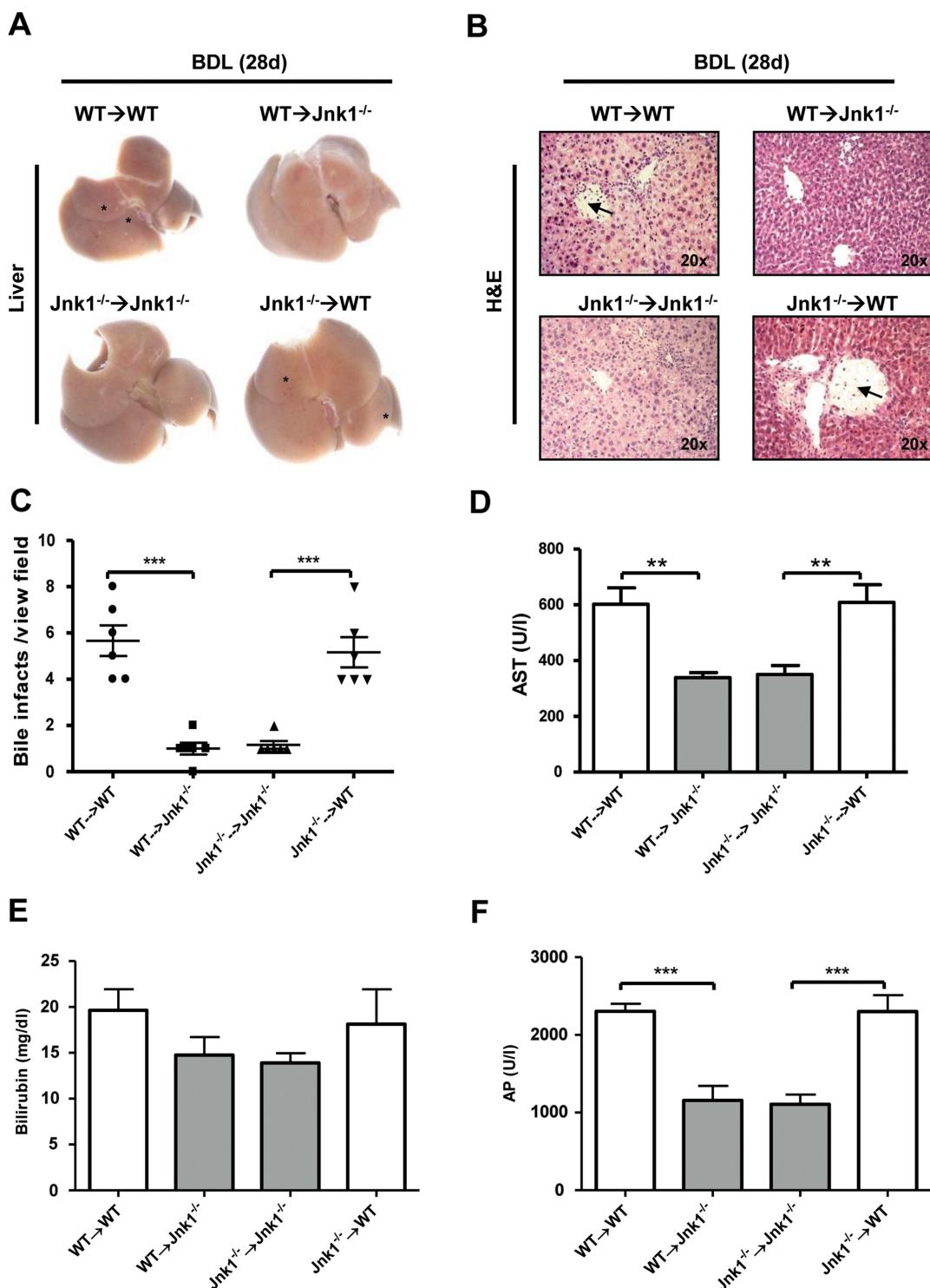
Additionally, we performed CK19 staining in liver sections to investigate the pattern of ductular reaction during BDL-triggered liver injury (figure 6D). WT and *Jnk1*<sup>Δhepa</sup> mice presented larger extension of the portal tract compared with the *Jnk1*<sup>−/−</sup> mice. Moreover, necrotic areas and periportal CK-19-positive hepatocytes, a sign of periportal cholestasis, were present in WT and *Jnk1*<sup>Δhepa</sup> mice but not in *Jnk1*<sup>−/−</sup> mice (figure 6D).



