ACTIVIN B PROMOTES HEPATIC FIBROGENESIS

by

Yan Wang

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STATEMENT OF COMMITTEE APPROVAL

Dr. Guoli Dai, Chair

Department of Biology - Indianapolis

Dr. Benjamin Yaden

Department of Biology – Indianapolis

Dr. David G. Skalnik

Department of Biology - Indianapolis

Dr. Nicolas Berbari

Department of Biology - Indianapolis

Dr. Suthat Liangpunsakul

Department of Biochemistry & Molecular Biology - Indianapolis

Approved by:

Dr. Theodore R. Cummins

Head of the Graduate Program

Dedicated to Xuekun, Angela, and Edward

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LIST OF ABBREVIATIONS

ActRII	Activin type II receptor
ActRIIB-Fc	ActRIIB Fc fusion protein
ALD	Alcoholic liver disease
ALK	Activin receptor-like kinase
ALT	Alanine transaminase
APRI	Aspartate transaminase to platelet ratio index
AST	Aspartate transaminase
α-SMA	Alpha smooth muscle actin
BDL	Bile duct ligation
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CCl_4	Carbon tetrachloride
CCL2	Chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor type 2
CK19	Cytokeratin 19
CTGF	Connective tissue growth factor
CXCL	Chemokine (C-X-C motif) ligand
CXCR3	Chemokine (C-X-C motif) receptor 3
CYP2E1	Cytochrome P450 Family 2 Subfamily E Member 1
DAMP	Damage-associated molecular pattern
DapB	Dihydrodipicolinate reductase
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
EndoMT	Endothelial–mesenchymal transition
ERK	Extracellular signal regulated kinase
ESLD	End stage of liver diseases
FBS	Fetal bovine serum
Fn14	Fibroblast growth factor-inducible 14
FSP1	Fibroblast-specific protein 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF	Growth differentiation factor
GFAP	Glial fibrillary acidic protein
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
HMGB1	High-mobility group protein 1
HSC	Hepatic stellate cell
IgG	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase

NAFLD	Nonalcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
PDGF	Platelet-derived growth factor
PIIINP	N-terminal propeptide of type III collagen
PPIB	Peptidylprolyl isomerase B
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
ROS	Reactive oxygen species
RPLOP	Ribosomal protein lateral stalk subunit P0
Smad	Mothers against decapentaplegic homolog
SBE	Smad-binding element
TGFBRII	Transforming growth factor beta receptor II
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteinase
TWEAK	TNF-like weak inducer of apoptosis
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
Wnt	Wingless/Integrated

ABSTRACT

Author: Wang Yan, PhD
Institution: Purdue University
Degree Received: August 2019
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Committee Chair: Guoli Dai

Liver fibrosis is a common consequence of various chronic liver diseases. Although transforming growth factor β 1 (TGFβ1) expression is known to be associated with liver fibrosis, the reduced clinical efficacy of TGFβ1 inhibition or the inefficiency to completely prevent liver fibrosis in mice with liver-specific knockout of TGF receptor II suggests that other factors can mediate liver fibrogenesis. As a TGFβ superfamily ligand, activin A signaling modulates liver injury by prohibiting hepatocyte proliferation, mediating hepatocyte apoptosis, promoting Kupffer cell activation, and inducing hepatic stellate cell (HSC) activation *in vitro*. However, the mechanism of action and *in vivo* functional significance of activin A in liver fibrosis models remain uncertain. Moreover, whether activin B, another ligand structurally related to activin A, is involved in liver fibrogenesis is not yet known. This study aimed to investigate the role of activin A and B in liver fibrosis initiation and progression. The levels of hepatic and circulating activin B and A were analyzed in patients with various chronic liver diseases, including end-stage liver diseases (ESLD), non-alcoholic steatohepatitis (NASH), and alcoholic liver disease (ALD). In addition, their levels were measured in mouse carbon tetrachloride (CCl_4), bile duct ligation (BDL), and ALD liver injury models. Mouse primary hepatocytes, RAW264.7 cells, and LX-2 cells were used as *in vitro* models of hepatocytes, macrophages, and HSCs, respectively. The specificity and potency of anti-activin B monoclonal antibody (mAb) and anti-activin A mAb were evaluated using Smad2/3 luciferase assay. Activin A, activin B, or their combination were immunologically inactivated by

the neutralizing mAbs in mice with progressive or established liver fibrosis induced by CCl_4 or with developing cholestatic liver fibrosis induced by BDL surgery. In patients with ESLD, NASH, and ALD, increases in hepatic and circulating activin B, but not activin A, were associated with liver fibrosis, irrespective of etiology. In mice with CCl_4-, BDL-, or alcohol-induced liver injury, activin B was persistently elevated in the liver and circulation, whereas activin A showed only transient increases. Activin B was expressed and secreted mainly by the hepatocytes and other cells, including cholangiocytes, activated HSCs, and immune cells. Exogenous administration of activin B promoted hepatocyte injury, activated macrophages to release cytokines, and induced a pro-fibrotic expression profile and septa formation in HSCs. Co-treatment of activin A and B interdependently activated the chemokine (C-X-C motif) ligand 1 (CXCL1)/inducible nitric oxide synthase (iNOS) pathway in macrophages and additively upregulated connective tissue growth factor expression in HSCs. Activin B and A had redundant, unique, and interactive effects on the transcripts related to HSC activation. The neutralization of activin B attenuated the development of liver fibrosis and improved liver function in mice with CCl_4- or BDL-induced liver fibrosis and largely reversed the already established liver fibrosis in the CCl_4 mouse model. These effects were improved by the administration of additional anti-activin A antibody. Combination of both antibodies also inhibited hepatic and circulating inflammatory cytokine production in the BDL mouse model. In conclusion, activin B is a potential circulating biomarker and potent promotor of liver fibrosis. Its levels in the liver and circulation increase significantly in both acute and chronic states of liver injury. Activin B might additively or interdependently cooperate with activin A, which directly acts on multiple liver cell populations during liver injury and fibrosis, as the combination of both proteins increases pro-inflammatory and pro-fibrotic responses *in vitro.* In addition, the neutralization of both activin A and activin B *in vivo* enhances the preventive and

reversible effects of liver injury and fibrosis compared to that when activin B alone is neutralized. Our data reveal a novel target of liver fibrosis and the mechanism of activin B-mediated initiation of this process by damaging hepatocytes and activating macrophages and HSCs. Our findings show that activin B promotes hepatic fibrogenesis, and that targeting of activin B has anti-inflammatory and anti-fibrotic effects, which ameliorate liver injury by preventing or regressing liver fibrosis. Antagonizing either activin B alone or in combination with activin A prevents and regresses liver fibrosis in multiple animal studies, paving way for future clinical studies.

CHAPTER 1. INTRODUCTION

1.1 Clinical aspects of liver fibrosis

Liver diseases include a wide spectrum of progressive conditions such as hepatic steatosis, hepatic inflammation, viral hepatitis, alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH)[1], hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma[2]. Liver disease is one of the leading causes of mortality in the US with 3.9 million people (1.6% adult population) diagnosed with chronic liver disease in 2015[3]. Liver fibrosis is the common consequence of various chronic liver diseases and results from the abnormal wound healing response with progressive and excessive production and deposition of extracellular matrix (ECM) or connective tissue along with chronic inflammation[1]. Infection of hepatitis C and B viruses is the most common cause of liver fibrosis. The other causes include alcohol consumption, obesity, insulin resistance or type II diabetes, hypertension, hyperlipidemia, autoimmune hepatitis, cholestasis, and chronic cholangiophaties[4]. The accumulation of ECM proteins distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes characterizes advanced liver fibrosis or cirrhosis. The major clinical consequences of cirrhosis are hepatocyte dysfunction, portal hypertension, and hepatocellular carcinoma. Its severe or life-threatening complications include ascites, variceal hemorrhage, spontaneous bacterial peritonitis, coagulation disorders, and hepatic encephalopathy[5]. Therefore, the assessment of liver fibrosis and quantification of hepatic fibrotic tissue have become important for diagnosis, defining etiologies, guiding therapeutic strategies, and predicting prognosis. At present, the three major clinical approaches for measuring liver fibrosis are liver biopsy assessment, liver stiffness measurement, and circulating biomarker detection in the blood. Clinically, liver biopsy is considered the golden standard to assess the stage and grade

of liver fibrosis for diagnosis and therapeutic efficacy evaluation. The histological scoring systems include the five-stage meta-analysis of histological data in viral hepatitis (METAVIR), seven-stage Ishak, and three-stage Knodell fibrosis scoring systems[6]. The most commonly used METAVIR fibrosis scoring system includes F0 to F4 stages of liver fibrosis: F0, normal liver or no fibrosis; F1, portal fibrosis without septa formation; F2, portal fibrosis with few bridging or septa; F3, portal fibrosis with numerous portal to portal or portal to central bridging; F4, portal fibrosis with numerous portal to portal or portal to central bridging and nodule formation or cirrhosis. Portal to central fibrous bridging is associated with chronic biliary disease-related fibrosis, and portal to portal fibrous septa formation in liver fibrosis is caused by chronic viral hepatitis[7]. The histological patterns of liver fibrosis depend on their etiologies. In chronic viral hepatitis, liver fibrosis starts with portal enlargement as mild fibrosis; peri-portal fibrosis and portal to portal fibrous bridging as advanced fibrosis; and regenerative nodule formation as cirrhosis. In chronic ALD and NASH, liver fibrosis is characterized by peri-venular and peri-sinusoidal fibrosis distributed in the centrilobular areas. The histological changes are hepatocyte feathery degeneration, cholangiocytes, or bile ductular hyperplasia in cholestasis liver fibrosis[8]. However, the invasive histological assessment method is limited by patient unwillingness, pain, expense, associated complications, and sampling error. The blood tests for liver fibrosis include N-terminal propeptide of type III collagen (PIIINP), hyaluronic acid, and other circulating biomarkers, which might be contributed by extrahepatic diseases[9]. Measurement of liver stiffness by using elastography is a potential noninvasive alternative to assess liver fibrosis, although the application of this method is limited by its reliability and portability[5 10]. Clinically, there is an urgent need to identify an invasive circulating biomarker associated with severity of liver fibrosis which could assist diagnosis and guide therapeutic strategies.

