

ORIGINAL ARTICLE

Activin B promotes the initiation and progression of liver fibrosis

Yan Wang¹ | Matthew Hamang¹ | Alexander Culver¹  | Huaizhou Jiang¹ | Jennifer Yanum¹ | Veronica Garcia¹ | Joonyong Lee¹  | Emily White² | Praveen Kusumanchi³ | Naga Chalasani³  | Suthat Liangpunsakul^{3,4,5}  | Benjamin C. Yaden¹ | Guoli Dai¹ 

¹Department of Biology, School of Science, Center for Developmental and Regenerative Biology, Indiana University–Purdue University Indianapolis, Indianapolis, Indiana, USA

²College of Science, Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA

³Division of Gastroenterology and Hepatology, Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA

⁴Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana, USA

⁵Roudebush Veterans Administration Medical Center, Indianapolis, Indiana, USA

Correspondence

Benjamin Yaden, Department of Biology, School of Science, Indiana University–Purdue University Indianapolis, Indianapolis, Indiana. Lilly Research Laboratories, Eli Lilly & Company, Lilly Corporate Center, Indianapolis, Indiana, USA.

Email: yaden_benjamin_c@lilly.com

Guoli Dai, Department of Biology, School of Science, Center for Developmental and Regenerative Biology, Indiana University–Purdue University Indianapolis, Indiana, USA.

Email: gdai@iupui.edu

Funding information

Translational Research Pilot Grant from Indiana University and Lilly Graduate Research Advanced Degree Program

Abstract

The role of activin B, a transforming growth factor β (TGF β) superfamily cytokine, in liver health and disease is largely unknown. We aimed to investigate whether activin B modulates liver fibrogenesis. Liver and serum activin B, along with its analog activin A, were analyzed in patients with liver fibrosis from different etiologies and in mouse acute and chronic liver injury models. Activin B, activin A, or both was immunologically neutralized in mice with progressive or established carbon tetrachloride (CCl₄)–induced liver fibrosis. Hepatic and circulating activin B was increased in human patients with liver fibrosis caused by several liver diseases. In mice, hepatic and circulating activin B exhibited persistent elevation following the onset of several types of liver injury, whereas activin A displayed transient increases. The results revealed a close correlation of activin B with liver injury regardless of etiology and species. Injured hepatocytes produced excessive activin B. Neutralizing activin B largely prevented, as well as improved, CCl₄–induced liver fibrosis, which was augmented by co-neutralizing activin A. Mechanistically, activin B mediated the activation of c-Jun-N-terminal kinase (JNK), the induction of inducible nitric oxide synthase (iNOS) expression, and the maintenance of poly (ADP-ribose) polymerase 1 (PARP1) expression in injured livers. Moreover, activin B directly induced a profibrotic expression profile in hepatic stellate cells (HSCs) and stimulated these cells to form a septa structure. **Conclusions:** We demonstrate that activin B, cooperating with activin A, mediates the activation or expression of JNK, iNOS, and PARP1 and the activation of HSCs, driving the initiation and progression of liver fibrosis.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. *Hepatology Communications* published by Wiley Periodicals LLC on behalf of American Association for the Study of Liver Diseases.

INTRODUCTION

Liver fibrosis is the common consequence of liver injury secondary to a variety of repeated insults and/or injuries including alcohol-associated liver disease (ALD), nonalcoholic steatohepatitis (NASH), viral hepatitis, and autoimmune liver disease.^[1] The initiation and progression of liver fibrosis is driven by complicated cellular and molecular mechanisms, which include a variety of different cell types.^[2–5] Damaged hepatocytes and cytokines released from the resident liver inflammatory cells can directly or indirectly activate hepatic stellate cells (HSCs) into myofibroblasts, leading to the accumulation of collagen I/III and deposition of other extracellular matrix components, and thus resulting in liver fibrosis.^[1,6,7]

Activin proteins consist of dimers formed by four inhibin subunits (inhibin β A, inhibin β B, inhibin β C, and inhibin β E) in mammals.^[8] Widely expressed inhibin β A and inhibin β B genes are essential for inducing mesoderm formation during development and for follicle stimulating hormone production.^[9–11] The inhibin β C and inhibin β E are expressed predominantly in the liver, and are dispensable during development and for maintaining adult homeostasis.^[12] Activins A, B, AB, C, and E represent homo or hetero-dimers of inhibin β A β A, β B β B, β A β B, β C β C, and β E β E, respectively.^[8] Activins A, B, and AB signal through activin receptors/mothers against decapentaplegic homolog 2/3 (Smad2/3) pathway, whereas activins C and E may not.^[13] Activin A is expressed and secreted by hepatocytes and non-parenchymal cells such as HSCs, cholangiocytes, and endothelial cells in the liver.^[14–16] Several studies demonstrated that activin A induces the activation of HSCs and macrophages and the apoptosis of hepatocytes *in vitro*.^[14–20] An *in vivo* study showed that neutralizing activin A mildly reduced CCl₄-induced acute liver injury in mice.^[21] However, the association of activins with human liver disease/fibrosis/cirrhosis has yet to be determined.

As structurally related proteins, activin B shares 63% identity and 87% similarity to activin A.^[8] Both ligands bind to the activin receptors II and I, and multiple common activator protein 1 sites in the promoters of both *inhibin β A* and *inhibin β B* have been identified, which suggests that activin B may act similarly to activin A in mediating liver pathogenesis.^[8,22–24] Hepatocytes constitutively express abundant inhibin β A but relatively low inhibin β B.^[14] However, hepatic *inhibin β B* gene expression is highly up-regulated, while *inhibin β A* was down-regulated, in response to CCl₄-induced acute liver injury.^[25] Moreover, activin B up-regulated hepcidin expression in hepatocytes via Smad1/5/8 signaling in response to several inflammatory insults in mice, whereas activin A did not. These findings suggest a unique role for activin B in mediating liver injury in comparison to activin A.^[26] Taken together, it remains

unclear whether activin B is involved in the initiation and progression of liver fibrotic response to liver injury. Thus, the objective of this study was to ascertain the role for activin B to modulate liver fibrogenesis.

MATERIALS AND METHODS

Human liver and serum samples

The hepatic messenger RNA (mRNA) and protein expression of activin A and B was first determined from normal liver (n = 5) and the explant, which was obtained during liver transplantation from patients with cirrhosis secondary to NASH (n = 8). We further examined the levels of mRNA and protein expression of both activins in patients with different stages (F0–F4) of NASH (n = 21). Serum levels of activins were measured in patients with NASH (n = 44), excessive drinkers without liver disease (n = 36), and those with alcoholic cirrhosis (n = 15) compared with normal controls (n = 16). All samples were collected under the Indiana University–Purdue University Indianapolis (IUPUI) Institutional Review Board–approved protocols.

Mouse liver injury models

All mouse experiments were performed with the approval of Institutional Animal Care and Use Committee of Eli Lilly and Company and IUPUI. Several mouse models were used for our study. For acute liver injury models, C57BL/6 female mice at the age of 10–12 weeks (Envigo) received a single intraperitoneal administration of CCl₄ (Sigma Aldrich) (1:10 dilution in corn oil, 10 ml/kg) for 1.5 h, 3 h, 6 h, 24 h, and 3 days. For liver fibrosis models, mice were intraperitoneally injected of CCl₄ twice a week for 4 weeks or 10 weeks.^[27,28] For the ALD model, ethanol oral feeding lasted for 10 days plus binge, as described previously.^[29] Bile duct ligation (BDL) model was also used. Briefly, under iso-flurane anesthesia (2%–4% vol/vol), male C57BL/6 mice were placed on a heat pad and laparatomized. The common bile duct was exposed, isolated, ligated two times with non-resorbable sutures (polyester 6–0; Catgut). Sham-operated mice underwent a laparotomy with exposure but not ligation of the bile duct. The abdominal muscle and skin layers were stitched, and the mice were treated with ketoprofen as an analgesic. The animals were allowed to recover from anesthesia and surgery under a red warming lamp and were held in single cages. After 14 days, mice were euthanized to obtain blood and liver samples. Activin A antibody and activin B antibody were purchased from R&D System (Cat#MAB3381) and Ansh Labs (Cat#AB-306-AA042), respectively, and were used for neutralizing activin A and activin B in CCl₄ chronic liver injury model.

Blood biochemistry

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, and total bilirubin levels were measured with a Hitachi Modular Analyzer (Roche Diagnostics). Total bilirubin levels below the limit of detection (0.14 mg/dl) were replaced by half of the limit of detection (0.07 mg/dl) for statistical analysis.

Histology and immunohistochemistry

Formalin-fixed and paraffin-embedded liver sections were subjected to a standard procedure of immunohistochemistry with primary antibodies against F4/80 (eBioscience), myeloperoxidase (MPO; R&D System), or activin B (AB-306-AA042; Ansh Labs). The liver sections were subjected to Masson's trichrome staining. Images were acquired using digital slide scanning (Aperio Technologies), and the stained area percentage was analyzed by ImageJ (National Institutes of Health).

Enzyme-linked immunosorbent assay of activin A and activin B

Activin A and activin B proteins in liver tissue, serum, or cell culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA) methods (Activin A ELISA kit, Sigma; Activin B ELISA kit, Ansh Labs) according to the protocols provided by the manufacturers.

SMAD-binding element 12 luciferase-based reporter gene assay

Human embryonic kidney 293 (HEK293) cells stably expressing the SMAD-binding element 12 (SBE12) luciferase system were purchased from Qiagen. They were seeded about 75,000 cells/well/100 μ l Dulbecco's modified Eagle's medium/Ham's F12 (Life Technologies) with 10% fetal bovine serum (FBS) into a 96-well plate. After overnight incubation at 37°C with 5% CO₂, the medium was replaced with fresh medium containing 1% FBS. Activin A or activin B antibody was serially diluted (1:3) in the medium containing 1% FBS to produce the following titration range: 0.01 ng/ml to 1771.5 ng/ml. A total of 50 μ l of each concentration was then mixed with an equal volume of 30 ng/ml of activin A or activin B (R&D Systems) and incubated at room temperature for 30 min. After that, the mixture was added to individual wells and incubated for 24 h. Subsequently, cells were washed once with phosphate-buffered saline and subjected to lysis. Luminescence was measured using a GeniosPRO instrument (Tecan) with substrate injection (Luciferase Reporter Gene Assay Kit; Roche). Relative luciferase

units were measured, and IC₅₀ curves were fitted using GraphPad Prism software (GraphPad Software).

Cell culture, microarray analysis, and quantitative real-time polymerase chain reaction are described in the [Supporting Information](#).

Western blot analysis

Liver homogenates were separated using polyacrylamide gel electrophoresis under reducing conditions (10 μ g per lane). Proteins from the gels were electrophoretically transferred to polyvinylidene difluoride membranes. Antibodies against phosphorylated c-Jun-N-terminal kinase (p-JNK; 4668), JNK (9252), poly (ADP-ribose) polymerase (PARP; 9542), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 5174) were purchased from Cell Signaling Technology. Nitric oxide synthase 2 antibody (sc-7271) was purchased from Santa Cruz Biotechnology. Immune complexes were detected by SuperSignal West Pico PLUS Chemiluminescent Substrate (34,577; Thermo Fisher Scientific). Signals were detected using ImageQuant LAS 4000 Mini (General Electric Life Sciences) and quantified using ImageJ software.