**Figure 3** Non-hepatocyte Jnk1 controls proliferation during chronic liver injury. (A and B) Extracts from wildtype (WT), Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers were prepared 28 days after CCl<sub>4</sub> treatment (A) or bile duct ligation (BDL) (B) and were examined by immunoblot analysis using proliferating cell nuclear antigen (PCNA), Cyclin A, cyclin D and GAPDH antibodies as indicated. (C and D) mRNA expression analysis of PCNA was quantified by RT-PCR of samples taken from WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers sacrificed after CCl<sub>4</sub> treatment (C) or BDL (D). (E and F) The same samples were subjected to RT-PCR to analyse the expression of cyclin D in WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers sacrificed 28 days after CCl<sub>4</sub> treatment (E) or BDL (F). (G and H) Representative immunofluorescence for Ki-67 of liver cryosections from WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers after 4 weeks of repeated CCl<sub>4</sub> injections (G) or BDL (H). Quantification of Ki-67-positive cells was performed using Image J Software and represented as Ki-67-positive cells relative to DAPI nuclei and powerfield. Arrows (→) indicate Ki-67-positive cells. Values are mean±SEM from at least 8–10 mice per group (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).



**Figure 4** Jnk1 in non-parenchymal cells but not in hepatocytes promotes liver fibrogenesis. (A and B) Extracts from wildtype (WT), Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers prepared 28 days after CCl<sub>4</sub> treatment (A) or bile duct ligation (BDL) (B) were examined by immunoblot analysis by probing with antibodies to α-smooth muscle actin (αSMA), collagen IA1 and GAPDH. (C and D) mRNA expression analysis of αSMA was quantified by RT-PCR of samples taken from WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers sacrificed after CCl<sub>4</sub> treatment (C) or BDL (D). (E and F) mRNA expression analysis of collagen IA1 was quantified by RT-PCR in the same livers that underwent CCl<sub>4</sub> treatment (E) or BDL (F). (G and H) Representative Sirius red staining of paraffin sections from WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers after 4 weeks of CCl<sub>4</sub> treatment (G) or BDL (H). Quantification of Sirius red staining was performed using Image J Software and represented as percentage of area fraction. Values are mean±SEM from at least 8–10 mice per group (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).