In clinical patients and experimental models, liver fibrosis is reversible if the underlying liver injury causes are eliminated[11]. Its reversibility allows the possibility to develop therapeutic strategies to treat this disease. To understand the mechanism of regression of liver fibrosis, ECM producing myofibroblasts and activated macrophages are two main cell populations which have been focused to investigate. Myofibroblasts apoptosis, reduced fibrotic cytokines, upregulated increased collagenase activity and elimination of fibrous scars are major characters of regressed liver fibrosis[12]. Presently, although multiple factors have been recognized as pro-fibrotic regulator, clinical trials have not shown notable anti-fibrotic effect or causes severe adverse conditions [13 14]. Liver fibrosis is a complicated process contributed by multiple cell populations, cross-talk of various signaling pathways and modified by epigenetic regulation. At present, no effective medicines or Food and Drug Administration-approved molecules are available to treat liver fibrosis. Thus far, the only remedial treatment for late-stage cirrhosis is liver transplantation. However, this treatment is highly restricted because of the lack of donor organs and the incompatible clinical conditions of patients. Thus, developing an effective approach for preventing and regressing liver fibrosis has become an urgent and unmet clinical need.

1.2 Cellular mechanism of liver fibrosis

The liver is constituted by parenchymal cells (hepatocytes and cholangiocytes) and non-parenchymal cells, including hepatic stellate cells (HSCs), macrophages, neutrophils, T cells, natural killer cells, liver sinusoidal endothelial cells, and progenitor cells[15]. The cellular and molecular mechanisms underlying the initiation and progression of liver fibrosis are not completely understood. Multiple cell populations, including hepatocytes, macrophages, and, particularly, HSCs, cooperatively modulate the formation and resolution of liver fibrosis. The crosstalk between various liver cell types and between different molecular signaling pathways has

drawn more attention recently in order to identify the key mediators and thus the potential therapeutic targets of this pathogenesis[16]. Irrespective of the etiologies, including hepatitis virus infection and alcohol consumption, hepatocytes, cholangiocytes, and endothelial cells are damaged and release damage-associated molecular patterns (DAMPs) and reactive oxygen species (ROS), which directly activate HSCs, Kupffer cells, and other immune cells[17 18]. Accumulating evidence shows that myofibroblasts, as liver fibrogenic cells, drive the fibrogenic response and play a key role in hepatic fibrogenesis[19]. Hepatic myofibroblasts are mainly derived from activated HSCs and portal fibroblasts, whereas a small population originates from extrahepatic precursors. Activated Kupffer cells and other immune cells secrete cytokines and growth factors, including transforming growth factor beat (TGFβ), tumor necrosis factor (TNFα), interleukin 1 beta (IL-1β), interleukin-6 (IL-6), and chemokine (C-C motif) ligand 2 (CCL2), which along with ROS and DAMPs activate HSCs and trans-differentiate them to myofibroblasts to produce fibrillar collagen, fibronectin, and laminin[20]. These cytokines and growth factors also suppress matrix metalloproteinase (MMP) expression and promote tissue inhibitor of metalloproteinase (TIMP-1) transcripts to inhibit ECM degradation[21]. Excessive ECM accumulation and fibrous scar formation are the characteristic features of liver fibrosis. Fibrous scar tissue formation, hepatocyte loss, chronic inflammation, and liver architecture destruction eventually lead to liver dysfunction or failure[15]. While myofibroblasts are the central effectors of fibrogenesis, injured hepatocytes, endothelial cells, or bile duct epithelial cells, as well as activated macrophages and other immune cells, participate in the initiation and progression of liver fibrosis (Figure 1.1). Herein, we focus on reviewing the roles of hepatocytes, macrophages, and myofibroblasts in liver fibrosis development.

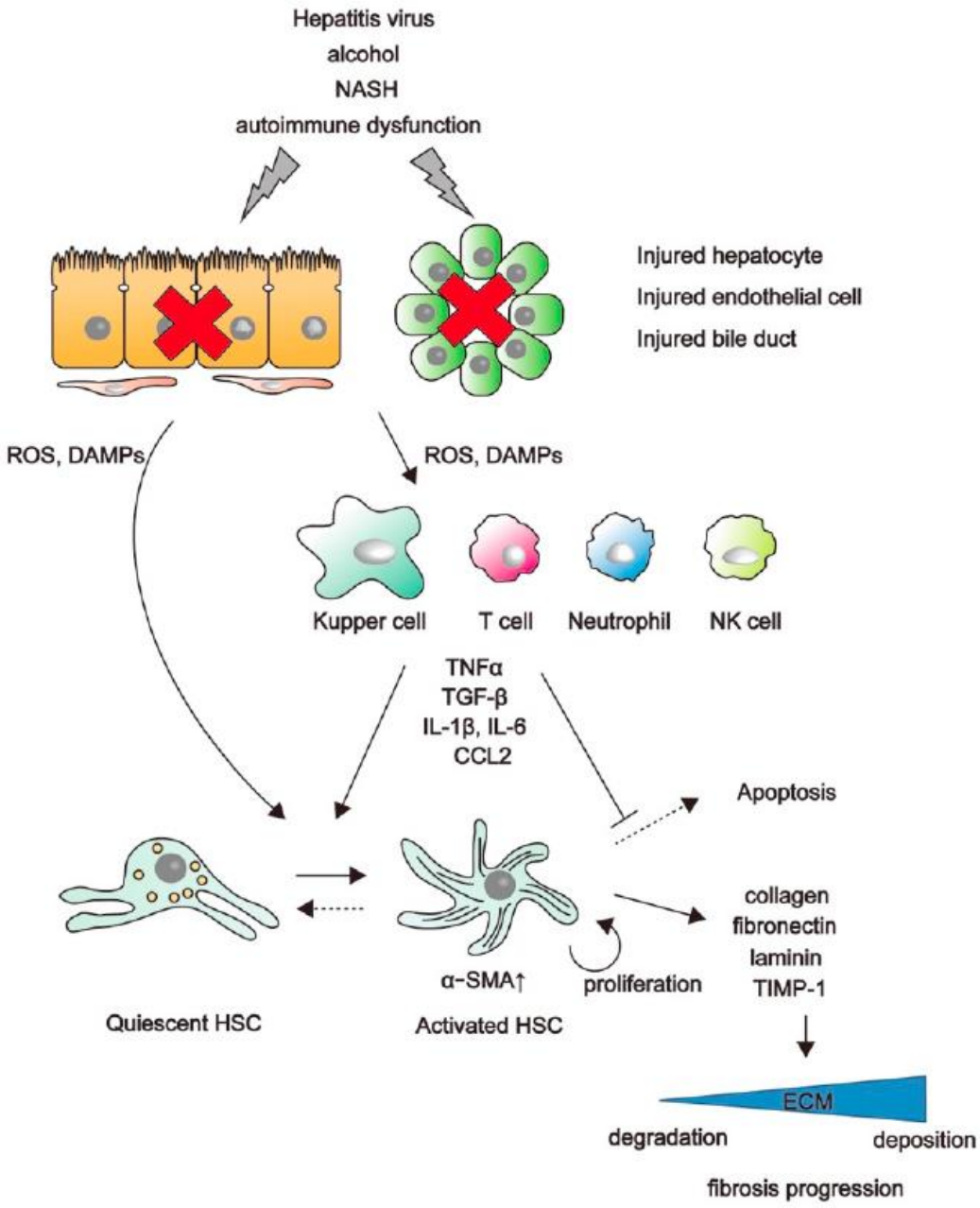


Figure 1.1 The cellular mechanism of liver fibrosis.
Nishikawa K, Osawa Y, Kimura K. *Int. J. Mol. Sci. 2018*

Hepatocytes

Hepatocytes, as liver parenchymal cells, are the major cell type in the liver and constitute approximately 60% of liver cells and up to 80% of liver mass. Another 40% of liver cells are non-

parenchymal cells, including Kupffer cells, HSCs, natural killer cells, lymphocytes, and liver sinusoidal endothelial cells[22,23]. The fundamental function of hepatocytes is to mediate lipid, carbohydrate, and protein metabolism to maintain homeostasis. Hepatocytes also produce albumin, clotting factors, lipoproteins, and bile acids. In addition, they detoxify toxic compounds, modify chemicals, and mediate urea metabolism and gluconeogenesis[23].