Statistical analysis

Statistical significance, $p < 0.05$, was determined by ordinary one-way or two-way analysis of variance tests followed by Dunnett's to compare the differences between experimental and control groups. The data were expressed as means \pm SEM. GraphPad Prism Software 9.0 was used for data analysis and figure preparation.

RESULTS

Levels of hepatic and circulating activin B are significantly increased in patients with liver fibrosis

We first determined whether activin B and A are clinically relevant to different etiologies of liver fibrosis. We found that, in patients with advanced liver fibrosis or cirrhosis, hepatic activin B mRNA and protein exhibited marked increases relative to healthy controls ([Figure 1A,B](#)). Circulating activin B did not increase in excessive alcohol users without liver disease but was potently elevated more than 5-fold in patients with alcoholic cirrhosis ([Figure 1C](#)). In patients with NASH, hepatic levels of activin B significantly increased only in those patients with F3 and F4 biopsy-confirmed fibrosis compared with F0 controls ([Figure 1D](#)), while serum levels of activin

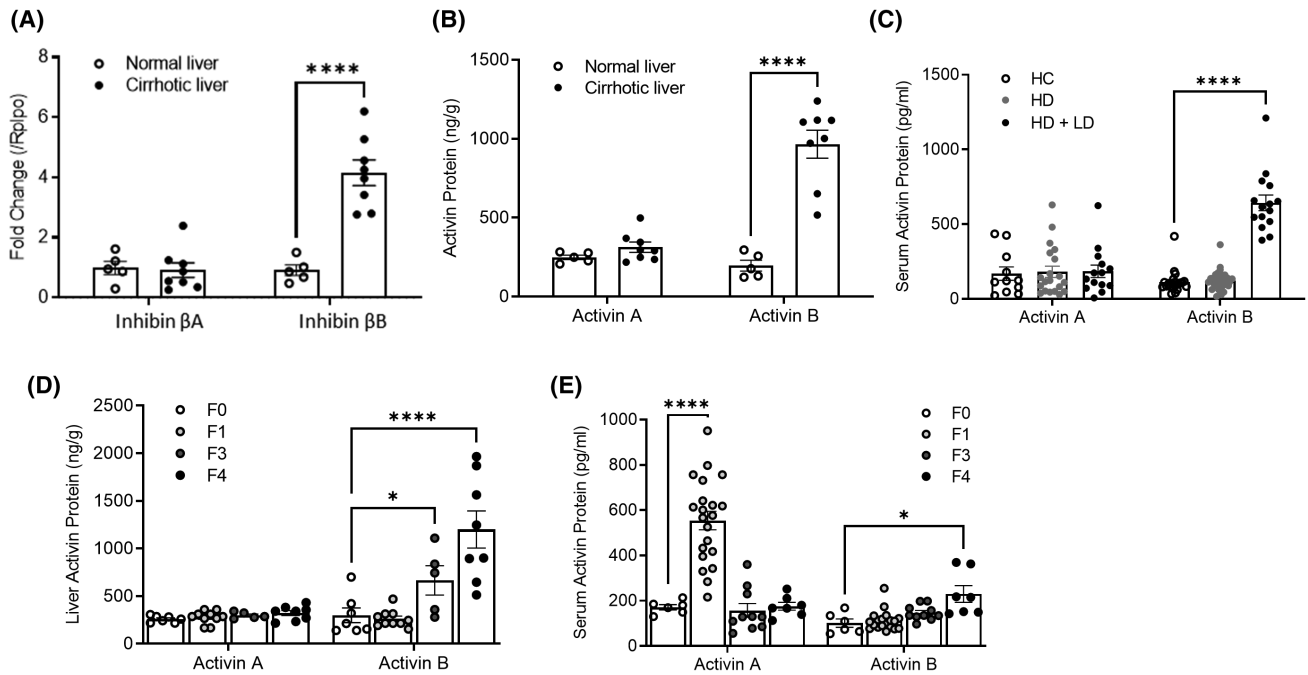


FIGURE 1 Liver and serum activin B increase in the patients with liver fibrosis. The messenger RNA (mRNA) expression of hepatic inhibin β A and inhibin β B (A) and proteins of hepatic activin A and B (B) in patients with cirrhosis (n = 8) and healthy controls (n = 5) were analyzed by quantitative real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. (C) The concentrations of serum activin A and B proteins were determined with ELISA in healthy controls (HC; n = 16), heavy drinkers without liver diseases (HD; n = 36), and heavy drinkers with liver disease (HD+LD; n = 15). Activin A and B proteins were evaluated by ELISA in the livers (D) and serum (E) of patients with different stages of nonalcoholic steatohepatitis (F0, n = 4; F1, n = 6; F3, n = 5; and F4, n = 6). For all of these assays, data are expressed as means \pm SEM. * p < 0.05, **** p < 0.0001 compared with HC or F0 group via two-way analysis of variance (ANOVA). Note that inhibin β A and β B encode the subunit of activin A and B respectively.

B were increased in those patients with F4 fibrosis (Figure 1E). Additionally, we found that the serum level of activin A markedly increased in those with F1 fibrosis (Figure 1E). Taken together, we demonstrate that the expression of activin B is closely correlated with advanced fibrosis/cirrhosis, regardless of underlying disease etiologies.

Levels of hepatic and circulating activin B are significantly elevated in mouse models of liver injury

To further investigate the expression patterns and cellular sources of activin B and A in liver injury, we performed acute and chronic liver injury studies in mice. In an acute liver injury, mice were administered a single dose of CCl_4 . We found up-regulated hepatic *inhibin β B* mRNA expression up to 3 days following injection (Figure 2B), concomitant with the increases in activin B protein concentrations in the sera (Figure 2D) and livers (Figure 2F). In vehicle-treated livers, activin B was weakly detected primarily in pericentral hepatocytes. In sharp contrast, activin B was strongly expressed in these cells in CCl_4 -treated livers (Figure 2G). It is well known that CCl_4 damages pericentral hepatocytes.^[30] Thus, our data demonstrate that injured hepatocytes

produce excessive activin B. In accordance with activin B expression, we observed increases in hepatic mRNA expression, hepatic protein concentration, and serum level of activin A at 24 h following CCl_4 injection (Figure 2A,C,E). To determine these effects following chronic liver injury, we administered mice with CCl_4 twice weekly for 4 weeks along with a separate ALD model of chronic alcohol plus binge. In the CCl_4 model, we observed increases in hepatic mRNA expression and serum concentration only for activin B, but not activin A (Figure 3A,B). Similar findings were found in mice fed with chronic alcohol plus binge (Figure 3C,D). Activin B expression was quantified in the BDL model to further characterize activin B response to liver damage. As quickly as 6 h (0.25 days) following surgery, mice had elevated circulating activin B protein, and this response persisted up to 7 days after BDL (Figure S1A). Similarly, hepatic *inhibin β B* exhibited persistent up-regulation during the first 7 days after surgery (Figure S1B). Hepatic activin B protein concentrations remained higher up to 14 days following BDL compared with sham controls (Figure S1C). In contrast, during the same period, circulating activin A protein levels were not significantly altered (Figure S1D), and hepatic *inhibin β A* mRNA expression showed a transient increase at 3 days following surgery (Figure S1E). Collectively, we demonstrate that, regardless of liver

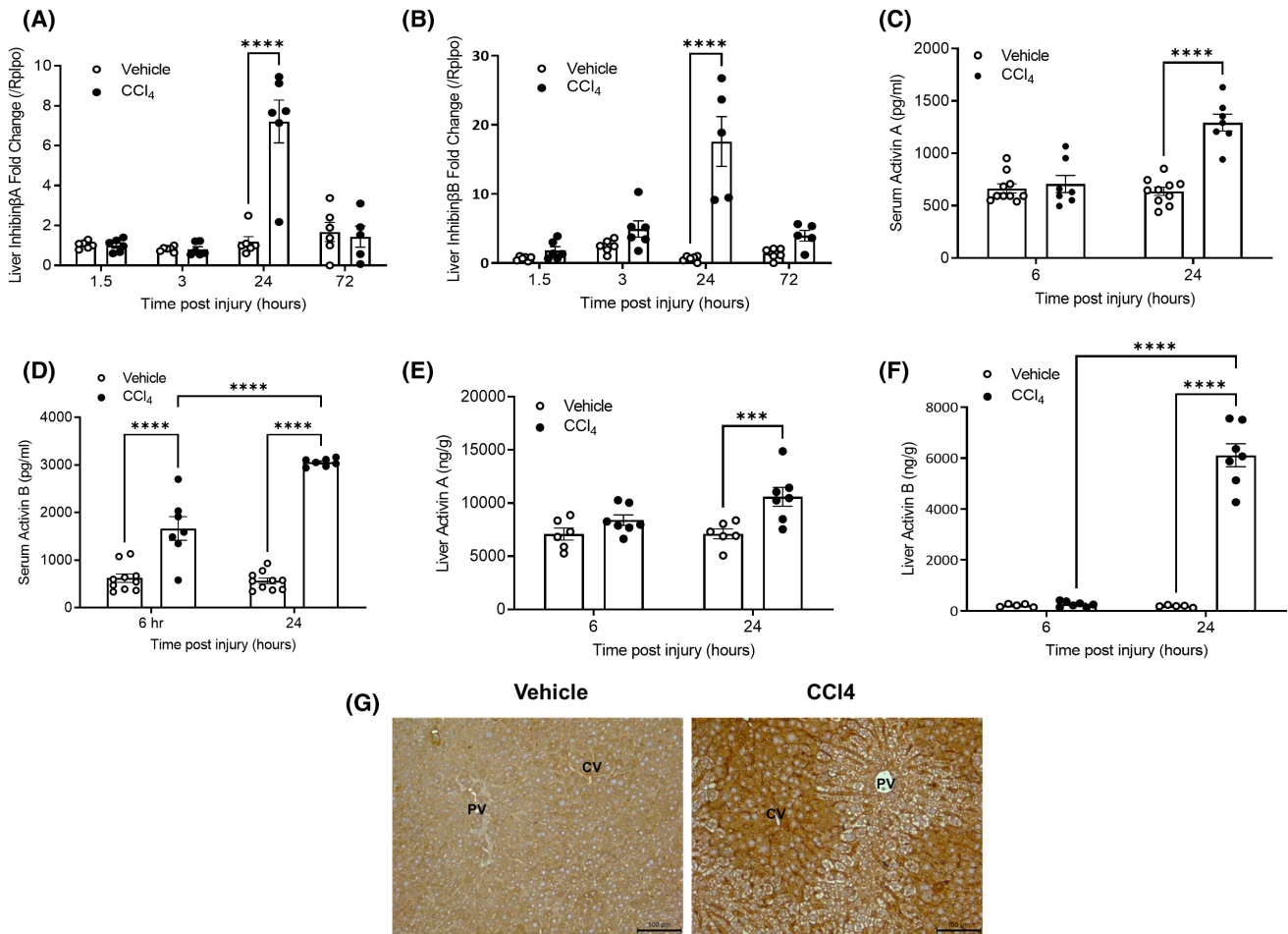


FIGURE 2 Liver and serum activin B increases in mice following acute liver injury. Female mice were given a single administration of carbon tetrachloride (CCl₄) or vehicle, and tissues were collected at the indicated times following injury. The mRNA expression of liver inhibin βA (A) and inhibin βB (B) was analyzed by quantitative real-time PCR at indicated time points (n = 6). Serum (C,D) and liver (E,F) activin proteins were quantified via ELISA at the indicated time points (n = 5–8). For all of these quantitative assays, data are expressed as means ± SEM. ****p* < 0.001, *****p* < 0.0001 via two-way ANOVA relative to vehicle controls. (G) Immunostaining of activin B in the livers of mice 24 h after CCl₄ or vehicle treatment.

injury types, activin B is persistently associated with liver disease progression from acute phase to chronic phase, whereas activin A is transiently relevant to the acute phase. Moreover, the association of activin B with liver fibrosis is highly conserved between humans and mice.