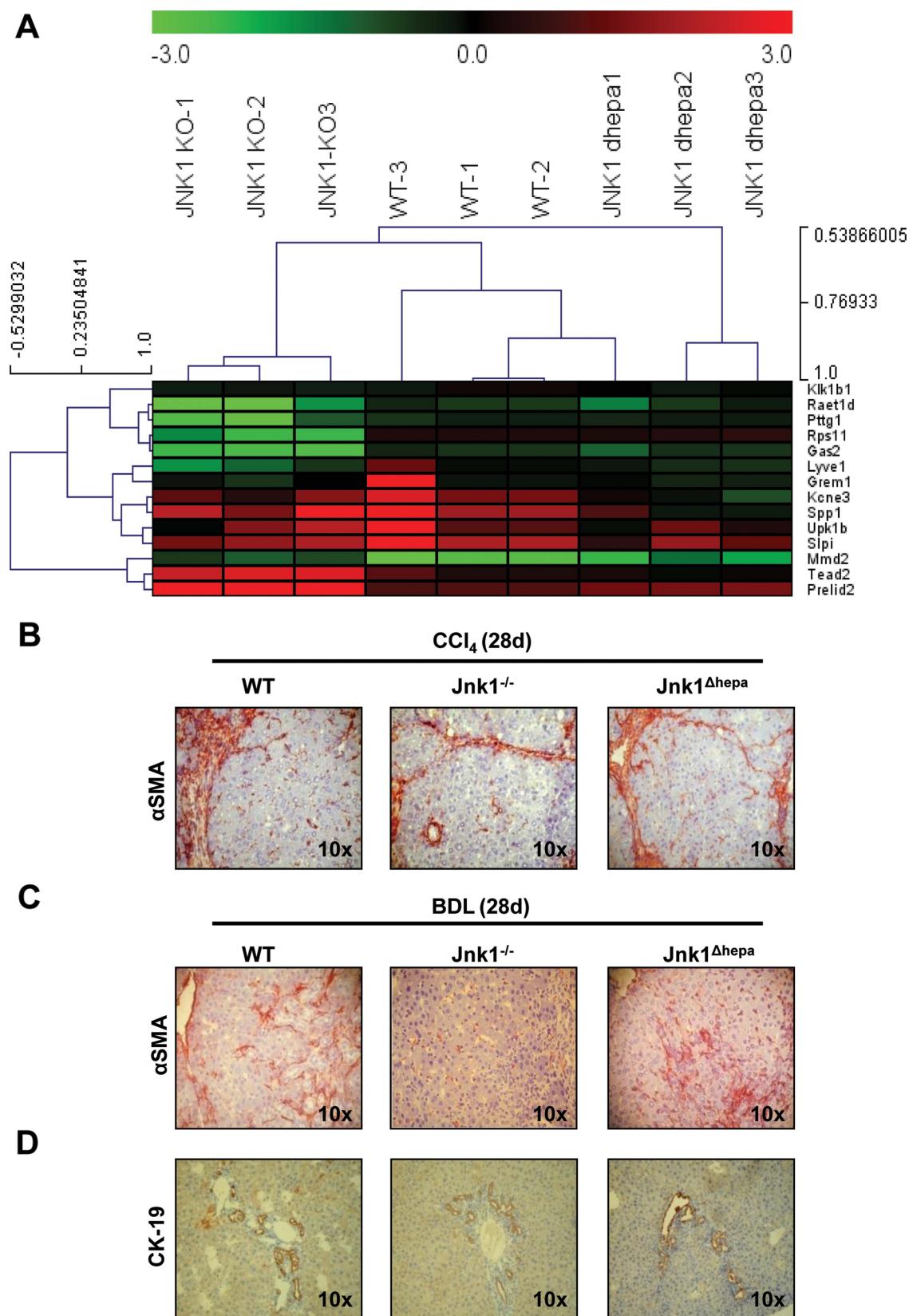


**Figure 5** Jnk1 in haematopoietic cells is not a source of liver injury. (A and B) Macroscopic appearance of wildtype (WT) or Jnk1<sup>-/-</sup> mice transplanted with Jnk1<sup>-/-</sup> or WT bone marrow cells. (A) Mice were subjected to bile duct ligation (BDL) and were sacrificed 28 days later. \* indicates yellow dots on liver surface. (B) Representative H&E staining of liver sections from these mice is shown. (C) Bile infarcts corresponding to the yellow dots observed on the liver surface and histologically assigned as necrotic foci by two independent expert liver pathologists. (D) Quantification of bile infarcts in WT and Jnk1<sup>-/-</sup> livers, transplanted with Jnk1<sup>-/-</sup> or WT bone marrow cells and consecutively BDL for 28 days, was performed blinded by a pathologist. (E) Serum levels of aspartate aminotransferase were determined after bone marrow transplantations (BMT)+BDL in Jnk1<sup>-/-</sup> or WT mice. (F) Bilirubin and (G) alkaline phosphatase serum levels were determined after BMT+BDL in Jnk1<sup>-/-</sup> or WT mice as indicated. Values are mean±SEM from at least 6–8 mice per group (\*\*p<0.01; \*\*\*p<0.001).

#### Jnk1 is essential for HSCs transdifferentiation

The array analysis and immunohistochemistry suggested differences in HSCs activation during chronic liver injury; we thus

tested the importance of Jnk1 for HSCs transdifferentiation into myofibroblasts. Primary HSCs from WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> mice were isolated using a FACSaria-based protocol (see online



**Figure 6** Microarray of genes involved in matrix formation and deposition are decreased in *Jnk1*<sup>-/-</sup> mice. (A) Microarray was performed from wildtype (WT), *Jnk1*<sup>-/-</sup> and *Jnk1*<sup>Δhepa</sup> livers 28 days after bile duct ligation (BDL). Log2 expression values of the individual mice were divided by the mean of the sham-operated mice. Log ratios were saved in a .txt file and analysed with the Multiple Experiment Viewer. The selected top-regulated, upregulated and downregulated genes are shown (red: upregulated; green: downregulated, n=3, fold change -3.0 to 3.0). (B and C) Immunohistochemistry was performed on paraffin sections for α-smooth muscle actin (αSMA) in livers of WT, *Jnk1*<sup>-/-</sup> and *Jnk1*<sup>Δhepa</sup> 28 days after CCl<sub>4</sub> (B) or BDL treatment (C). (D) Immunohistochemistry staining on paraffin sections for CK-19 of WT, *Jnk1*<sup>-/-</sup> and *Jnk1*<sup>Δhepa</sup> livers 28 days after BDL treatment.

supplementary figure S6A).<sup>14</sup> In these cells, proliferation, apoptosis and transdifferentiation were studied in vitro over a time period of 10 days. We first investigated pJnk and  $\alpha$ SMA expression in WT HSCs. Our results clearly demonstrated that  $\alpha$ SMA expression and Jnk activation were associated with HSCs transdifferentiation (figure 7A).