Hepatocytes participate in all the three phases of the liver injury process: initiation, perpetuation, and resolution[24]. The initial response to liver injury is hepatocyte stress and death, which leads to the release of ROS and DAMPs. Hepatocyte death is reflected by elevated serum alanine transaminase (ALT) and aspartate transaminase (AST), which are clinically used liver injury markers. ROS are the major stimuli to activate HSCs and macrophages. They also stimulate hepatocytes to increase TGFβ expression. As a representative ROS, hydrogen peroxide induces the activation of mitogen-activated protein kinase (MAPK) signaling pathway to activate multiple nuclear factors, including nuclear factor κB and nuclear factor erythroid-derived factor 2, in hepatocytes[25 26]. In chronic liver diseases, DAMPs from dying hepatocytes prompt inflammation and are represented by high-mobility group protein 1 (HMGB1) that in turn recruit inflammatory cells. DAMPs are mainly released when the membrane integrity is lost during hepatocyte necrosis and necroptosis. Hepatocyte death can be caused by apoptosis, necrosis, or necroptosis in response to diverse liver injuries, and the most common type of hepatocyte death is apoptosis. Hepatocyte apoptosis is the characteristic of liver injury and intrahepatic inflammation. Ballooning hepatocytes are a histological feature of apoptotic liver cells in NASH[27]. The death receptor Fas-FasL, death receptor 5, caspase 8, and mitochondria have been shown to be involved in hepatocyte apoptosis[28-30]. It is induced by multiple insults, including hepatitis virus infection and alcohol

consumption, and occurs in NAFLD and cholestatic diseases[31]. Carbon tetrachloride (CCl_4) and paracetamol induce hepatocyte necrosis, which is characterized by cell swelling, increased cell and organelle membrane permeability, and leakage of intracellular contents and inflammatory factors, including DAMPs, interleukin 33 (IL-33), TNFα, IL-6, and CCL2. These factors target neighboring or surrounding cells to induce liver inflammation[27], which plays a critical role in liver fibrogenesis. Hepatocyte swelling is mediated by mitochondrial dysfunction, calcium ion elevation, and adenosine triphosphate exhaustion. Hepatocyte necrosis is a predominant inducer of liver failure and an effective suppressant of liver regeneration[32]. Hepatocyte necroptosis is controlled necrosis. Alcohol, paracetamol, and TNF-mediated damage induce hepatic necroptosis in the liver. Intrahepatic lipid loading along with cell death is a unique feature of necroptosis. Upregulation of receptor interacting serine/threonine protein kinase 3 (RIP3) in response to NASH is important for the induction of necroptosis signaling[33]. Depending on the injury type, hepatocyte death occurs via the apoptosis, necrosis, or necroptosis pathway. Apoptotic and necrotic hepatocytes release ROS, DAMPs, apoptotic bodies, and necrotic fragments, which activate hepatic macrophages and HSCs to promote liver fibrosis during the initiation and perpetuation phases of liver injury[34 35]. During the perpetuation phase, non-parenchymal cell activation leads to increased hepatocyte death that prolongs liver injury. Activated HSCs and hepatic macrophages release chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, CXCL8, and IL-1β to attract neutrophils that secrete ROS and proteases to cause hepatocyte necrosis. Another mechanism of liver injury perpetuation is hypoxia due to blood flow interruption, ischemia, and ECM deposition. Hypoxia induces the death of hepatocytes surrounded by fibrous tissue and leakage of DAMPs[34 36]. In addition to releasing ROS and cell debris, hepatocytes mediate ECM remodeling by secreting MMPs and TIMPs. During the resolution phase, liver regeneration mainly involves two cell populations. Hepatocytes

repopulate to compensate for the lost liver mass and function, and HSC activation mediates ECM remodeling and repair. Liver regeneration is driven by multiple mechanisms: local hepatocyte proliferation to replace neighboring dead hepatocytes in response to trivial liver damage; hypertrophy of healthy hepatocytes if less than 10% of the liver cells are impaired; extensive hepatocyte mitosis if more than 10% liver cells are damaged; and activation of hepatic progenitor cells to supplement hepatocyte repopulation if the liver is severely damaged[37-39]. Cholangiopathies, which lead to cholestatic liver fibrosis, are the main indication for liver transplantation in up to 80% of children with liver diseases and in approximately 20% of adult patients[40]. The main target of cholangiopathies is cholangiocytes, which proliferate, secrete inflammatory cytokines, and mediate biliary apoptosis and liver fibrosis in response to cholestatic liver injury[41].

Macrophages

Liver macrophages include residential Kupffer cells and monocyte-derived macrophages. The former physiologically self-renew and are non-migratory phagocytes. They reside in hepatic sinusoids and have high phagocytic capability to maintain liver homeostasis by recognizing, ingesting, and degrading pathogens or cellular debris[20]. Liver insults activate Kupffer cells to secret inflammatory mediators, which in turn attract circulating monocytes to infiltrate into the liver. Activation of Kupffer cells and recruitment of monocytes are the key steps in liver fibrosis initiation and progression. Kupffer cells release cytokines and chemokines to induce the conversion of circulating monocytes to numerous monocyte-derived macrophages. The circulating Ly-6C^{hi} and Ly-6C^{lo} monocytes originate from the bone marrow or spleen, respectively, and each of them expresses specific receptors. Liver injury-induced chemokines such as CCL2 attract Ly-6C^{hi} monocyte infiltration from the circulation as they express C-C chemokine receptor type 2

(CCR2). In addition, phagocytes can infiltrate from the peritoneal cavity and specifically express GATA6 transcription factor[42]. The phenotypes of Kupffer cells and monocyte-derived macrophages adapt to the hepatic microenvironment[43-45]. Macrophage polarization is defined *in vitro* by M1 and M2 macrophage subsets. In response to different stimulators, Kupffer cells and infiltrating macrophages either undergo M1 or M2 activation during the initiation and progression of liver fibrosis. Classic M1 activation mediated by lipopolysaccharide (LPS) or interferon γ shows a pro-inflammatory phenotype by releasing mediators, including TNF-a, IL-6, and IL-1β, whereas the alternate M2 activation by IL-4 or IL-10 exhibits an anti-inflammatory phenotype by secreting IL-4, IL-10, TGFβ1, and IL-13 (Figure 1.2)[20 46]. M1 macrophages induce hepatic steatosis, hepatocyte apoptosis, and inflammation through these inflammatory cytokines, whereas M2 macrophages mediate wound repair and fibrogenesis. In the liver, the balance of M1 and M2 macrophages is pivotal in hepatic pathogenesis and can be regulated by multiple mediators. IL-10 secreted by M2 macrophages induces M1 macrophage apoptosis to prevent hepatic steatosis and liver damage in mice with ALD and NAFLD[47]. The phenotypes of liver macrophages *in vivo* are more complicated than this binary classification. In the resolution phase of liver fibrosis, an additional macrophage phenotype that expresses mixed markers of M1 and M2 macrophages promotes HSC apoptosis[48].

The heterogenic phenotypes of liver macrophages are rapidly altered and switched in response to a myriad of insults. In acetaminophen-induced liver injury, macrophages express both inflammatory and resolution markers[49]. Various insults activate macrophages, resulting in their secretion of cytokines and chemokines, which in turn causes monocyte infiltration to augment the number of macrophages in the liver. Monocyte-derived macrophages perform distinct functions,

depending on the stage of injury. In addition, microRNAs are another group of mediators that regulate the balance of M1/M2 macrophages. For example, micr-155 directly targets and downregulates IL-13Ra1 and several IL-13-related genes on M2 macrophages and promotes classic M1 activation[50]. Thus, microRNAs can exacerbate injury or provoke tissue repair events. Overall, the central function of macrophages is to mediate hepatocyte injury, advance inflammation, and activate HSCs to induce fibrosis or promote resolution by releasing specific cytokines and chemokines.

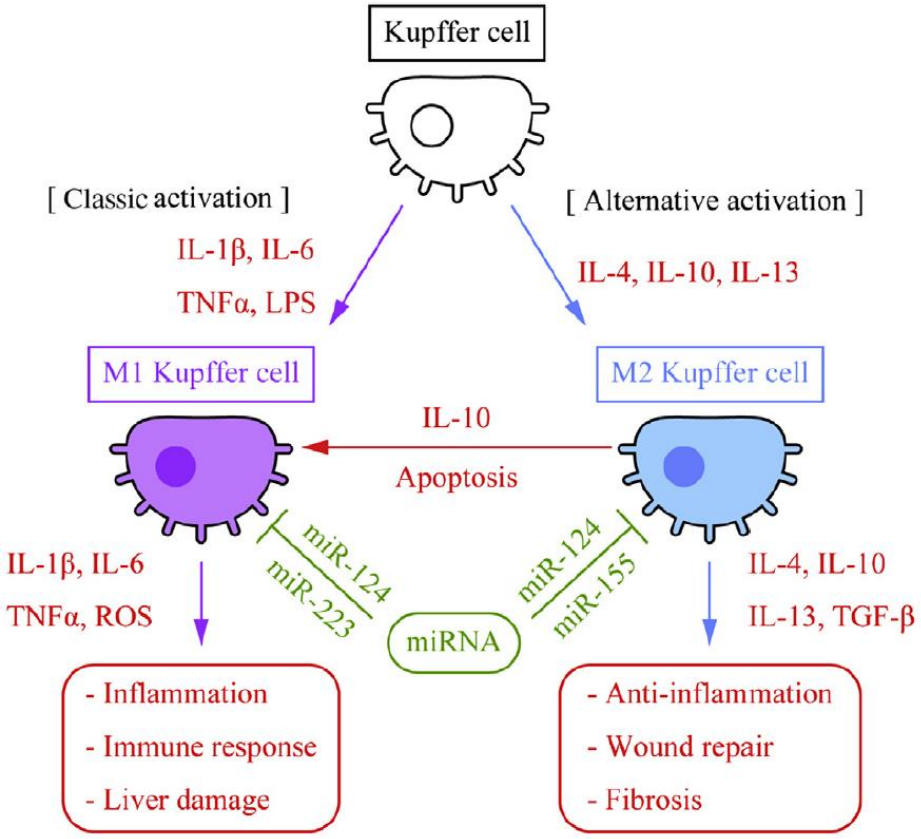


Figure 1.2 The activation of M1 and M2 Kupffer cells.
Sato K, Hall C, Glaser S, Francis H, Meng F, Alpini G. Am. J. Pathol. 2016