Neutralization of activin B prevents CCl₄-induced liver fibrosis

These association studies in humans and mice strongly suggested that activin B and A participate in mediating liver fibrogenic response to liver injury. Therefore, we set out to test inhibition of each individual ligand pharmacologically to evaluate their separate and combined contributions to hepatic fibrogenesis. Global gene knockouts of these two widely produced activin ligands cause developmental defects, reproductive failure, or postnatal death in mice.^[9–11] We thereby used

neutralizing antibodies to systemically inactivate these two proteins and subsequently examine their effects on the initiation of CCl₄-induced liver fibrosis. There were five treatment groups: (1) vehicle; (2) immunoglobulin G (IgG)+CCl₄; (3) activin A antibody + CCl₄; (4) activin B antibody + CCl₄; and (5) combination of both antibodies + CCl₄. In the initial association studies, we found time windows during which both activin A and B were induced in the acute phase of liver injuries (Figure 2A–F). This co-induction suggested a possible spatiotemporal coordination between the two activin ligands, warranting combination antibody treatment in this study. Antibodies were initially dosed half an hour before the first CCl₄ injection and were dosed weekly thereafter. In a Smad2/3 binding element promoter luciferase assay, we showed that activin A antibody did not cross-react to activin B, and activin B antibody did not cross-react with activin A (Figure S2). The data demonstrated the specificity of these two antibodies. In addition, the half maximal inhibitory concentration

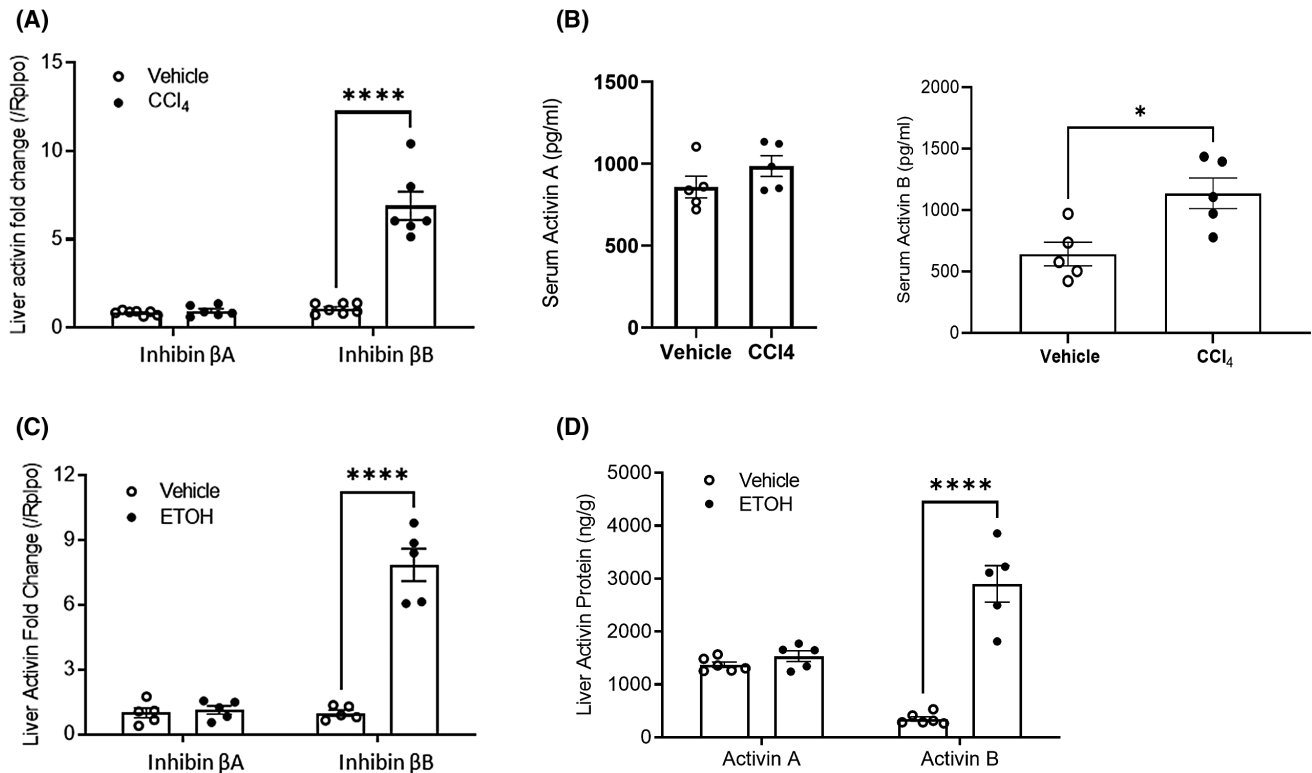


FIGURE 3 mRNA and protein levels of activin B and A in CCl₄-induced chronic liver injury. CCl₄ or vehicle was dosed twice per week for 4 weeks in female mice. (A) mRNA expression of hepatic inhibin β A and inhibin β B was assessed by quantitative real-time PCR (n = 5–7). (B) Concentrations of serum activin A and activin B protein were quantified via ELISA (n = 5). In a separate study, following 10 days of oral alcohol (ETOH) plus binge administration in male mice, hepatic inhibin β A and inhibin β B transcript levels were determined by quantitative real-time PCR (n = 5) (C), and hepatic activin A and activin B protein contents were quantified via ELISA (n = 5) (D). For all of these quantitative assays, data are expressed as means \pm SEM. * p < 0.05, **** p < 0.0001 via two-way ANOVA relative to vehicle controls.

(IC₅₀) of activin B antibody (325 ng/ml) is about 5 times less potent than that of activin A antibody (54.6 ng/ml) (Figure S2). A dosage of 10 mg/kg of activin A antibody was used weekly because our previous study demonstrated the greatest efficacy of this regimen in regressing degeneration of injured skeletal muscle in mice.^[31] We administered 50 mg/kg once per week of activin B antibody, to attempt to neutralize activin B with an equal amount to activin A, based on their IC₅₀.

As a result, activin B antibody exerted extensive beneficial effects, including reduced liver injury indicated by serum ALT and AST (Figure 4A,B), improved liver functions estimated by serum glucose and total bilirubin levels (Figure 4C,D), and decreased liver fibrosis analyzed by collagen staining and collagen type I alpha 1 (COL1 α 1) mRNA expression (Figure 4E–G). Activin A antibody treatment reduced liver injury and improved liver functions to a lesser extent compared with activin B antibody, but did not decrease total bilirubin and liver fibrosis, although collagen 1 α 1 mRNA expression was inhibited (Figure 4A–G). The dual antibodies showed beneficial effects equivalent to, or in some cases greater than, activin B antibody alone (Figure 4A–G). In CCl₄ chronically damaged livers, activin B and A are essential collaborators to induce profibrotic genes chemokine (C-X-C motif) ligand 1 (CXCL1), cytokine-inducible nitric

oxide synthase (iNOS), connective tissue growth factor (CTGF), and transforming growth factor β 1 (TGF β 1), because neutralizing either one of them prevented or inhibited the up-regulation of these genes (Figure 4H). It has been reported that lipocalin-2 (LCN2) is secreted by immune cells and hepatocytes and promotes CCl₄-induced liver fibrosis.^[32] As expected, the expression of LCN2 is elevated in the livers chronically damaged by CCl₄. Neutralizing activin B fully, but neutralizing activin A mildly, inhibited the up-regulation of LCN2. However, neutralizing both activin A and activin B did not show additive effects (Figure S3). Taken together, these data demonstrate that (1) activin B and, to a much lesser extent, activin A, mediate the initiation of liver fibrosis; and (2) activin B inhibition or, even better, both activin B and A inhibition, largely prevents liver fibrosis.

Neutralization of activin B improves established CCl₄-induced liver fibrosis

The superior effects of antibody-mediated inactivation of activin B or both activin B and A in preventing liver fibrosis prompted us to test the same strategy to reverse existing liver fibrosis. Following the same study design as the prevention study, CCl₄ was