Next, we quantified the number of HSCs throughout time in vitro. The total number of living WT and Jnk1 $^{\Delta\text{hepa}}$  HSCs increased continuously up to 10 days, whereas proliferation in Jnk1 $^{-/-}$  HSCs was significantly attenuated (figure 7B, see online supplementary figure S6B). These differences were further confirmed using immunofluorescence stainings for Ki-67 and  $\alpha$ SMA. HSCs derived from Jnk1 $^{-/-}$  mice showed significantly less Ki-67 and  $\alpha$ SMA expression compared with HSCs derived from WT or Jnk1 $^{\Delta\text{hepa}}$  livers (figure 7B). Additionally, we performed TUNEL analysis of seeded HSCs up to 10 days after isolation. In these experiments, HSCs derived from Jnk1 $^{-/-}$  livers were more prone to cell death compared with Jnk1 $^{\Delta\text{hepa}}$  or WT cells (see online supplementary figure 6C,D).

Subsequently, we quantified mRNA expression of  $\alpha$ SMA, collagen IA1 and PDGFR- $\beta$ , which are all known to be activated during HSCs transdifferentiation. HSCs from Jnk1 $^{-/-}$  mice showed downregulation of  $\alpha$ SMA (see online supplementary figure S6E), collagen IA1 and PDGFR- $\beta$  compared with WT and Jnk1 $^{\Delta\text{hepa}}$  animals (figure 7C,D). Altogether, in vitro experiments with primary HSCs confirmed the in vivo studies defining Jnk1 as an essential factor involved in HSCs activation and transdifferentiation.

### **Adenoviral-mediated Jnk1 deletion in primary HSCs prevents transdifferentiation and promotes apoptosis**

We next aimed to delete Jnk1 expression in HSCs to better define its relevance for transdifferentiation into myofibroblasts. To this end, we isolated primary HSCs from Jnk1 floxed mice (Jnk1 $^{ff}$ ) and infected these cells 2 days after plating with adenoviruses coexpressing either cre-recombinase and enhanced green fluorescent protein (EGFP) (AdvCRE) or EGFP only (AdvCTRL). Transactivation and differentiation of HSCs into myofibroblasts were studied 7 and 10 days after plating, respectively (figure 8A,F, see online supplementary figure 7A,C).

AdvCRE-mediated deletion of Jnk1 resulted in poor survival of HSCs, which was most evident 10 days after plating (see online supplementary figure S7A) but already visible after 7 days (figure 8A). Efficient adenoviral infection was confirmed by determining EGFP fluorescence (figure 8B). Targeted AdvCRE-mediated deletion of Jnk1 in HSCs was associated with significantly reduced transactivation but strongly enhanced apoptosis as evidenced by measurement of Ki-67 and  $\alpha$ SMA expression and TUNEL analysis (figure 8C,D, see online supplementary figure 7B,C). Gene expression analysis from AdvCRE- or AdvCTRL-infected Jnk1 $^{ff}$  HSCs and untreated controls confirmed that targeted deletion of Jnk1 in HSCs resulted in a dramatic reduction of  $\alpha$ SMA (figure 8E) and collagen IA1 (figure 8F) mRNA expression and thus prevented the transactivation into myofibroblasts.

Finally, to investigate the functional consequences of Jnk1 deletion in the liver, we analysed the expression profile of downstream targets of the Jnk signalling pathway (see online supplementary figure S7D). In particular, *stat3*, the transcription factors *jnk2* and *jund* and genes related to inflammation such as *nfatc1* were downregulated in Jnk1 $^{-/-}$  compared with Jnk1 $^{\Delta\text{hepa}}$  and WT mice (see online supplementary figure S7D).

### **DISCUSSION**

One of the tasks to truly understand the role of Jnk for liver physiology and pathophysiology is to characterise and identify their cell-type-specific functions under different conditions. Parenchymal and non-parenchymal liver cells as well as immune infiltrating cells contribute to mechanisms of liver injury and repair. In this context, cell-type-specific activation of Jnk family members might be relevant to determine the outcome of liver disease.