Myofibroblasts

Excessive ECM accumulation is a key characteristic of liver fibrosis, and the major cellular source of ECM is hepatic myofibroblasts, which consist of a heterogeneous population of liver fibrogenic cells. They mainly originate from resident mesenchymal cells of activated HSCs and portal fibroblasts. Extrahepatic precursors, including bone marrow-derived cells and circulating fibrocytes, are the minor contributors. Whether myofibroblasts are derived from epithelial or endothelial cells through EMT or endothelial–mesenchymal transition (EndoMT), respectively, is still debated (Figure 1.3)[51]. The variety of etiologies and different hepatic fibrotic sites might determine the contribution of myofibroblasts from distinct origins. A common characteristic of HSCs and portal fibroblasts is the production of fibrillar collagens, which represent the main components in the ECM. Vitamin A is contained in HSCs, but not in portal fibroblasts. Phenotype analysis showed that HSCs are the main cellular source of myofibroblasts in hepatotoxic liver fibrosis. Portal fibroblasts are the major contributors of myofibroblasts in early cholestatic liver injury, whereas HSCs become the main source in late-stage cholestatic liver disease[52]. In hepatotoxin-induced liver injury, HSCs produce more than 80% of the myofibroblasts[53]. HSCs are the first recognized fibrogenic cell population; in the normal liver, they are quiescent mesenchymal cells, rich in lipid droplets containing retinoid (vitamin A), expressing glial fibrillary acidic proteins (GFAPs) and adipogenic genes, and residing in the Disse space between hepatocytes and sinusoidal endothelial cells[54]. Therefore, quiescent HSCs are characterized by peri-sinusoidal location and vitamin A and GFAP staining. Various liver injuries induce the activation of HSCs and their adoption of fibrotic phenotype; HSCs are the main contributors of myofibroblasts. The trans-differentiation of quiescent HSCs to myofibroblasts is induced by a group of fibrogenic factors in response to various liver injuries. Myofibroblasts attain a fibrotic phenotype through the

expression of specific fibrogenic markers such as α-smooth muscle actin, reducing GFAP and adipogenic gene expression, and losing vitamin A and lipid droplet content. Activation of HSCs comprises initiation and perpetuation phases. The initiation phase is the pre-inflammatory stage, in which HSCs undergo transcriptional changes mainly in response to paracrine cytokines, lipid peroxide, or stimuli released by injured hepatocytes, infiltrated and activated Kupffer cells, endothelial cells, and platelets. Perpetuation is the maintenance stage of the activated HSC phenotype and leads to fibrosis regulated by paracrine and autocrine cytokines or growth factors. HSCs migrate toward the inflammatory sites via platelet derived growth factor (PDGF), CCL2, and CXCR3 chemotaxis. HSC proliferation is mediated by PDGF, vascular endothelial growth factor (VEGF), and other mitogens, which increases the HSC cell number to promote fibrosis. TGFβ1 and connective tissue growth factor (CTGF) are the two potent fibrogenic factors that induce the generation of ECM, including collagen types I and III, fibronectin, and proteoglycans. The alpha-smooth muscle actin (α-SMA) is a cytoskeletal protein and the hallmark of myofibroblasts, which acquire contractility from increased α-SMA expression. HSC contractility relies on calcium signaling as well as endothelin-1 and nitric oxide[55]. The balance of matrix production and degradation is the key event in matrix remodeling to maintain fibrosis. The MMPs can degrade both collagen and non-collagen components of the ECM. The TIMPs inhibit MMP activity by binding to them. The fibrotic phenotype of activated HSCs is maintained through complicated processes, including chemotaxis, proliferation, fibrogenesis, matrix degradation, and contractility.

Portal fibroblasts, which are also liver-resident mesenchymal cells, are another source of liver fibrogenic cells. In cholestatic and biliary liver diseases, portal fibroblasts trans-differentiate into

myofibroblasts and are the main cellular sources of myofibroblasts in the early phase of cholestatic fibrosis development. The trans-differentiation of portal fibroblasts also contributes to the formation of peri-portal fibrotic septa associated with chronic liver injury. Activated HSCs and portal myofibroblasts share some fibrotic markers such as α-SMA and collagens, but also express distinct markers. During the differentiation of HSCs to myofibroblasts, target gene expression is regulated by transcription factor activation or transcriptional suppression, and post-transcriptional regulation is altered by epigenetic modifications, including microRNA regulation, DNA methylation, and histone acetylation[56]. Activated HSCs specifically express desmin, cytoglobin, and synaptophysin, and portal myofibroblasts are characterized by IL-6, fibulin-1, and elastin. Both activated HSCs and portal myofibroblasts have common fibrotic functions, although portal myofibroblasts have superior proliferation potential and higher resistance to apoptosis than activated HSCs. Bone marrow transplant studies indicated that bone marrow stem cells and circulating fibrocytes are the minor contributors of myofibroblasts. Previous *in vitro* studies suggested that TGFβ induces hepatocytes and cholangiocytes to differentiate into a mesenchymal phenotype with fibroblast-specific protein 1 (FSP-1) expression via EMT[57]. However, *in vivo* cell fate mapping studies showed that epithelial and mesenchymal markers do not co-localize in injured livers[58 59]. All myofibroblasts are effector cells of liver fibrogenesis characterized by fibrillar collagen-rich ECM production and have the ability to migrate, proliferate, and contract. In response to numerous fibrogenic factors, including cytokines and growth factors, from injured liver, hepatic myofibroblasts proliferate, migrate to the injury sites, and accumulate there. Subsequently, hepatic myofibroblasts abundantly synthesize and produce a group of ECM proteins that mainly consists of fibrillar collagens I and III. Simultaneously, these cells secrete MMPs such

as ECM-degrading enzymes, MMP activators, and TIMPs. In chronic liver diseases, ECM remodeling is balanced by the secretion of MMPs and TIMPs in response to HSC activation[60 61].

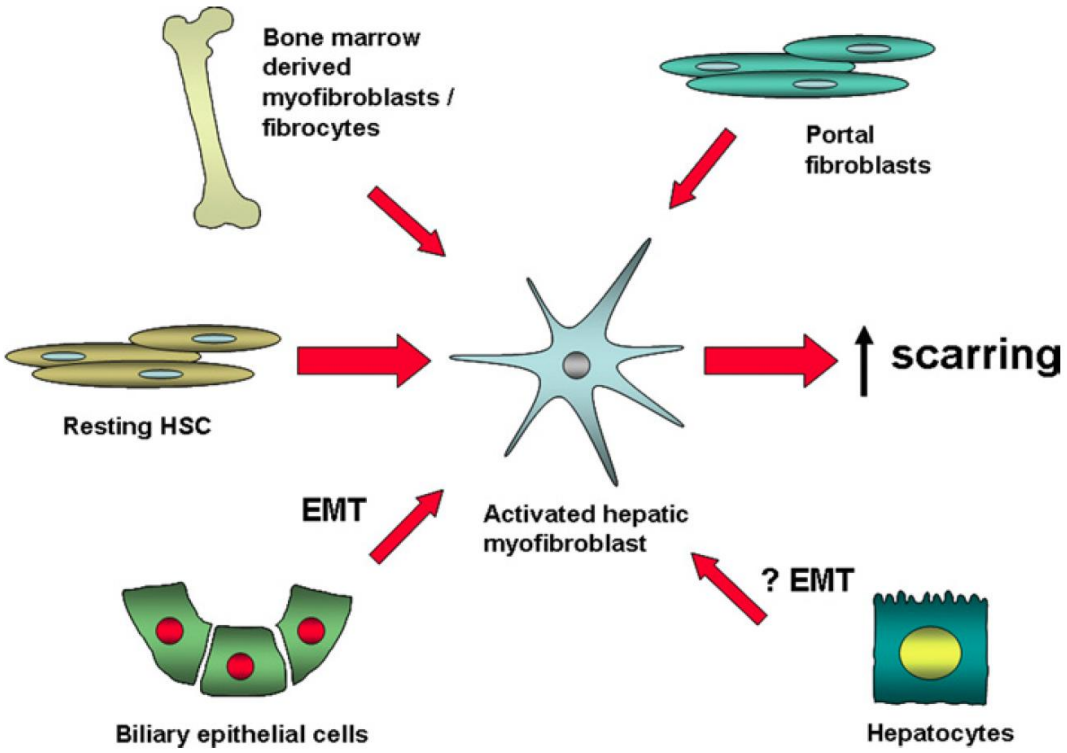


Figure 1.3 Heterogeneity of hepatic myofibroblasts.
Mallat, L. Am. J. Physiol. Cell Physiol. 2013