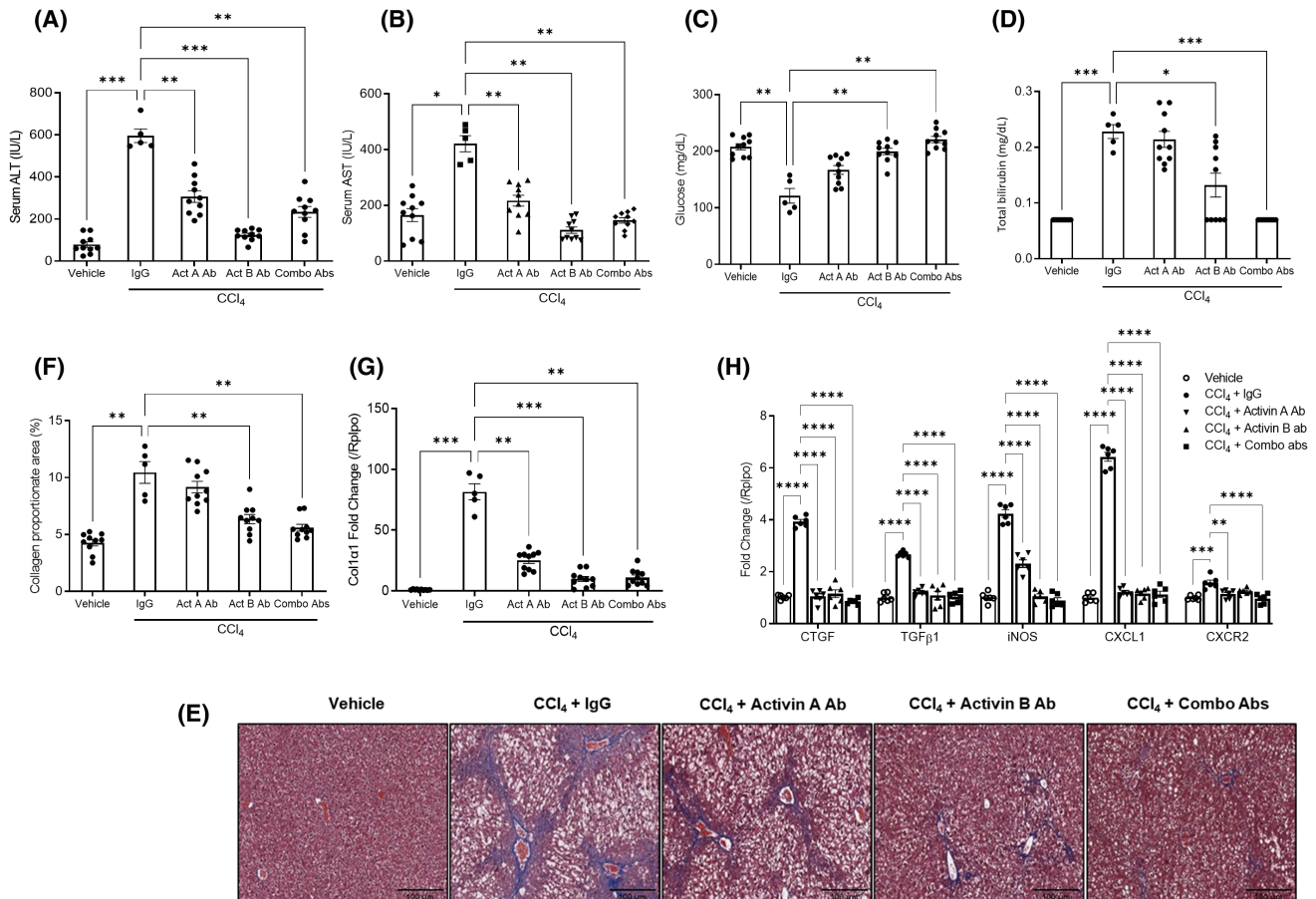


FIGURE 4 Treatments with activin B antibody (Ab), activin A antibody, or combination of them display distinct effects in preventing liver fibrosis induced by CCl₄ in mice. Adult female mice were administered CCl₄ or corn oil vehicle twice per week for 4 weeks. Half an hour before the first CCl₄ injection each week, mice were treated with immunoglobulin G (IgG; 60 mg/kg), activin A antibody (10 mg/kg of activin A antibody + 50 mg/kg of IgG), activin B antibody (50 mg/kg of activin B antibody + 10 mg/kg of IgG), or combination of activin A and activin B antibodies (10 mg/kg of activin A antibody + 50 mg/kg of activin B antibody). Four weeks after the initial CCl₄ injection, alanine aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B), glucose (C), and total bilirubin (D) were analyzed in blood. (E) Representative liver sections stained with Masson's trichrome. (F) Percent Masson's trichrome staining areas were quantified by ImajJ. (G) The mRNA expression of hepatic collagen type I alpha 1 (Col1α1) was evaluated by quantitative real-time PCR. (H) Transcripts of the genes indicated were quantified by quantitative real-time PCR in liver tissues. Data are expressed as means ± SEM (n = 5 for IgG control group; n = 10 for all other groups). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 compared with IgG controls via ordinary one-way ANOVA for (A)–(D), (F), and (G). Statistical analysis was performed via two-way ANOVA following by Dunnett's multiple comparisons test relative to IgG controls for (H). Total bilirubin levels below the limit of detection (0.14 mg/dl) were replaced by half of the limit of detection (0.07 mg/dl) for statistical analysis. Abbreviations: CTGF, connective tissue growth factor; CXCL, chemokine (C-X-C motif) ligand 1; CXCR2, chemokine (C-X-C motif) receptor 2.

injected twice per week for 10 continuous weeks. Starting at the seventh week when liver fibrosis was fully established, antibodies were dosed weekly for the remaining 4 weeks. Consequently, we found distinct reversal effects among the antibody treatment groups. The reversal effects followed the sequence of magnitude of effect where inactivating both activin B and A had greater effect than inactivating activin B alone, and inactivating activin A had the lowest effect. The combinational inactivation exerted the most beneficial effects across all assessments, including reduced liver injury assessed by serum ALT and AST (Figure 5A,B), improved liver function estimated by serum glucose and total bilirubin levels (Figure 5C,D), decreased collagen deposition (Figure 5E,F), and

less macrophage infiltration (Figure 5G,I). Inactivating activin B alone generated a stronger antifibrotic effect (Figure 5E,F), but nearly equal effects in other assessments (Figure 5A–D,G–I), compared with inactivating activin A alone. Of note, inactivating activin B alone and inactivating both activin B and A equivalently regressed liver fibrosis (Figure 5E,F). Neutrophils (MPO-positive cells) and Kupffer cells (F4/8-positive cells) were similarly distributed in fibrotic livers, concentrating in septa. Neutralizing activin A, activin B, or both did not alter the total number of infiltrated neutrophils, but almost equally reduced the total number of Kupffer cells (Figure 5G–I). Collectively, these results demonstrate that (1) activin B is a stronger driver of liver fibrogenesis than activin

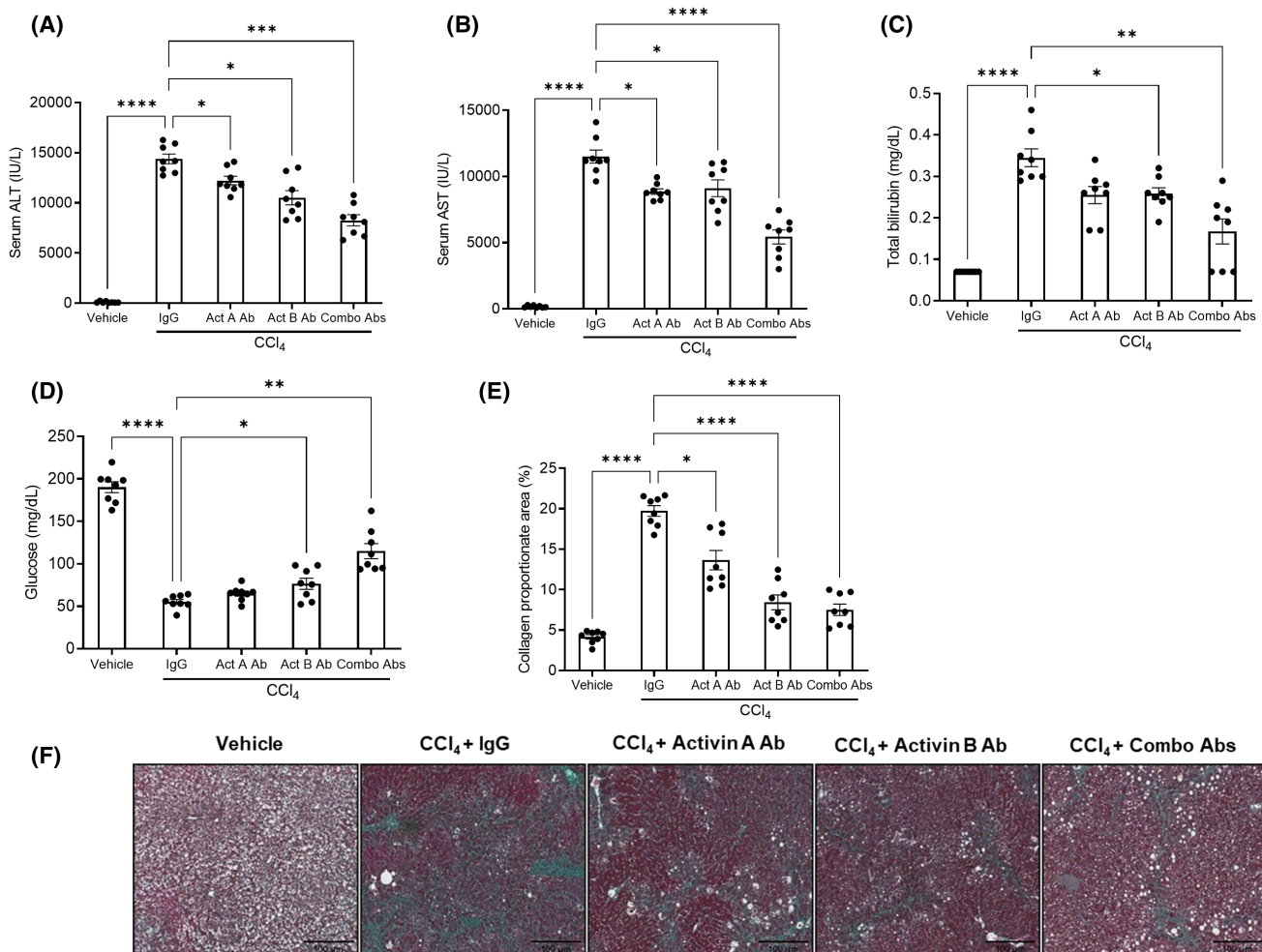


FIGURE 5 Activin B antibody, activin A antibody, and a combination of them display different effects in regressing liver fibrosis induced by CCl₄ in mice. Adult female mice were subjected to CCl₄ or vehicle injection twice per week for 10 weeks. Starting from the seventh week, these mice were treated with IgG (60 mg/kg), activin A antibody (10 mg/kg of activin A antibody + 50 mg/kg of IgG), activin B antibody (50 mg/kg of activin B antibody + 10 mg/kg of IgG), or combination of activin A and activin B antibodies (10 mg/kg of activin A antibody + 50 mg/kg of activin B antibody) weekly. Ten weeks after the initial CCl₄ injection, ALT (A), AST (B), glucose (C), and total bilirubin (D) were analyzed in blood, and Masson's trichrome collagen staining areas were quantified by ImageJ (E). (F) Representative liver sections stained with Masson's trichrome. (G) Representative liver sections immunostained with myeloperoxidase (MPO) or F4/80 antibody. (H,I) Quantification of percent positive staining area of MPO (H) and F4/80 (I). Data are presented as means \pm SEM (n = 8). * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001 compared with IgG controls via ordinary one-way ANOVA relative to IgG controls. Total bilirubin levels below the limit of detection (0.14 mg/dl) were replaced by half of the limit of detection (0.07 mg/dl) for statistical analysis.

A; (2) these two activin ligands may act cooperatively to promote the progression of chronic liver injury; and (3) neutralization of activin B, or both of these ligands, improves liver fibrosis.