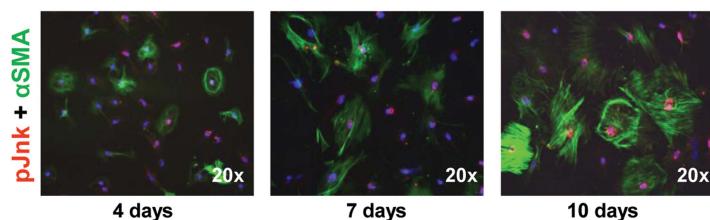
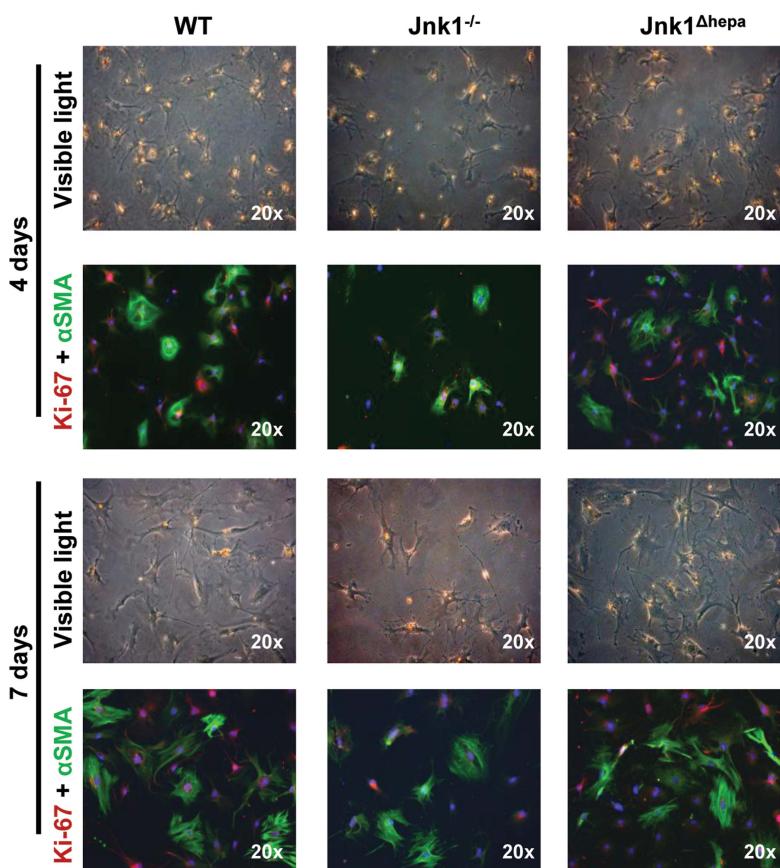
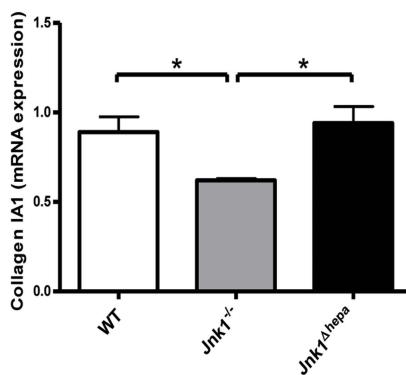
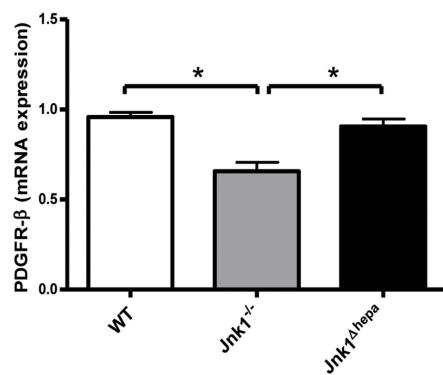
In the present study, we were interested in defining the relevance of Jnk1 activation in different liver cell compartments during liver fibrosis. We used two widely accepted in vivo experimental approaches<sup>15 16</sup>: (i) the surgical BDL model of biliary fibrosis and (ii) the toxic CCl<sub>4</sub> model of liver fibrosis. To identify the most relevant target cell of Jnk1-mediated profibrotic effects, we compared constitutive Jnk1 knockout mice with mice lacking Jnk1 specifically in hepatocytes or in BMDC after BMT and explored the contribution of Jnk1 in vivo and in vitro in HSCs to define their function during liver fibrogenesis.