1.3 TGFβ superfamily signaling in liver fibrosis

Irrespective of etiology, the common mechanisms of hepatic fibrogenesis include the following cellular events: (1) hepatocytes and cholangiocytes undergo apoptosis, necrosis, or necroptosis and release ROS and DAMPs, growth factors, cytokines, and chemokines in response to liver injury; (2) monocytes, macrophages, and neutrophils are attracted by these factors, migrate to the injury sites, and secrete additional growth factors and cytokines/chemokines; and (3) hepatic myofibroblasts are trans-differentiated from HSCs, portal fibroblasts, bone marrow-derived cells,

and circulating fibrocytes possibly via EMT by a collection of these factors. The pro-fibrotic cells possess the properties of high ECM production and deposition, proliferation, migration, and contractility. Multiple inflammatory and fibrogenic signaling pathways promote the activation of HSCs and portal fibroblasts. TGFβ1 is the major growth factor to activate HSCs and portal fibroblasts. PDGF, cytokines (e.g., IL-17, IL-22, and IL-33), chemokines (e.g., CCl2, CXCL2, and CXCL10), adipokines (e.g., leptin, adiponectin, and resistin), pro-angiogenic factors (e.g., VEGF-A and angiopoietin), intracellular signaling, and transcription factors all contribute to this process[17] [62].

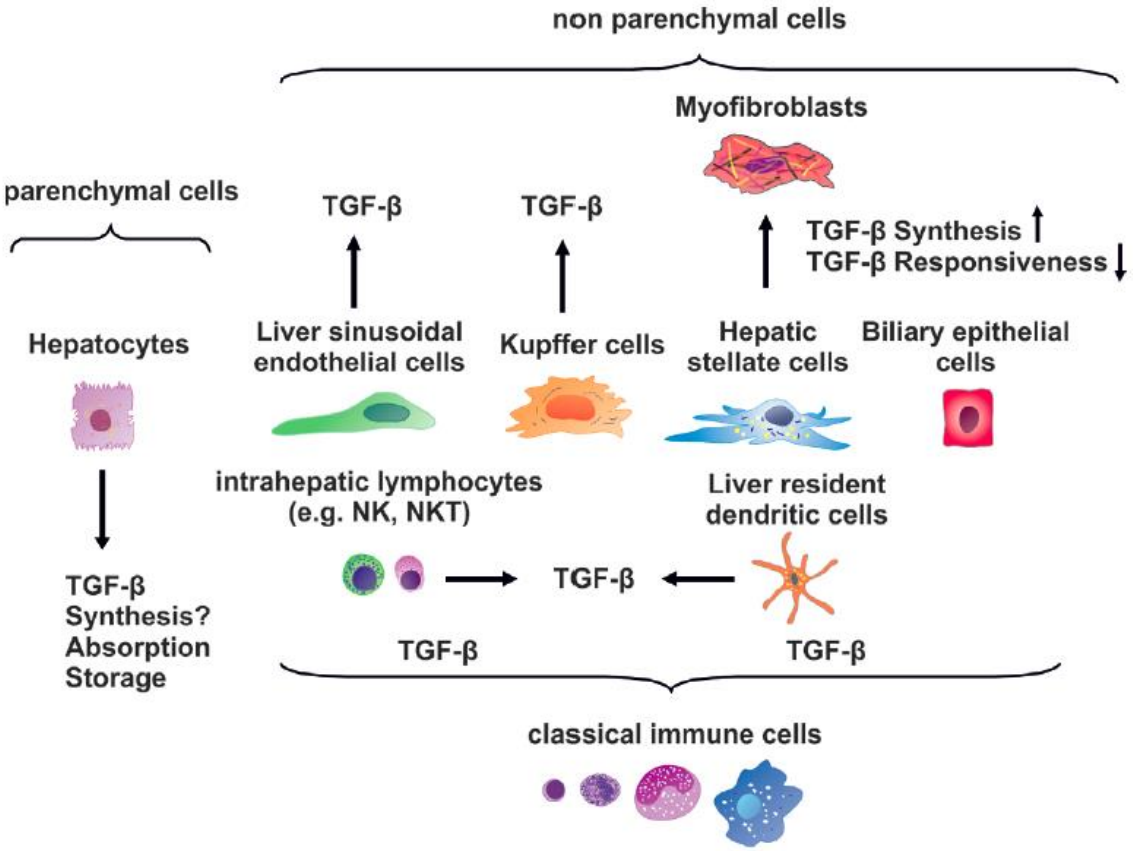


Figure 1.4 Hepatic TGF-β cellular sources.

Schon H, Weiskirchen R. Hepatobiliary Surg. Nutr. 2014

TGFβ signaling molecules constitute one of the most sophisticated signaling pathways known[63-65]. The TGFβ superfamily consists of over 50 secretory, structurally related ligands, which are divided into four subfamilies: the TGFβ subfamily, the bone morphogenetic protein (BMP) and growth differentiation factor (GDF) subfamily, the activin and inhibin subfamily, and a group of various divergent ligands. The majority of TGFβ superfamily ligands are secreted as active dimers that are inhibited by several secreted proteins, including follistatin, except for GDF8 and TGFβ, which are secreted as immature or inactive precursors[66]. TGFβ superfamily ligands bind to two transmembrane receptors: type I and type II receptors[67 68]. Type II receptors include TGFβ receptor II (TGFBRII), activin receptor II A (ActRIIA), and ActRIIB. Soluble ActRIIB binds to various TGFβ superfamily ligands, including GDF5, GDF8, GDF11, activin A, activin B, activin C, activin E, Nodal, BMP2, BMP4, BMP6/7, BMP9, and BMP10[69-71]. TGFβ signaling pathways have multi-functional effects and complicated intracellular interacting components. The TGFβ superfamily is known to play critical roles in embryogenesis, reproduction, and adult tissue homeostasis. TGFβ signaling critically modulates the initiation and progression of fibrosis in several organs, including the liver[64]. Hepatic TGFβ is expressed in diverse non-parenchymal cells, including HSCs, Kupffer cells, cholangiocytes, liver sinusoidal endothelial cells, lymphocytes, and liver-resident dendritic cells (Figure 1.4). Liver macrophages and HSCs are the two major cellular sources of TGFβ1. TGFβ1 stimulates hepatic fibrosis through multiple mechanisms. TGFβ signaling is involved in approximately all the stages of liver disease progression, from the initiation of liver injury through hepatic inflammation, liver fibrogenesis, cirrhosis, and even hepatocellular carcinoma. The TGFβ effect is also context-dependent. Primarily, bioactive TGFβ1 first binds to TGFβ receptor II, and then phosphorylates TGFβ type I receptor; next, intracellular events activate Smad2/3 and non-Smad signaling pathways to initiate or suppress target gene transcription (Figure 1.5)[17]. Presently,

although TGFβ1 have been recognized as pro-fibrotic regulator, clinical trials have shown TGFβ1 inhibition does not offer preventive or regressive effects on liver fibrosis progression, but, in turn, causes severe adverse effects such as inflammation and ontogenesis[13].

In TGFβ superfamily, different subfamily ligands generally signal via distinct, but overlapping, subsets of receptors and Smads. BMPs bind BMP-type II receptors (BMPR2), ActRIIA, and ActRIIB; type I receptors ALK1, ALK2, ALK3, and ALK6; and Smads 1, 5, and 8, whereas TGF-βs and activins use their respective type II receptor TGFBR2 or ActRIIA and ActRIIB, but share the type I receptors ALK4, ALK5, ALK7 and Smads 2 and 3[72 73]. Interaction is known to occur between BMPs and TGFβ/activin signaling. In particular, BMP7 is a negative regulator for TGFβ signaling[74]. In developmental biology, activin A synergistically acts with BMP4, basic fibroblast growth factor, and Wnt to drive embryonic stem cells to the definite endoderm[75 76]. In the central nervous system, activin B and TGF synergistically induce the proliferation and maturation of oligodendrocytes during myelin formation[77]. In HSCs, the activated target genes of TGFβ1 include fibrillar collagen I and III and other ECM genes, which contribute to ECM accumulation and liver fibrosis, whereas suppressed target genes consist of MMP transcripts that mediate ECM degradation. In hepatocytes, TGF-β1 inhibits growth and division by causing G1 phase cell cycle arrest and inducing hepatocyte cell death via the apoptosis response. The cytostatic and apoptotic effects of TGFβ1 on hepatocytes are pivotal for liver mass homeostasis and tumor development. In addition, TGF-β1 causes the trans-differentiation of hepatocytes to myofibroblasts via EMT[78]. In hepatic tumorigenesis, TGFβ1 might exhibit tumor suppressive effects at the early stages and promote tumor progression in the late stages when hepatocytes become resistant to the previous suppressive effect.