Neutralization of activin B suppresses the activity of JNK, the expression of iNOS, and PARP1 in CCl₄-induced liver injury

To gain mechanistic insights, we examined effects of inactivation of activin A, activin B, or both of them on the activity or expression of several known mediators of liver injury using the liver samples collected from

the preventive study (Figure 6). JNK activation leads to hepatocyte necrosis during a variety of liver injuries.^[33] As expected, CCl₄ induced the activation of both JNK1 and JNK2. Neutralizing activin A alone reduced the activity of JNK1, but not JNK2. Neutralizing activin B alone prevented the activation of both JNK1 and JNK2. Neutralizing both activin A and B severely suppressed the activities of both JNK1 and JNK2 to the extent even below the basal levels in uninjured livers. The results indicate that activin B potently activates JNK, synergistically with activin A, during liver injury. iNOS is known to be expressed mainly in hepatocytes and Kupffer cells in the liver and enhance CCl₄-induced liver necrosis and fibrosis.^[34] CCl₄ drastically induced hepatic iNOS expression, which was markedly and equivalently

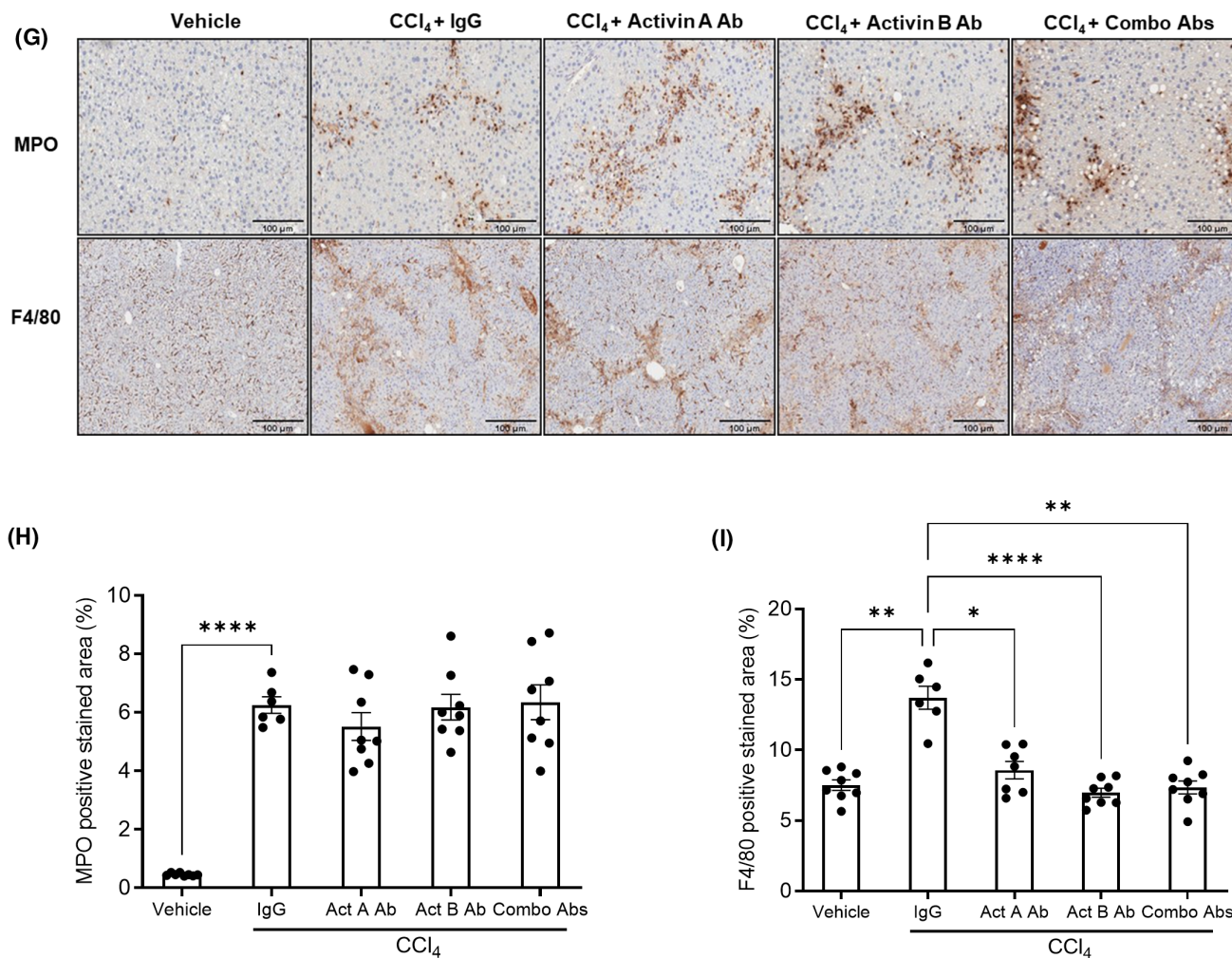


FIGURE 5 (Continued)

inhibited by inactivating activin A, activin B, or both of them. Activin A neutralization seemed to have a stronger inhibitory effect compared with other antibody treatments, without reaching statistical significance. Poly (ADP-ribose) polymerase 1 (PARP1) has been shown to mediate CCl₄-induced hepatocyte death, inflammation, and fibrosis.^[35] We found that CCl₄ did not increase hepatic PARP1 expression. However, neutralization of activin B, but not activin A, suppressed PARP1 expression in injured livers, and neutralization of both activin A and B displayed further suppression. Taken together, we demonstrate that activin B mediates the activation of JNK, the induction of iNOS expression, and the maintenance of PARP1 expression in the livers damaged by CCl₄.

Activin B directly promotes HSC activation

Myofibroblasts centrally drive liver fibrogenesis and are primarily differentiated from activated HSCs.^[36] LX-2

cells, a human HSC cell line, largely mimic the behavior of primary HSCs and thereby have been used widely to study the property of HSCs.^[37] Because LX-2 cells are more relevant to humans than mouse HSCs, we tested the behavioral responses of LX-2 cells to activin A, activin B, combination of both, and TGFβ1, a recognized regulator of HSC activity. We found that LX-2 cells formed a septa-like structure following 24 h of exposure to these three ligands (Figure 7A), mimicking the behavior of HSCs during liver fibrogenesis. This observation demonstrates that activin B and A directly activate HSCs. This finding also urged us to examine whether these activin ligands redundantly act on HSCs at a molecular level. Hence, we treated LX-2 cells with activin A, activin B, or TGFβ1 protein for 6 h, and profiled their early responsive genes by microarray analysis. As a result, these three proteins regulate overlapping but differential gene networks in these cells (Figure 7B). The overlapping 877 genes were associated predominately with HSC activation and hepatic fibrosis, including up-regulated TGFβ signaling negative feedback modulator TMEPAI (transmembrane prostate androgen-induced

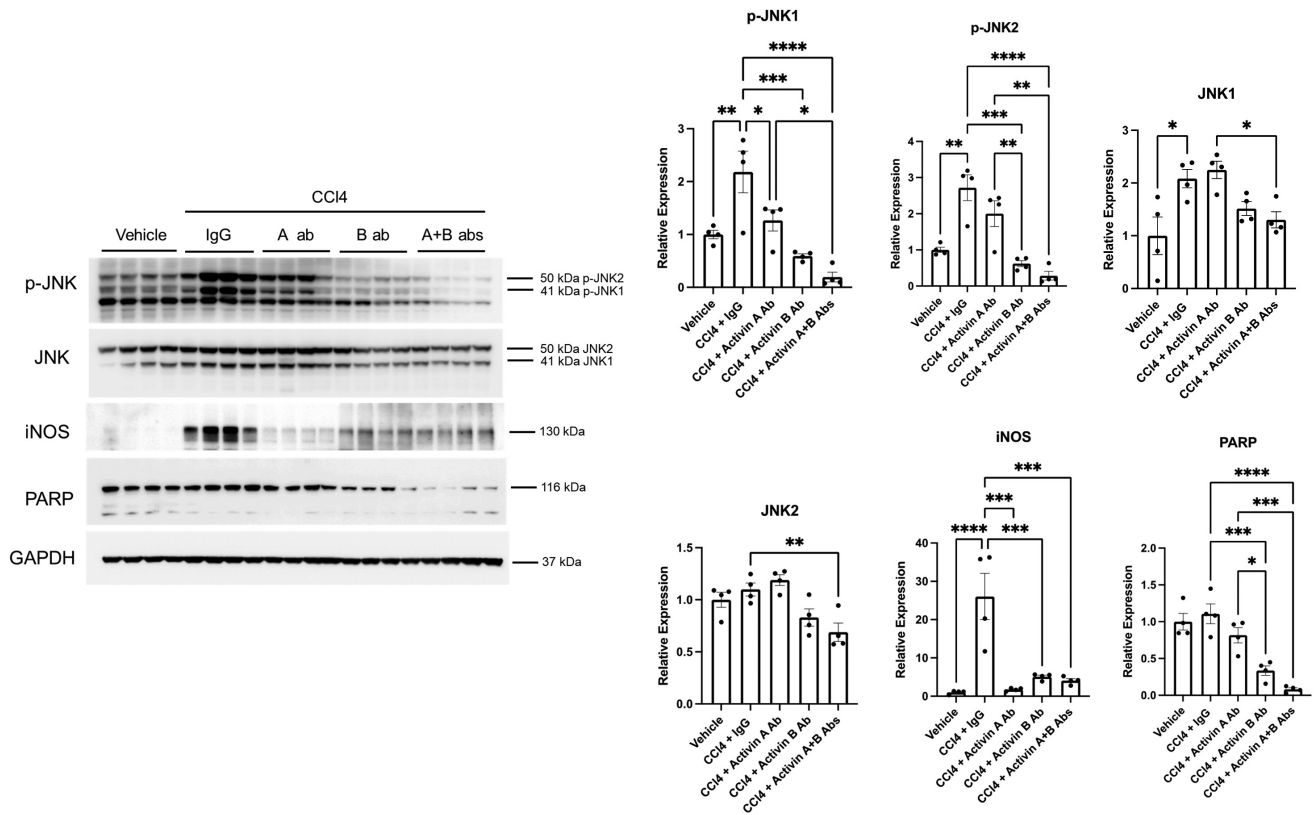


FIGURE 6 The effects of antibody treatments on the expression of hepatic c-Jun-N-terminal kinase (JNK), inducible nitric oxide synthase (iNOS), and poly (ADP-ribose) polymerase 1 (PARP1) in the livers chronically injured by CCl₄. Liver lysates were prepared from the liver samples collected in the preventive study described in Figure 5. Western blotting was performed using antibodies against the proteins indicated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Relative densitometry was normalized against GAPDH. Data are presented as the mean fold changes relative to vehicle controls \pm SEM (n = 4). * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

protein), EGR2 (early growth response protein 2), calcium ion-binding protein MGP (matrix Gla protein), and down-regulated BMP4 (bone morphogenetic protein 4), DUSP6 (dual-specificity phosphatase 6), extracellular matrix glycoprotein tenascin-X (TNXB), IL (interleukin)-8, and IL-17 receptor C (Figure 7C,D). These data suggest that activin signaling dictates a spectrum of HSC properties redundantly via multiple ligands, including activin A and B. On the other hand, each of these individual ligands has a large and unique set of target genes associated with critical cellular functions. These data together suggest that activin B is a direct regulator of HSCs and that activin ligands distinctly but coordinately modulate the transcriptome of HSCs.