Earlier studies have examined the relevance of Jnk activation not only in acute but also in chronic liver injury.<sup>17</sup> Especially, Jnk1 has been reported to play a crucial role in response to TNF $\alpha$ ,<sup>18</sup> concanavalin A,<sup>19</sup> ischaemia-reperfusion,<sup>20 21</sup> high-fat diet<sup>22 23</sup> and hepatic carcinogens.<sup>24</sup> Therefore, we first investigated the role of hepatocyte-specific Jnk1 during liver injury in the CCl<sub>4</sub> and BDL models. Unexpectedly, Jnk1 in hepatocytes had no major impact on the progression of liver disease, while both models of experimental liver fibrosis clearly indicated that Jnk1 in non-hepatocytes is of major relevance. In Jnk1 $^{-/-}$ , but not in Jnk1 $^{\Delta\text{hepa}}$  livers, the number of bile infarcts or necrotic foci as well as markers of liver injury—AST, ALT, AP and bilirubin—and apoptotic cell death was reduced.

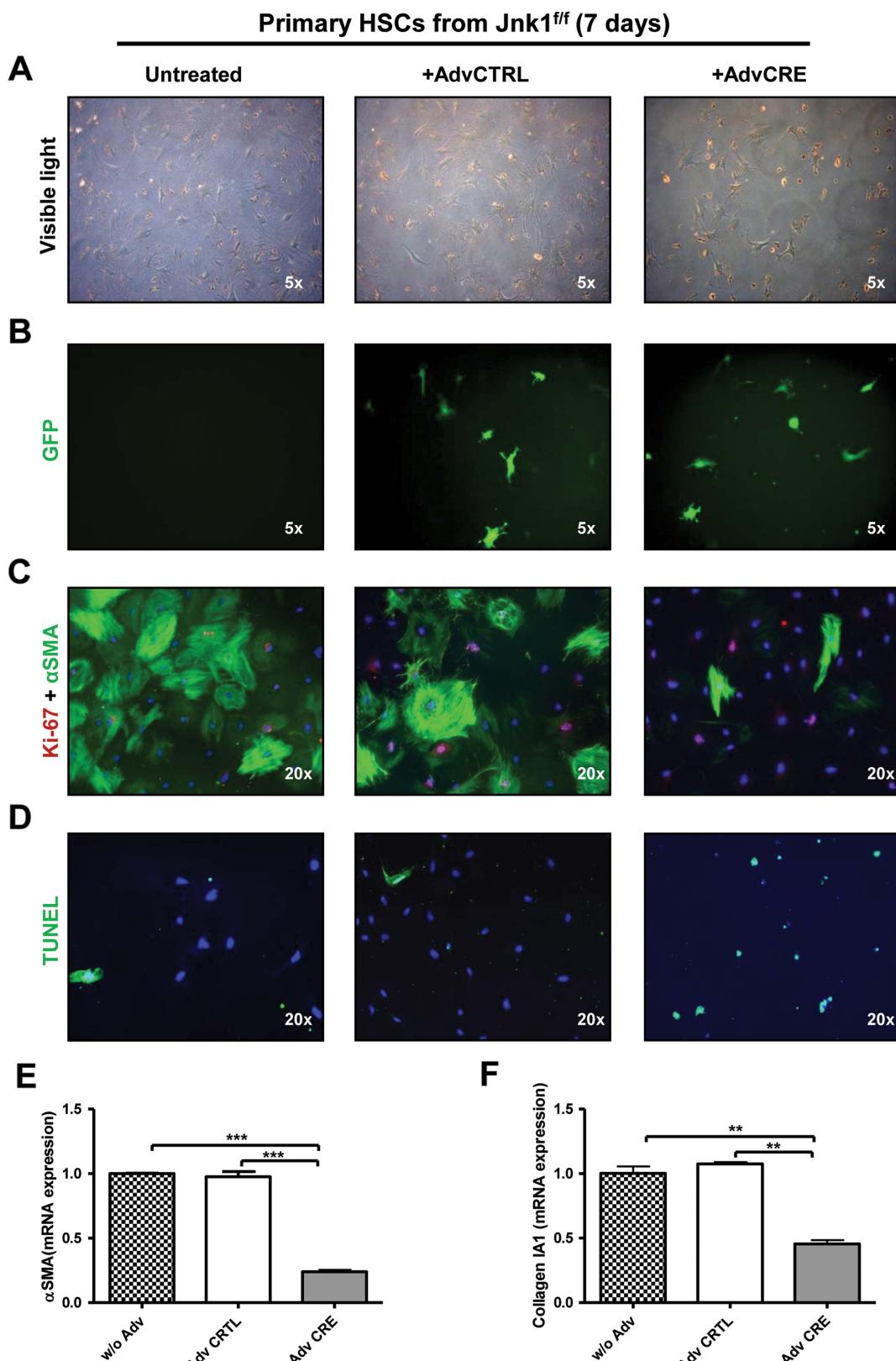
However, the role of Jnk in apoptosis remains highly controversial.<sup>25–28</sup> Some authors suggested a proapoptotic role of Jnk1 during TNF-induced apoptosis in vitro<sup>26</sup> and in vivo.<sup>21</sup> Under normal circumstances, transient Jnk activation confers cytoprotection.<sup>29 30</sup> However, our preliminary results suggest that NF- $\kappa$ B influences the role of Jnk1 during chronic liver injury, suggesting that its prolonged activation can be harmful (Cubero *et al*, unpublished data).