Therefore, the clinical application of TGFβ signaling inhibition is limited by the complex role of TGFβ in liver fibrogenesis, carcinogenesis, and immune modulation. Moreover, liver TGFBRII knockout only partially prevented liver fibrosis in the CCl_4-treated mouse model[79]. Even with such high structural similarities of 74% conservation of sequence identifies between TGFβ1 and TGFβ2, 78% between TGFβ1 and TGFβ3, 82% between TGFβ2 and TGFβ3, TGFβ1 and TGFβ2 act as pro-fibrotic factors[80], whereas TGFβ3 acts oppositely[81 82]. Ligand-dependent consequences are exemplified by BMP7 counteracting TGFβ1 activities during organ fibrosis[65 83]. Individual investigations of TGFβ superfamily ligands are required for determining their roles in organ fibrogenesis.

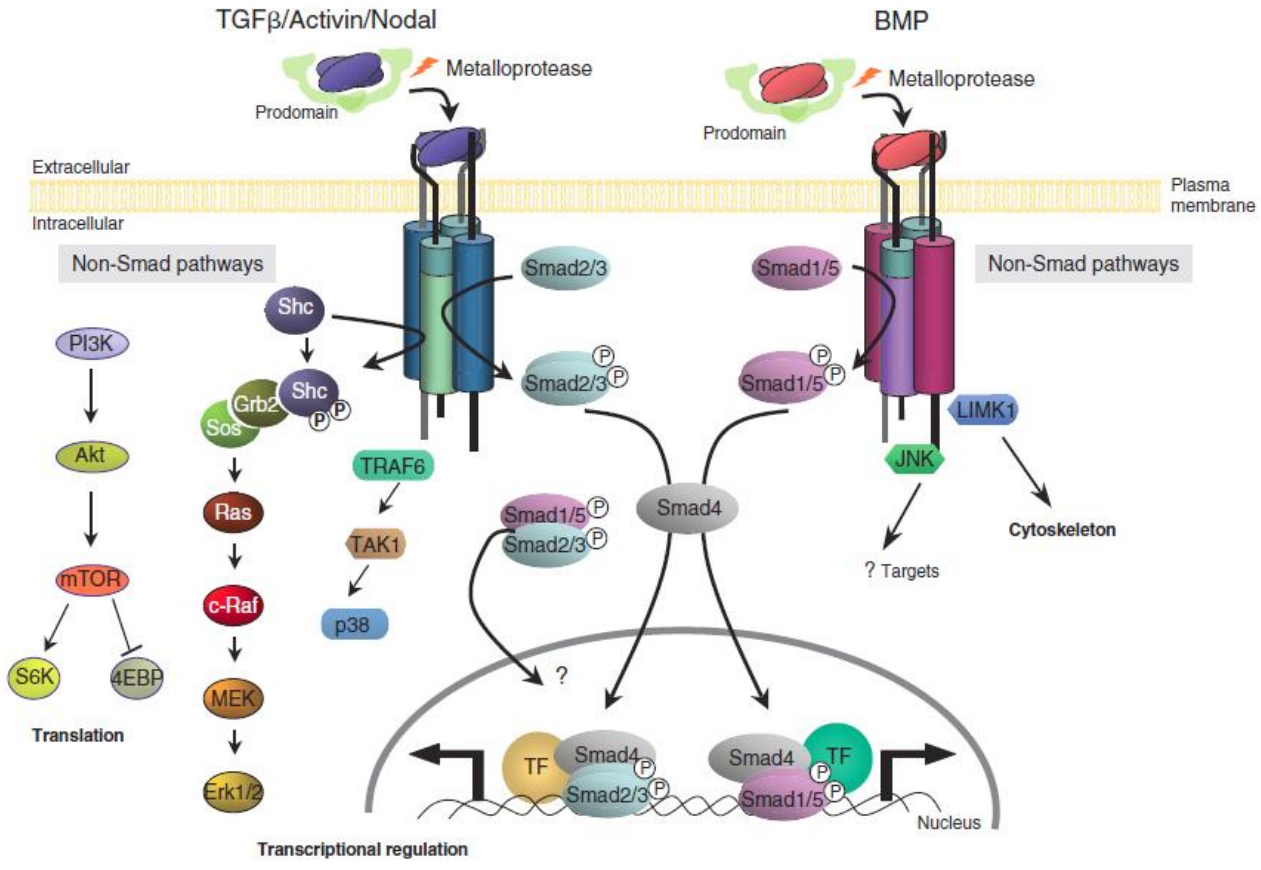


Figure 1.5 TGFβ superfamily canonical Smad and non-Smad signaling pathways.
Wharton K, Derynck R. Development 2009

1.4 Activins in liver fibrosis

Activin was discovered approximately 40 years ago; activins are homo- or heterodimers formed by four inhibin subunits—inhibin βA, inhibin βB, inhibin βC, and inhibin βE—in mammals[84]. At present, the homo-dimers of activins include activin A, activin B, activin C, and activin E, whereas the heterodimers are activin AB, activin AC, activin BC, and activin AE[22]. The inhibin βA or inhibin βB subunit can also form inhibin A or inhibin B protein by dimerization with the inhibin α subunit. Inhibins function as antagonists of activins in the reproductive system[85]. The inhibin βA and inhibin βB genes are widely and relatively highly expressed in the reproductive system. Activin A and B are essential for inducing mesoderm formation during development and stimulating follicle stimulating hormone production in the reproductive system[86-88]. Inhibin βC and inhibin βE are expressed primarily in the liver and are dispensable for development and adult homeostasis[89]. Activin A is a homodimer of inhibin βA–inhibin βA, and activin B is a homodimer of inhibin βB–inhibin βB (Figure 1.6)[84]. Activins A, B, and AB signal through activin receptors II and I[90], whereas activins C and E might not[91]. Thus far, two type II receptors (ActRIIA and ActRIIB) and three type I receptors (activin receptor like kinase, ALK4, ALK5, and ALK7) have been identified for activin A and B. Both activin receptors II and I are single transmembrane serine threonine kinase receptors that are shared with other TGFβ superfamily ligands[92].

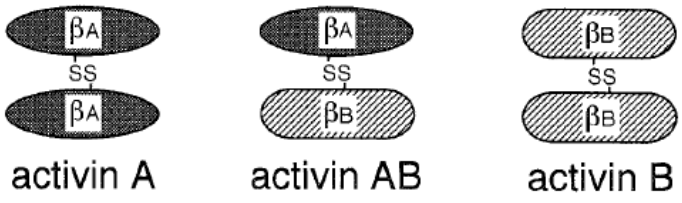


Figure 1.6 Structure of activin as dimers of inhibin βA and inhibin βB subunits.
Asashima M, Ariizumi T. Zoological Science 1995

Activin A and B act through the activin type II receptor and type I receptor pathways. They bind to one of ActRIIA and ActRIIB expressed on target cell surfaces, and the type II receptor then interacts with an activin type I receptor. Activin A binds to ALK4 and ALK5, whereas activin B might act via ALK4 and ALK7 (Figure 1.7)[93 94]. Binding of the activin type II receptor to activin type I receptor results in the phosphorylation of intracellular domains of activin type I receptor, which in turn phosphorylates the intracellular proteins Smad2 and Smad3. These phosphorylated proteins bind with Smad4, a common mediator, to form a protein complex that translocates to the nucleus to act as a transcription factor to regulate target gene expression and affect cellular proliferation, differentiation, or apoptosis[95]. Furthermore, other Smad-independent intracellular signaling events via activins/ActRII/ActRI include p38 MAPK, JNK, and ERK1/2[96 97]. Intracellularly, Smad2 interacts with other components in wingless/integratiob-1 (Wnt) signaling and the β-catenin pathway[98]. The ability of activins to signal through canonical Smad signaling, Smad-independent transduction, and crosstalk of Smad with other signaling pathways lead to a complex signaling behavior. None of the activins has been extensively investigated in liver homeostasis and disease. Relatively, activin A is the most studied activin in this regard. Activin A is expressed and secreted by hepatocytes and other non-parenchymal cells such as HSCs, cholangiocytes, and endothelial cells[99-101]. Several lines of evidence show that activin A is associated with liver regeneration, inflammation, fibrosis, and hepatocellular carcinoma[22 90 102-104]. Notably, activin A inhibits hepatocyte DNA synthesis and proliferation and induces hepatocyte apoptosis, indicating that it is a negative regulator of liver homeostasis[100 101 105]. Furthermore, activin A stimulates the activation of *in vitro* cultured HSCs and induces TGFβ1 production in fibroblasts from different organs (lung, kidney, and pancreas). TGFβ1 in turn stimulates activin A secretion from fibroblasts. These findings imply that activin A is involved in the hepatic fibrogenic

response[99 102 106]. Activin A can stimulate Kupffer cells to release TNFα and TGFβ1, which then can activate HSCs[107]. Activin A is induced in acute liver injury and blocking activin A ameliorates CCl_4-induced liver injury[108]. Hepatocytes express abundant inhibin βA and relatively low amount of inhibin βB in rodent livers[99]. CCl_4 upregulated hepatic inhibin βB expression in acute liver injury[109]. As two structurally related proteins, activin A and activin B share 63% identity and 87% similarity, and both bind to the same activin receptors II and I. Moreover, multiple common AP-1 sites have been identified in the promoters of inhibin βA and inhibin βB. This suggests that activin B might mediate liver pathogenesis in a similar manner as activin A[84 95 110 111]. Recently, activin B was shown to activate the ALK2/ALK3/Smad1/5/8 signaling pathway and thus upregulate hepcidin expression in the hepatocytes of mice subjected to several inflammatory insults, suggesting a role of activin B in mediating the hepatic inflammatory response[112]. Because of its ability to bind multiple type I receptors (ALK2, ALK3, ALK4, and ALK7) to activate both Smad2/3 and Smad 1/5/8 signaling pathways, activin B might be considered to possess a broad spectrum of biological functions, unlike activin A. Whether activin B plays a role in liver fibrogenesis is not yet known. This is the major question addressed in this study.