To gain insight into how activin A and B interactively act on HSCs, we exposed LX-2 cells to activin A or B alone or both and subsequently examined how a group of genes known to regulate HSC activity respond transcriptionally. We observed four scenarios: (1) *ACVR1* (activin A receptor type 1) and *CKDN1B* (cyclin-dependent kinase inhibitor 1B) equivalently responded to individual ligand (Figure 8E); (2) *CASP6* (caspase-6) solely responded to activin A (Figure 7E); (3) *CASP3*, *GDNF* (glial cell line-derived neurotrophic

factor), and *CXCL1* specifically responded to dual ligands (Figure 7F); and (4) *CTGF* equally responded to individual ligands but synergistically to dual ligands (Figure 7F). The results indicate that activin B and A have redundant, unique, and interactive effects on HSCs. CTGF is a potent driver of HSC activation and liver fibrogenesis.^[38] Activin A and activin B equivalently, and a combination of them additively, increased the expression of CTGF together with ACTA2 and COL1A1 in both LX-2 cells and mouse primary HSCs (Figure 7G,H). Taken together, these *in vitro* data demonstrate that activin B and A directly, redundantly, as well as cooperatively promote the activation of HSCs.

DISCUSSION

Our studies revealed that circulating activin B is closely associated with liver injury and fibrosis regardless of etiologies and species. These data suggest a highly conserved, activin B-mediated mechanism of response to liver insults in mammals. This response is activated rapidly following liver injury, operates stably as the progression of disease, and predominates throughout liver

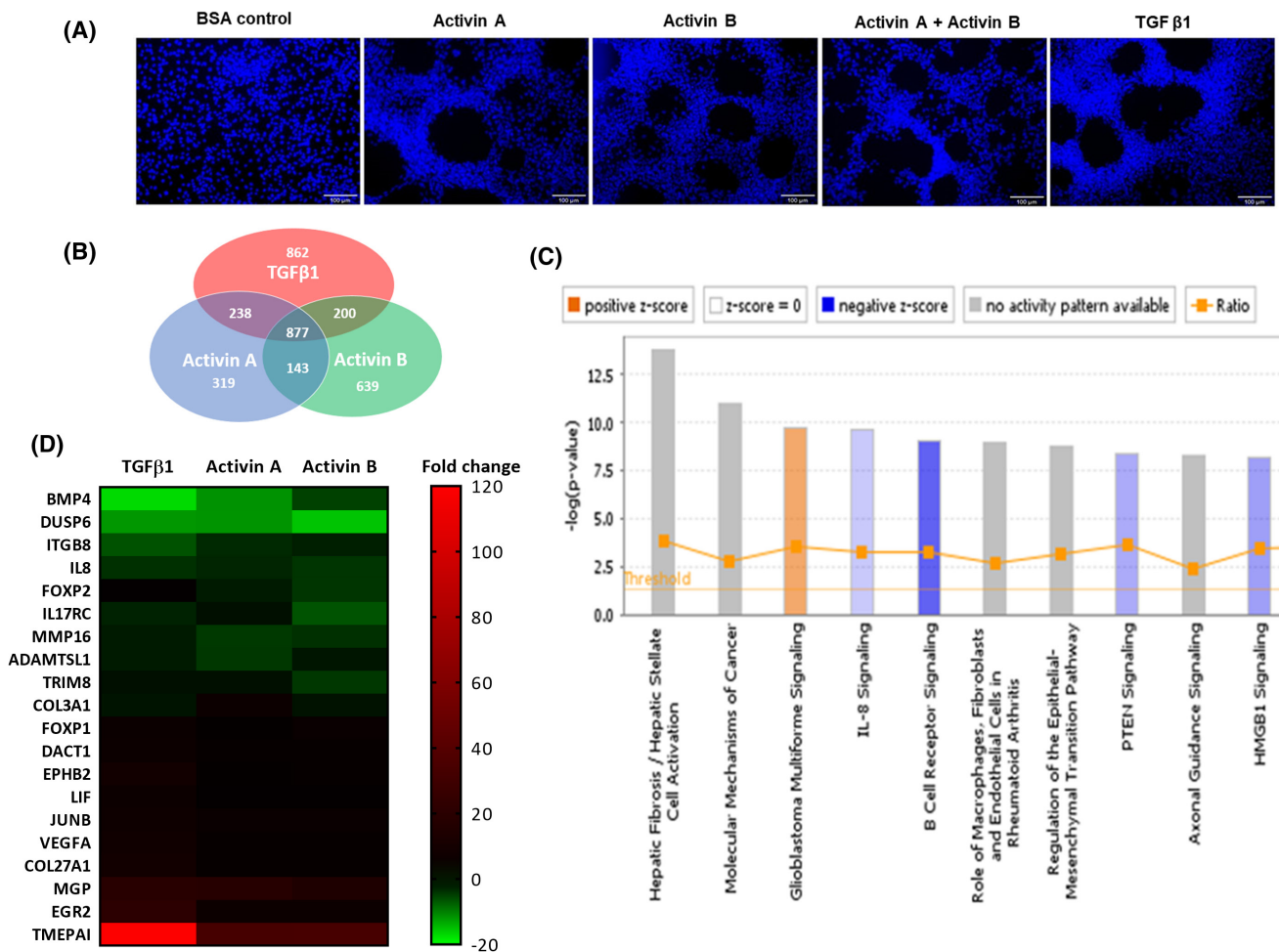


FIGURE 7 Activin B morphologically and molecularly activates hepatic stellate cells (HSCs). (A) LX-2 cells were treated with bovine serum albumin (BSA; 100 ng/ml), activin A (100 ng/ml), activin B (100 ng/ml), their combination (100 ng/ml each), or transforming growth factor β 1 (TGF β 1; 5 ng/ml) for 24 h and then underwent 4', 6-diamidino-2-phenylindole (DAPI) staining. (B) LX-2 cells were treated with activin A (100 ng/ml), activin B (100 ng/ml), or TGF β 1 (5 ng/ml) for 6 h. Total RNAs were isolated, reverse-transcribed to complementary DNA, and then subjected to microarray analysis using HG-U133 plus two chips ($n = 6$). Pie chart shows the numbers of genes commonly or uniquely regulated by the individual ligands. (C) The top 10 signaling pathways revealed by Ingenuity Canonical Pathway analysis of the 877 target genes shared by these three ligands. (D) Heat map of the 20 genes exhibiting the highest magnitudes of up-regulation or down-regulation in response to these three ligands. (E–G) LX-2 cells were treated with vehicle, activin A (100 ng/ml), activin B (100 ng/ml), or their combination (100 ng/ml of each) for 24 h. (H) Mouse primary HSCs were exposed to vehicle, activin A (100 ng/ml), activin B (100 ng/ml), or their combination (100 ng/ml of each) for 24 h. The expression of the genes indicated was assessed with quantitative real-time PCR. Data are shown as means of fold changes relative to vehicle controls \pm SEM ($n = 4$ –6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ via two-way ANOVA compared with vehicle controls. Abbreviations: ACTA2, smooth muscle alpha-2 actin; ACVR, activin A receptor; ACVR1, activin A receptor type 1; ADAMTSL, a disintegrin and metalloproteinase with thrombospondin motif-like protein; BMP, bone morphogenetic protein; CASP, caspase; CASP3, caspase-3; CDKN, cyclin-dependent kinase inhibitor; CDKNB, cyclin-dependent kinase inhibitor; Col1 α 1, collagen type I alpha 1; COL3A1, collagen type III alpha 1; DACT, dishevelled binding antagonist of beta catenin; DUSP, dual specificity phosphatase; DUSP6, dual-specificity phosphatase 6; EGR2, early growth response protein 2; EPHB, ephrin receptor B; FOXP, forkhead box P; GDNF, glial cell line–derived neurotrophic factor; IL, interleukin; IL17RC, interleukin 17 receptor C; ITGB, integrin beta; JUNB, jun B proto-oncogene; LIF, leukemia inhibitory factor; MGP, matrix Gla protein; MMP, matrix metalloproteinase; TMEPAI, transmembrane prostate androgen–induced protein; TRIM, tripartite motif family; VEGFA, vascular endothelial growth factor alpha.

fibrosis. Increased mRNA and protein levels of hepatic activin B is always concomitant with enriched circulating activin B in patients and mice with liver injury. Therefore, it is likely that increased production of hepatic activin B largely contributes to its systemic elevation during liver fibrosis development. This warrants further investigation to potentially develop activin B as a reliable and sensitive serum marker for monitoring liver injury progression.

The regulation of transcription, translation, and secretion of activin B remains largely unknown. We found that activin B is induced in a variety of liver injuries, suggesting the existence of common mediators. Others have shown that lipopolysaccharide stimulates the expression of activin B, which in turn elevates hepcidin expression, in mouse livers.^[39] This suggests that activin B is induced by pro-inflammatory factors in injured livers. There

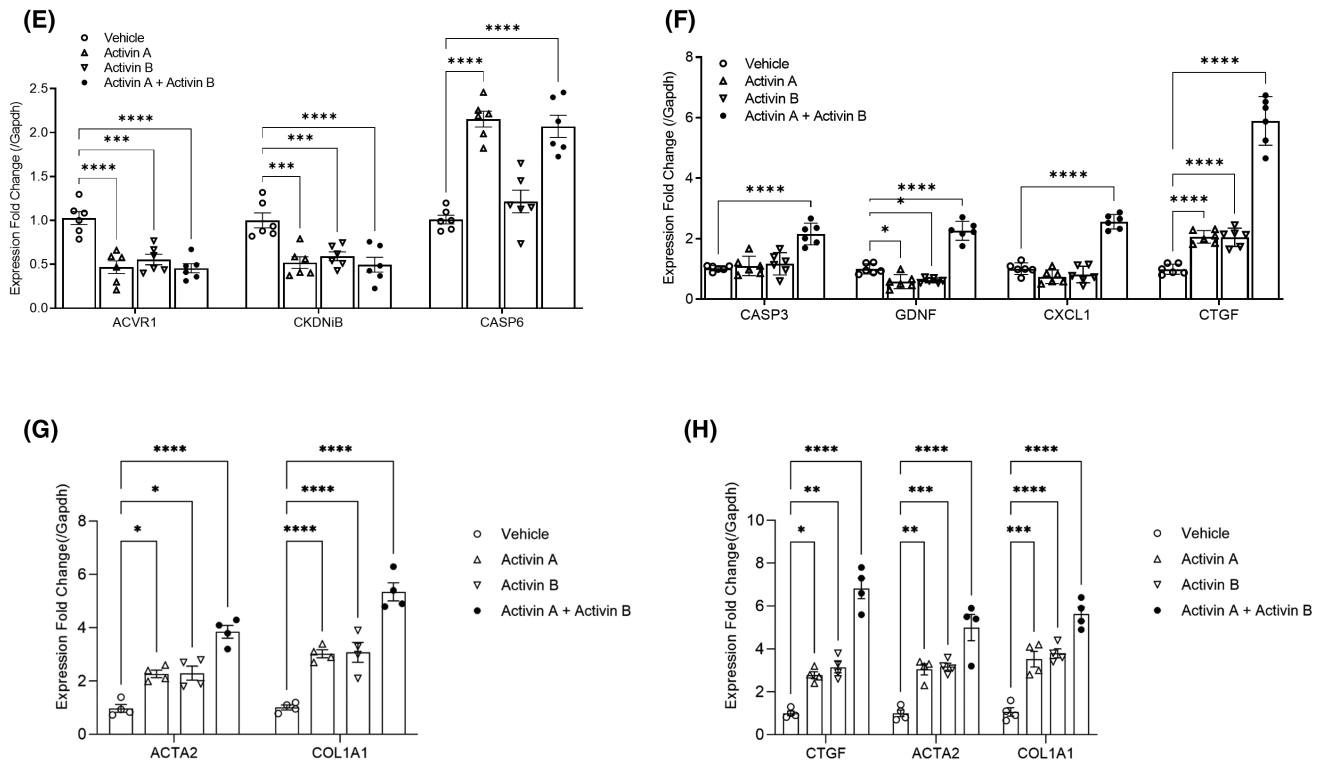


FIGURE 7 (Continued)

are two genes encoding hepcidin in mice (hepcidin 1 [*Hepc1*] and hepcidin 2 [*Hepc2*]).^[40] We found that the expression of *Hepc2*, but not *Hepc1*, was significantly reduced in CCl₄-damaged livers. Neutralizing activin A, activin B, or both activin A and activin B did not affect the expression of these two genes (Figure S3). The results suggest that hepcidin 2 is related to liver injury but activin B may not regulate hepcidin in injured livers.