Several reports have supported the hypothesis that Jnk1 is necessary for the development of fibrosis in organs such as lung and kidney.<sup>31 32</sup> In the liver, our results show that already during acute hepatocellular damage, the response to BDL-associated biliary hyperplasia is Jnk1-dependent but not mediated by hepatocytes. Moreover, after 4 weeks of CCl<sub>4</sub> or BDL treatment, liver injury was strongly reduced in Jnk1 $^{-/-}$  animals.

Since the experiments using Jnk1 $^{\Delta\text{hepa}}$  mice clearly demonstrated that Jnk1 in hepatocytes has no impact on the progression of liver fibrosis in both models of injury, we next investigated the contribution of BMDC. Previous studies revealed that Jnk1 activation, for example, in Kupffer cells (KC), is relevant during hepatic inflammation, and thus, finally might contribute to liver fibrosis.<sup>33 34</sup> In particular, the study of Kodama<sup>34</sup> showed that Jnk1 in KCs promotes the progression from hepatic steatosis to steatohepatitis and fibrosis in mice. However, they used a choline-deficient L-amino acid defined diet, a model of mitochondrial-derived reactive oxygen species carcinogenesis, which does not mimic the pathophysiology of pharmacological fibrosis or biliary hyperplasia caused by CCl<sub>4</sub> and BDL. Indeed, the paracrine interactions between cholangiocytes and fibroblasts seem especially important during

**A Primary HSC from WT mice****B Primary HSCs****C****D**

**Figure 7** Jnk1 in hepatic stellate cells (HSCs) is essential for the pathogenesis of liver fibrosis. Primary HSCs were isolated from wildtype (WT), Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> mice. A total of 150,000 cells were seeded in six-well plates and cultivated for up to 10 days. (A) Co-immunofluorescence staining for pJnk (red) and α-smooth muscle actin (αSMA) (green) was performed in WT cells day 4 to day 10 after isolation. (B) Morphology and cell density was confirmed by phase contrast microscopy at day 4 and day 7 after isolation. Primary HSCs isolated from WT, Jnk1<sup>-/-</sup> and Jnk1<sup>Δhepa</sup> livers were costained for Ki-67 and αSMA at day 4 and day 7 after isolation. (C+D) mRNA expression analysis of collagen IA1 (C) and PDGFR-β (D) was quantified by RT-PCR of samples taken from isolated primary HSCs at day 7 of culture. Values are mean±SEM from at least six mice per group (\*p<0.05).



**Figure 8** Targeted deletion of *Jnk1* in synchronised primary hepatic stellate cells (HSCs) prevents transactivation and promotes cell death. Primary HSCs were isolated from *Jnk1<sup>ff</sup>* mice. A total of 150,000 cells were seeded in six-well plates for 7 days. On day 2, HSCs were infected with adenovirus coexpressing cre-recombinase and EGFP (AdvCRE) or EGFP (AdvCTRL) using  $2 \times 10^8$  virions/ml or left completely untreated. Representative images of the morphology and cell density were obtained by phase contrast microscopy at day 7 in culture. (A) Phase contrast microscopy showing density and morphology of HSCs. (B) The efficiency of adenoviral infection was confirmed by EGFP-mediated fluorescence (green). (C) Primary HSCs were costained with Ki-67 (red) and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) (green) 7 days after plating. (D) Representative TdT-mediated dUTP-biotin nick end labelling (TUNEL) stainings of primary HSCs from *Jnk1<sup>ff</sup>* mice at day 7 of culture. Apoptotic cells are stained green; total cells were counterstained with DAPI (blue). (E and F) mRNA expression of  $\alpha$ SMA (E) and collagen IA1 (F) were quantified by RT-PCR of samples taken from Adv-infected primary HSCs after 7 days in culture. Values are mean  $\pm$  SEM from at least 3–4 mice per group (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001).