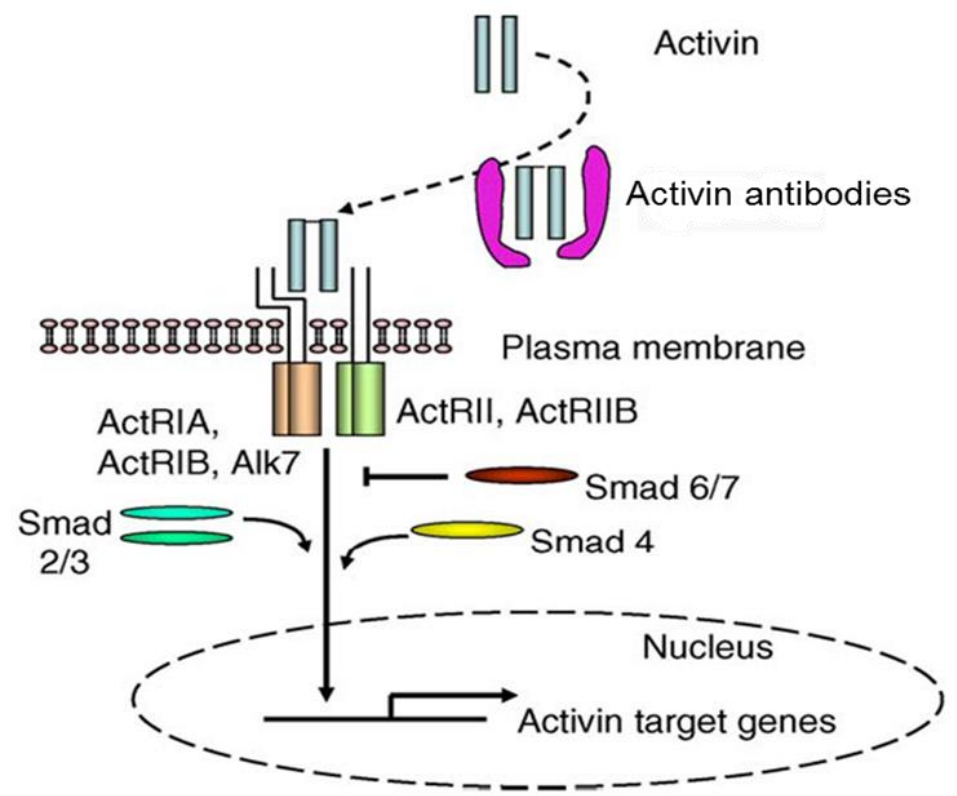


Figure 1.7 Modified schematic representation of activin signaling pathway.
Werner S, Alzheimer C. Cytokine & Growth Factor Reviews. 2006

1.5 Objectives of the study

Based on the literature and our preliminary studies, we measured the mRNA and protein expression levels of activin B and A in the liver and blood of patients with human liver fibrosis and mouse models of various liver fibrosis. We also delineated the detailed molecular mechanisms by which activin B directly stimulates different liver cells, including hepatocytes, macrophages, and HSCs, along with the well-known pro-fibrotic regulator TGFβ1 in liver cells. Finally, we investigated the effect of the inhibition of activin B and A individually or in combination by using specific monoclonal antibodies (mAbs) on liver function and fibrosis in CCl4- and BDL-induced liver fibrosis models. We hypothesize that activin B participates in modulating the initiation and progression of liver fibrogenesis. To test this hypothesis, we defined the following objectives:

1. Determine whether activin B is associated with liver fibrosis in human patients.
2. Determine whether activin B is associated with liver fibrosis in mouse liver injury and fibrosis models.
3. Determine whether activin B directly acts on hepatocytes, macrophages, and HSCs.
4. Determine whether activin B modulates the initiation of CCl_4-induced liver fibrosis.
5. Determine whether activin B modulates the progression of CCl_4-induced liver fibrosis.
6. Determine whether activin B modulates the initiation of bile duct ligation (BDL)-induced liver fibrosis.

The outcomes of this study might enable to potentially consider activin B as a novel and critical regulator of liver fibrogenesis, providing new mechanistic insight into this pathogenesis. Moreover, future studies can be directed to evaluate whether activin B is an effective target for the prevention and treatment of liver fibrosis.

CHAPTER 2. MATERIALS AND METHODS

2.1 Human liver and serum samples

Human liver and serum samples were provided by the Division of Gastroenterology and Hepatology of Indiana University School of Medicine; the study protocol was reviewed and approved by the Institutional Review Board. Normal liver samples from healthy volunteers (n = 5) or from patients with ESLD with advanced fibrosis or established cirrhosis (n = 8) were obtained. Liver samples were collected from the patients with ESLD during their liver transplantation procedure. Demographic data, cirrhosis etiology, and other relevant information such as medication, alcohol use, and smoking history were obtained at the time of enrollment. Liver biopsy (n = 21) and blood samples (n = 44) were collected from patients with non-alcoholic steatohepatitis. NASH was staged based on the severity of scarring or fibrosis: F0, no scarring; F1, minimal scarring; F2, significant fibrosis; F3, severe fibrosis; and F4, cirrhosis or advanced scarring[113]. Blood samples were harvested from healthy controls (n = 16), heavy alcohol drinkers without liver disease (n = 36), and heavy alcohol drinkers with liver disease (n = 15). Liver tissue samples were snap frozen in liquid nitrogen. Serum and frozen liver samples were stored at -80 ºC until use.

2.2 CCl4 liver injury model

All mouse experiments were approved by the Institutional Animal Care and Use Committee of Eli Lilly and Company and Indiana University–Purdue University, Indianapolis. Animals were housed in a room with controlled temperature (22 ± 2 °C) and a 12-h light-dark cycle, with ad libitum access to food (Diet 2014; Envigo, Indianapolis, IN) and water. C57BL/6 female mice (10–12 weeks of age; Envigo) received intraperitoneal injection of CCl_4 (1:10 dilution in corn oil, 1 ml/kg; Sigma Aldrich, St. Louis, MO) once to induce acute liver injury or twice per week for 4 or 10

weeks to induce liver fibrosis[114 115]. Activin A mAb (10 mg/kg; MAB3381; R&D Systems, Minneapolis, MN), activin B mAb (50 mg/kg; AB-306-AI005; Ansh Labs, Webster, TX), or control mouse IgG were administered twice a week. CCl_4 is a widely used toxin that is very effective in inducing acute and chronic liver injury. It induces hepatotoxicity, hepatocyte necrosis, and eventually centrilobular liver fibrosis with repeated administration. CCl_4-induced liver fibrosis model in rodents is widely applied in pre-clinical studies owing to its high reproducibility and similarity to the mechanism of certain human liver diseases. The pathologic feature of CCl_4 liver fibrosis animal model is similar to those of toxin-induced human liver fibrosis. The mechanism of CCl_4-induced liver injury involves many complex reactions[116]. Initially, CCl_4 is transformed to trichloromethyl radical (CCl^*_3) in the liver by the cytochrome P450 Family 2 Subfamily E Member 1 (CYP2E1), CYP2B1, CYP2B2, and CYP3A of the cytochrome P450 superfamily monooxygenases. Subsequently, the highly reactive CCl^*_3 induces nucleic acid mutation, protein hypomethylation, and lipid peroxidation, leading to protein synthesis reduction and hepatic steatosis. CCl^*_3 is then oxidized to form another highly reactive trichloromethylperoxy radical (CCl_3OO^*), which can induce lipid peroxidation and polyunsaturated fatty acid destruction. These free radical reactions and pro-fibrotic cytokine and growth factor production result in centrilobular hepatocyte necrosis, Kupffer cell activation, inflammation, fibrosis, and cirrhosis[117].

2.3 Bile duct ligation liver injury model

The surgical procedure was performed under sterile conditions, as previously described[118]. Under isoflurane anesthesia, male C57bl/6 mice (n = 6 to 8) were placed on a heat pad; following a median abdominal incision or laparotomy, the common bile duct was exposed, isolated, and doubly ligated with non-resorbable sutures (polyester 6–0; Catgut, Markneukirchen, Germany). Sham-operated mice were subjected to laparotomy with exposure of the bile duct, but without its

ligation. The abdominal muscle and skin layers were stitched, and the mice were treated with ketoprofen as an analgesic. A common BDL model represents cholestatic fibrosis due to the obstruction of the common bile duct to induce peri-portal biliary fibrosis. The mechanism of BDL-induced liver fibrosis is the reaction of biliary epithelial cells or cholangiocytes to increased biliary pressure; the bile acids and other components secrete inflammatory cytokines, leading to cholestasis[117]. BDL-induced liver fibrosis is characterized by proliferation of cholangiocytes, upregulation of cholangiocyte-specific marker cytokeratin 19 (CK19), and increased expression of fibrogenic markers such as α-SMA, collagen I, and TGFβ1.