We demonstrated that activin B acts as a potent driver of the complications (hepatocyte injury and fibrosis) of chronic liver injury. Damaged hepatocytes produce activin B that may facilitate hepatocyte necrosis in an autocrine manner. This may explain why activin B inactivation reduced the release of ALT and AST from hepatocytes in injured liver. We also demonstrated that activin B acts as a strong profibrotic factor following liver injury. *In vitro*, activin B altered the transcriptome of HSCs toward a profibrotic myofibroblast-like profile. These effects were highlighted by up-regulated expression of genes encoding profibrotic factors including collagens, matrix metalloproteinase, and CTGF. Activin B also induced HSCs to form a septa-like structure *in vitro*. We revealed the property of these cells, offering an *in vitro* assay to investigate the behavior and morphology of these cells in fibrotic liver. *In vivo*, neutralizing activin B alone largely repressed septa formation, collagen deposition, and expression of fibrotic genes such as CTGF and TGFβ1 in chronically injured liver. Microarray data have provided us with a list of activin B target genes of interest for further investigation to

elucidate how activin B modulates HSC activity during liver injury. Collectively, these findings allow us to propose that activin B promotes the initiation and progression of liver fibrosis by augmenting hepatocyte death and sustaining HSC activation.

Our studies discovered that increased activin B requires the presence of activin A to optimally mediate liver injury progression. We showed that hepatic and circulating activin A were only transiently increased during the acute phase of liver injury and were kept at pre-injury levels throughout the long-term chronic phase. However, neutralizing activin A alone did produce some beneficial effects, although significantly less than neutralizing activin B alone, in both prevention and reversal studies. In addition, both activin B and A were required for the up-regulation of profibrotic factors CTGF and TGFβ1 in chronically damaged livers, because neutralizing either one of the two TGFβ ligands fully abolished the effects. Moreover, we observed several additive or interdependent effects between activin B and activin A *in vitro* on HSCs as well as *in vivo* in fibrotic livers. Most notably, inactivating both activin B and activin A gave rise to the most profound beneficial effects across hepatic structural and functional assessments compared with inactivating activin B or A alone. These observations enable us to reason that, as liver injury progresses, elevated activin B needs constitutive activin A for cooperative pathological actions, as both ligands are required for activating certain cellular programs that otherwise would not be initiated by a single

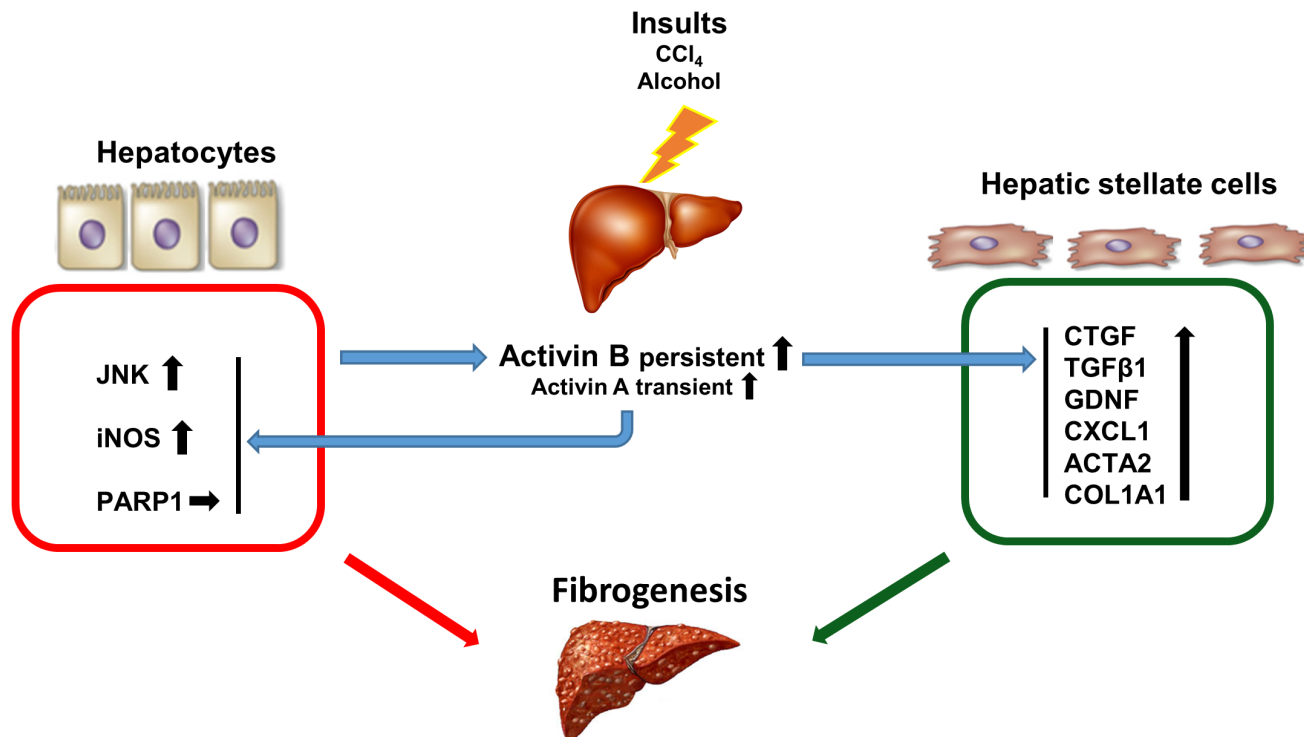


FIGURE 8 The mode of action of activin B in liver injury. Hepatocytes release activin B following injury. In an autocrine manner, activin B stimulates the activation of JNK and the expression of iNOS and PARP1, promoting hepatocyte death. In a paracrine manner, activin B induces HSCs to produce profibrotic factors including CTGF and CXCL1 and activates HSCs. As liver injury progresses, hepatocytes persistently produce activin B and transiently produce activin A. Activin B requires the presence of activin A for additive or synergistic effects on stimulating hepatocyte death and HSC activation. Thus, activin B acts as a key modulator, along with other profibrotic factors, to drive the initiation and progression of liver fibrosis.

ligand. This represents a mode of action of activin ligands and a mechanism governing the actions of activin B and A specifically. This discovery enables us to gain important mechanistic insight into the actions of activin B and A during fibrogenesis. Our preclinical studies demonstrate that targeting activin B, or ideally both activin B and A, is a promising strategy to prevent and even reverse liver fibrosis. This finding warrants future investigations to evaluate the efficacy of activin B blockade in diverse liver injury models.

We uncovered that activin B targets on multiple crucial modulators of liver injury. JNK critically mediates hepatocyte death and liver fibrosis induced by diverse insults such as cytokines, toxins, drugs, reactive oxygen species, and many others.^[33] Here we demonstrate that activin B inactivation fully prevented CCl₄-induced JNK activation. This revealed activin B as an essential and key activator of JNK in this setting. Moreover, the distinct extents of JNK suppression by inactivating activin A, activin B, or both of them are well correlated with the differing antifibrotic effects of these manipulations. This strongly suggests that activin B primarily activates JNK to promote liver fibrogenesis, cooperating with activin A for an optimal effect. Using *iNOS* knockout mice, several groups have demonstrated that iNOS facilitates hepatocyte necrosis and liver fibrosis in several liver injury models,^[34,41–43] although a report

showed a contrary result.^[44] Here we found that activin B is largely responsible for the induction of iNOS expression in response to liver injury. PARP1 is a nuclear enzyme. It regulates DNA repair, chromatin structure, and many other cellular processes. Pathological activation of PARP1 enhances cell death and inflammatory responses.^[45] Mukhopadhyay et al. reported that CCl₄ induces hepatic PARP1 expression, and *PARP1* deficiency or PARP1 pharmacological inhibition prevents or reverses CCl₄-induced hepatocyte death, inflammation, and fibrosis in male mice.^[35] Of note, here we observed that the basal expression of hepatic PARP1 appeared high and CCl₄ did not increase its expression in female mice. This could be a sex-dependent difference in basal and inducible expression of hepatic PARP1. We found that activin B is required for maintaining PARP1 expression in injured livers. This action of activin B needs the presence of activin A for a maximal effect. This can explain why the effects on suppressing PARP1 expression followed the pattern of inactivating both activin B and A > inactivating B > inactivating A. JNK can be activated by a spectrum of stimuli including cytokines such as TGFβ1.^[33] In addition, PARP1 activates JNK to induce cell necrosis.^[46] Here we show that neutralizing activin B vanished TGFβ1 induction (Figure 4H) and suppressed PARP1 expression (Figure 6) in injured livers. Therefore, our findings

strongly suggest that activin B regulates JNK activity via modulating the expression of TGF β 1 and PARP1. Notably, we show that neutralizing activin B is more potent than neutralizing activin A in suppressing PARP1 expression in injured livers. Correspondingly, neutralizing activin B is more effective than neutralizing activin A in reducing JNK activity (Figure 6). In addition, we found that neutralizing activin B, but not activin A, prevented the up-regulation of LCN2 (Figure S3), which is known to promote liver fibrogenesis.^[32] Thus, these observations may partially explain differential effects of targeting these two TGF β ligands during chronic liver injury. Collectively, we linked the action of activin B to JNK, iNOS, TGF β 1, PARP1, and LCN2 during liver injury and paved a way for further mechanistic studies.

Based our findings, we outline a working hypothesis for the mode of action of activin B in modulating hepatic injury response and fibrogenesis, as depicted in Figure 8. This hypothesis lays a foundation for our future investigations to further understand how activin B acts and evaluate activin B as a promising target for treating chronic liver diseases.

ACKNOWLEDGEMENT

We acknowledge the Histology Core of Indiana University-Purdue University Indianapolis for their technical assistance.