cholestatic liver disease,<sup>4</sup> whereas BMDC might be the main providers of the fibrogenic response in non-alcoholic steatohepatitis and HCC-producing diets. Moreover, KC can interact with HSCs, accelerate their activation and promote their fibrogenic responses in vitro.<sup>35</sup> To this end, WT or Jnk1<sup>-/-</sup> mice were reconstituted with BM from WT or Jnk1<sup>-/-</sup> donors. Unexpectedly, our results showed that Jnk1 in BMDC does not significantly contribute to liver fibrosis. These results were further confirmed by transplanting WT or Jnk1<sup>-/-</sup> BM into Jnk1<sup>Ahepa</sup> animals (data not shown).

The knockout and BM experiments strongly suggested that non-parenchymal liver cells are essential in mediating the Jnk1-dependent profibrogenic effect during chronic liver injury. Among non-parenchymal cells, cholangiocytes, non-BM-derived KC, endothelial cells or HSCs could be responsible for mediating the Jnk1-dependent effect on liver fibrosis. At this point, we followed two different strategies to identify the essential non-parenchymal liver cell. We included array analysis and immunohistochemistry studies, performed by an experienced pathologist, 4 weeks after chronic liver injury. We found differences in Jnk expression in the experimental models, dependent on the type of damage. After CCl<sub>4</sub>, Jnk expression was mostly found in αSMA-expressing cells, while after BDL additionally periportal areas were positive. This is also reflected in the human liver samples, where we found that apart from HSCs, other cells such as dying hepatocytes,<sup>12</sup> infiltrating cells or epithelial cells are also pJnk positive. Therefore, based on the array analysis and immunohistochemistry, we further investigated the mechanism of transdifferentiation in HSCs.

These results strengthened the in vivo findings and demonstrated that Jnk1 is crucial for HSCs activation and ECM formation. Lack of Jnk1 expression reduced proliferation and survival of HSCs associated with decreased expression of αSMA, collagen IA1 and PDGFR-β.

To further evaluate the exact function of Jnk1 in HSCs, we deleted the gene in synchronised primary cells 2 days after plating using an adenoviral approach. The transactivation kinetics of primary WT HSCs in vitro have been recently characterised in detail, demonstrating that HSCs start to proliferate approximately 3–4 days after plating while maximal αSMA expression, and consequently, full transactivation into myofibroblasts is reached after 7–10 days.<sup>36</sup> As target gene deletion after AdvCRE infection requires approximately 30 h (data not shown), from our data we conclude that Jnk1 activation mediates transactivation and inhibits apoptosis of HSCs. Accordingly, Jnk1 ablation caused a reduced lifespan and poor differentiation of HSCs into matrix-producing myofibroblasts, providing further evidence that HSCs-derived Jnk1 is crucial for the fibrogenic response.

In summary, we report that Jnk1 in hepatocytes and BM-derived cells is not important for the development of murine hepatic fibrosis. Our study identifies Jnk1 as an essential profibrogenic kinase in HSCs for the progression of liver fibrosis in two independent murine models. Thus, our results identify cell-type-specific Jnk1 inhibition in HSCs as an attractive concept to treat liver fibrosis.

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**Contributors** GZ and MH performed all the experiments and acquired all the data. YN participated in the in vitro experiments. JP performed immunohistochemical stainings. WH participated in the functional studies for Jnk1 in HSCs. MVB performed the microarray. OD contributed to the surgery. TR and NG collaborated with the histopathological studies and provided human liver samples, respectively. MM supervised all the microarray data. RJD created the Jnk1-floxed mice. CL provided his expertise with the adenoviral transfection, supervised the experiments and analysed the data. FJC set up the surgical procedure, performed experiments, analysed the microarray, wrote the manuscript and co-directed the study. CT conceived, supervised, codirected, wrote and raised the funds for the study.

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**Competing interests** None.

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## Jnk1 in murine hepatic stellate cells is a crucial mediator of liver fibrogenesis

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