FUNDING INFORMATION

Supported by Translational Research Pilot Grant from Indiana University and Lilly Graduate Research Advanced Degree Program.

CONFLICT OF INTEREST

The authors disclose that a patent entitled "Methods of treating or preventing liver fibrosis with inhibition of activins B and A" was published based on this manuscript (patent publication number: US2021/0009672 A1). Yan Wang, Matthew Hamang, and Alexander Culver are Lilly Graduate Research Advanced Degree Program graduate students in the Department of Biology of School of Science at Indiana University-Purdue University Indianapolis and are employees of Eli Lilly & Company. Benjamin C. Yaden is an adjunct professor in the Department of Biology of School of Science at Indiana University-Purdue University Indianapolis and is an employee of Eli Lilly & Company.

SYNOPSIS

We found that activin B promotes hepatocyte injury and activates hepatic stellate cells, mediating the initiation and progression of liver fibrosis. Thus, we demonstrate activin B as a strong profibrotic factor.

ORCID

Alexander Culver  <https://orcid.org/0000-0001-5740-6579>

Joonyong Lee  <https://orcid.org/0000-0002-2518-4286>

Naga Chalasani  <https://orcid.org/0000-0003-4082-3178>

Suthat Liangpunsakul  <https://orcid.org/0000-0002-6504-8123>

Guoli Dai  <https://orcid.org/0000-0002-3588-4796>

REFERENCES

- Higashi T, Friedman SL, Hoshida Y. Hepatic stellate cells as key target in liver fibrosis. *Adv Drug Deliv Rev.* 2017;121:27–42.
- Bitto N, Liguori E, La Mura V. Coagulation, microenvironment and liver fibrosis. *Cell.* 2018;7:85.
- Sanyal AJ. Novel therapeutic targets for steatohepatitis. *Clin Res Hepatol Gastroenterol.* 2015;39(Suppl 1):S46–50.
- Schuppan D, Ashfaq-Khan M, Yang AT, Kim YO. Liver fibrosis: direct antifibrotic agents and targeted therapies. *Matrix Biol.* 2018;68–69:435–51.
- Friedman SL. Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol.* 2010;7:425–36.
- Novo E, Cannito S, Paternostro C, Bocca C, Miglietta A, Parola M. Cellular and molecular mechanisms in liver fibrogenesis. *Arch Biochem Biophys.* 2014;548:20–37.
- Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol.* 2008;214:199–210.
- Namwanje M, Brown CW. Activins and inhibins: roles in development, physiology, and disease. *Cold Spring Harb Perspect Biol.* 2016;8:a021881.
- Matzuk MM, Kumar TR, Vassalli A, Bickenbach JR, Roop DR, Jaenisch R, et al. Functional analysis of activins during mammalian development. *Nature.* 1995;374:354–6.
- Schrewe H, Gendron-Maguire M, Harbison ML, Gridley T. Mice homozygous for a null mutation of activin beta B are viable and fertile. *Mech Dev.* 1994;47:43–51.
- Vassalli A, Matzuk MM, Gardner HA, Lee KF, Jaenisch R. Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev.* 1994;8:414–27.
- Lau AL, Kumar TR, Nishimori K, Bonadio J, Matzuk MM. Activin betaC and betaE genes are not essential for mouse liver growth, differentiation, and regeneration. *Mol Cell Biol.* 2000;20:6127–37.
- Mellor SL, Ball EM, O'Connor AE, Ethier JF, Cranfield M, Schmitt JF, et al. Activin betaC-subunit heterodimers provide a new mechanism of regulating activin levels in the prostate. *Endocrinology.* 2003;144:4410–9.
- De Bleser PJ, Niki T, Xu G, Rogiers V, Geerts A. Localization and cellular sources of activins in normal and fibrotic rat liver. *Hepatology.* 1997;26:905–12.
- Schwall RH, Robbins K, Jardieu P, Chang L, Lai C, Terrell TG. Activin induces cell death in hepatocytes in vivo and in vitro. *Hepatology.* 1993;18:347–56.
- Yasuda H, Mine T, Shibata H, Eto Y, Hasegawa Y, Takeuchi T, et al. Activin A: an autocrine inhibitor of initiation of DNA synthesis in rat hepatocytes. *J Clin Invest.* 1993;92:1491–6.
- Hully JR, Chang L, Schwall RH, Widmer HR, Terrell TG, Gillett NA. Induction of apoptosis in the murine liver with recombinant human activin A. *Hepatology.* 1994;20:854–62.
- Date M, Matsuzaki K, Matsushita M, Tahashi Y, Sakitani K, Inoue K. Differential regulation of activin A for hepatocyte growth and fibronectin synthesis in rat liver injury. *J Hepatol.* 2000;32:251–60.
- Wada W, Kuwano H, Hasegawa Y, Kojima I. The dependence of transforming growth factor-beta-induced collagen production on autocrine factor activin A in hepatic stellate cells. *Endocrinology.* 2004;145:2753–9.

20. Kiagiadaki F, Kampa M, Voumvouraki A, Castanas E, Kouroumalis E, Notas G. Activin-A causes hepatic stellate cell activation via the induction of TNF α and TGF β in Kupffer cells. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864:891–9.
21. Wang DH, Wang YN, Ge JY, Liu HY, Zhang HJ, Qi Y, et al. Role of activin A in carbon tetrachloride-induced acute liver injury. *World J Gastroenterol*. 2013;19:3802–9.
22. Hedger MP, Winnall WR, Phillips DJ, de Kretser DM. The regulation and functions of activin and follistatin in inflammation and immunity. *Vitam Horm*. 2011;85:255–97.
23. Hedger MP, de Kretser DM. The activins and their binding protein, follistatin—diagnostic and therapeutic targets in inflammatory disease and fibrosis. *Cytokine Growth Factor Rev*. 2013;24:285–95.
24. Dykema JC, Mayo KE. Two messenger ribonucleic acids encoding the common beta B-chain of inhibin and activin have distinct 5'-initiation sites and are differentially regulated in rat granulosa cells. *Endocrinology*. 1994;135:702–11.
25. Kobayashi T, Niimi S, Hashimoto O, Hayakawa T. Expression of inhibin betaA, betaB and follistatin mRNAs in the carbon tetrachloride induced rat liver regeneration model. *Biol Pharm Bull*. 2000;23:755–7.
26. Canali S, Core AB, Zumbrennen-Bullough KB, Merkulova M, Wang CY, Schneyer AL, et al. Activin B induces noncanonical SMAD1/5/8 signaling via BMP type I receptors in hepatocytes: evidence for a role in hepcidin induction by inflammation in male mice. *Endocrinology*. 2016;157:1146–62.
27. Lee H, Jeong H, Park S, Yoo W, Choi S, Choi K, et al. Fusion protein of retinol-binding protein and albumin domain III reduces liver fibrosis. *EMBO Mol Med*. 2015;7:819–30.
28. Knockaert L, Berson A, Ribault C, Prost PE, Fautrel A, Pajaud J, et al. Carbon tetrachloride-mediated lipid peroxidation induces early mitochondrial alterations in mouse liver. *Lab Invest*. 2012;92:396–410.
29. Lamas-Paz A, Hao F, Nelson LJ, Vazquez MT, Canals S, Gomez Del Moral M, et al. Alcoholic liver disease: utility of animal models. *World J Gastroenterol*. 2018;24:5063–75.
30. Liu Y, Meyer C, Xu C, Weng H, Hellerbrand C, ten Dijke P, et al. Animal models of chronic liver diseases. *Am J Physiol Gastrointest Liver Physiol*. 2013;304:G449–68.
31. Krishnan V. Inhibition of activin A ameliorates skeletal muscle injury and rescues contractile properties by inducing efficient remodeling in female mice. *J Pharmacol Exp Ther*. 2014;184:1152–66.
32. Chen J, Argemi J, Odena G, Xu MJ, Cai Y, Massey V, et al. Hepatic lipocalin 2 promotes liver fibrosis and portal hypertension. *Sci Rep*. 2020;10:15558.
33. Seki E, Brenner DA, Karin M. A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. *Gastroenterology*. 2012;143:307–20.
34. Aram G, Potter JJ, Liu X, Torbenson MS, Mezey E. Lack of inducible nitric oxide synthase leads to increased hepatic apoptosis and decreased fibrosis in mice after chronic carbon tetrachloride administration. *Hepatology*. 2008;47:2051–8.
35. Mukhopadhyay P, Rajesh M, Cao Z, Horvath B, Park O, Wang H, et al. Poly (ADP-ribose) polymerase-1 is a key mediator of liver inflammation and fibrosis. *Hepatology*. 2014;59:1998–2009.
36. Wells RG, Schwabe RF. Origin and function of myofibroblasts in the liver. *Semin Liver Dis*. 2015;35:e1.
37. Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut*. 2005;54:142–51.
38. Huang G, Brigstock DR. Regulation of hepatic stellate cells by connective tissue growth factor. *Front Biosci (Landmark Ed)*. 2012;17:2495–507.
39. Kanamori Y, Sugiyama M, Hashimoto O, Murakami M, Matsui T, Funaba M. Regulation of hepcidin expression by inflammation-induced activin B. *Sci Rep*. 2016;6:38702.
40. Lesbordes-Brion JC, Viatte L, Bennoun M, Lou DQ, Ramey G, Houbron C, et al. Targeted disruption of the hepcidin 1 gene results in severe hemochromatosis. *Blood*. 2006;108:1402–5.
41. Baldik Y, Diwan AD, Appleyard RC, Fang ZM, Wang Y, Murrell GA. Deletion of iNOS gene impairs mouse fracture healing. *Bone*. 2005;37:32–6.
42. Nozaki Y, Fujita K, Wada K, Yoneda M, Kessoku T, Shinohara Y, et al. Deficiency of iNOS-derived NO accelerates lipid accumulation-independent liver fibrosis in non-alcoholic steatohepatitis mouse model. *BMC Gastroenterol*. 2015;15:42.
43. Anavi S, Eisenberg-Bord M, Hahn-Obercyger M, Genin O, Pines M, Tirosh O. The role of iNOS in cholesterol-induced liver fibrosis. *Lab Invest*. 2015;95:914–24.
44. Moreno MG, Muriel P. Inducible nitric oxide synthase is not essential for the development of fibrosis and liver damage induced by CCl₄ in mice. *J Appl Toxicol*. 2006;26:326–32.
45. Hottiger MO, Boothby M, Koch-Nolte F, Luscher B, Martin NM, Plummer R, et al. Progress in the function and regulation of ADP-Ribosylation. *Sci Signal*. 2011;4:mr5.
46. Douglas DL, Baines CP. PARP1-mediated necrosis is dependent on parallel JNK and Ca(2+)-calpain pathways. *J Cell Sci*. 2014;127:4134–45.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Wang Y, Hamang M, Culver A, Jiang H, Yanum J, Garcia V, et al. Activin B promotes the initiation and progression of liver fibrosis. *Hepatol Commun*. 2022;6:2812–2826. <https://doi.org/10.1002/hep4.2037>