2.4 Alcohol liver injury model

Ten-week-old C57/B6 male mice were fed ethanol-containing Lieber-DeCarli liquid diet to study alcoholic liver disease. The mice were randomly divided into pair-fed and ethanol-fed groups. The mice were subjected to chronic feeding for 10 days with ethanol-containing (5%) Lieber-DeCarli liquid diet for 10 days plus binge with ethanol (5 g/kg), as described previously[119]. The animals were killed after 3 h. Chronic alcohol consumption induced liver diseases starting with liver steatosis and progression to fibrosis and cirrhosis. In the liver, ethanol is mainly metabolized through two oxidative pathways[120]. First, ethanol is oxidized by alcohol dehydrogenases, cytosolic aldehyde dehydrogenase 1, and mitochondrial aldehyde dehydrogenase 2 to generate reduced nicotinamide adenine dinucleotide[121]. Second, CYP2E1 and catalase oxidize ethanol to produce ROS, which induce hepatocyte damage. These oxidative stress metabolites induced by ethanol damage mitochondria and cause hepatocyte necrosis or apoptosis. In addition to oxidative stress, another mechanism of alcohol-induced liver fibrosis is peri-central hypoxia resulting from increased oxygen consumption. Sustained hypoxia has multiple effects on hepatocyte damage, including increased ROS production, impaired mitochondrial lipid metabolism, and hepatocyte

cell death[122]. Lipid synthesis dysregulation is another mechanism of alcohol-induced liver disease. Ethanol exposure leads to liver steatosis via the inhibition of sirtuin1 activity and other associated molecules, leading to abnormal lipogenesis[123]. Damaged hepatocytes and activated Kupffer cells release cytokines and chemokines to mediate inflammatory response and activate HSCs to differentiate into myofibroblasts[124]. These mechanisms induce hepatocyte apoptosis or necrosis, Kupffer cell-mediated inflammation, and HSC activation and differentiation in alcohol-induced liver disease.

2.5 Cell culture

Primary mouse hepatocytes (PMHs) were isolated and grown from adult male C57Bl/6 mice, as described previously[125]. Briefly, under anesthesia, the peritoneal cavity was opened, and the liver was perfused *in situ* via the portal vein for 4 min at 37 °C with calcium-magnesium (CM)-free HEPES buffer and for 7 min with CM-free HEPES buffer containing Type IV collagenase (35 mg/100 mL) and $CaCl_2$ (10 mM). Cells were used only if the cell viability was above 90% as assessed by trypan blue exclusion. After three centrifugations (44 *g* for 2 min) in Leibovitz's L-15 washing media supplemented with 0.2% bovine albumin, cells were plated onto 24-well or 96-well plates (26,000 cells/cm^2). Cells were cultured in high-glucose (25 mM) DMEM supplemented with 10% FBS. All culture media contained penicillin (100 units/ml) and streptomycin (100 μg/ml). After cell attachment for 2 h, the medium was replaced with fresh medium supplemented with 10% fetal bovine serum (FBS). PMH cultures were maintained under 5% CO_2 atmosphere at 37 °C. RAW264.7 cells, a mouse macrophage cell line, were purchased from American Type Culture Collection (Manassas, VA) and cultured following manufacturer's manual. LX-2 cells, a human hepatic stellate cell line, were cultured in DMEM supplemented with 2% FBS (Gibco, Invitrogen, Carlsbad, CA); they were a gift from Dr. Scott L. Friedman from the Mount Sinai School of

Medicine (New York, NY). These cells were treated with activin A, activin B, activin C, CXCL1, and TGFβ1 (Table 2.1).

Table 2.1 Proteins used in the in vitro studies

Protein	Catalogue number	Vendor
Activin A	338-AC	R & D systems
Activin B	8260-AB	R & D systems
Activin C	489-AC	R & D systems
TGF β1	7666-MB	R & D systems
CXCL1	453-KC	R & D systems

2.6 Smad2/3-binding element reporter assay

HEK293 cells stably expressing the Smad2/3-binding element (SBE)-12-luciferase system (Qiagen) were seeded at 50,000 to 100,000 cells/well/100 µL DMEM/F12 (Invitrogen) containing 10% FBS into a poly-D-lysine-coated 96-well plate. Following at least 16 h of incubation at 37 °C, the media was aspirated and replaced with 50 µL of 1% FBS-DMEM/F12. Anti-activin A mAb or anti-activin B mAb were serially diluted (1:2) with 1× PBS, pH 7.4 to produce the following titration range (3000 ng/mL to 23.4 ng/mL). Each concentration was then mixed with an equal volume of 15 ng/mL of activin A or activin B (R&D Systems) and incubated at room temperature for 30 min, after which 100 µL of the mixture was added to individual wells. The Smad reporter (I.E. 100% signal) was induced by either activin A or activin B alone, and negative controls (I.E. 0% background signal) were induced by vehicle alone. Plates were incubated at 37 °C for 20 h, followed by aspiration, and washed once with 1× PBS. Cells in individual wells were subjected to lysis, and luminescence was measured using a GeniosPRO instrument with substrate injection (Luciferase Reporter Gene Assay Kit, Roche). Values shown in the figures are representative of

experiments performed in triplicate. Relative luciferase units were measured, and IC_{50} curves were fitted using GraphPad Prism software (GraphPad Software, Inc.).

2.7 The in-situ hybridization assay

The cellular source of TGFβ ligands in injured liver tissue was determined by subjecting liver sections to *in situ* hybridization (ISH) for inhibin βA, inhibin βB, and TGFβ1. The *in-situ* detection of these transcripts was performed on paraffin-embedded tissue sections by using the RNAScope assay (Advanced Cell Diagnostics). Sections were pretreated using an extended protease treatment and hybridized under conditions as described (RNAScope Sample Preparation and Pretreatment Guide) by using automated RNAScope probes for activin A, activin B, and TGFβ1, as well as standard negative dihydrodipicolinate reductase (*DapB*; a bacterial gene) and positive peptidylprolyl isomerase B (*PPIB*) control probes (Table 2.2). The probes were detected using RNAScope LS 2.5 Duplex brown Assay for the Leica Bond RX auto-stainer (Cat. no. 322440) and Brown DAB (Cat. no.DS9800). Slides were counter-stained with hematoxylin.

Table 2.2 RNAscope probes used in the in-situ hybridization

RNAscope probe	**Catalogue number**	**Vendor**
Inhibin βA	455871	Advanced Cell Diagnostics
Inhibin βB	475271	Advanced Cell Diagnostics
TGF β1	407751	Advanced Cell Diagnostics
PPIB	321651	Advanced Cell Diagnostics
DapB	320759	Advanced Cell Diagnostics

2.8 Microarray and quantitative RT-PCR analysis

Human stellate cells (LX-2 cells) were cultured overnight in six-well dishes at 5×10^5 cells per well. The LX-2 cells were then treated with activin A, activin B, or TGFβ1 (R&D System, Minneapolis, MN) for 6 h. The RNA was extracted from treated LX-2 cells by using TRIzol reagent (Life Technologies, Waltham, MA). Following manufacturer's instructions, 1 mL of TRIzol was added to each cell pellet. Next, 500 µL of chloroform was added and mixed well, and then the samples were centrifuged at 12,000 *g* for 15 min at 4 °C. The aqueous phase was carefully removed and transferred to a new tube. RNA was precipitated with 100% isopropanol and centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was removed, and the RNA pellet was washed with 75% ethanol and centrifuged at 7500 *g* for 5 min at 4 °C. The pellet was air dried and re-suspended in RNase-free water. The absorbance at 260 and 280 nm was measured to determine the RNA yield.

Next, 2 µg of total RNAs was reverse transcribed using the High-capacity cDNA Archive Kit (Applied Biosystems, Beverly, MA) and then applied to HG-U133 plus 2 chips for microarray analysis. For real-time RT-PCR analysis, all cDNAs were assayed for house-keeping genes such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein lateral stalk subunit P0 (*RPLPO*) and genes of interest by using TaqMan Gene Expression Analysis and the Assay-On-Demand primer/probe sets (Applied Biosystems, Beverly, MA; Table 2.3). The mRNA levels of genes of interest were quantified by determining the cycle number at which amplification detection threshold was achieved. Real-time PCR analyses were performed in 10 µL reactions according to manufacturer's guidelines. Triplicate samples were subjected to quantitative PCR by using QuanStudio 7 Flex (Applied Biosystems, Beverly, MA) real-time PCR system with the maximum cycle number of 40. After the expression was normalized to the housekeeping gene, the

expression of the genes of interest was examined in the treated group compared to that in the vehicle control or sham group for subsequent analysis.

Table 2.3 Primers/probe sets used for RT-PCR

Gene	**Catalogue number**	**Amplicon length (base pair)**
GAPDH	Mm99999915_g1	109
RPLPO	Mm00725448_s1	124
Inhibin βA	Mm00434339_m1	65
Inhibin βB	Mm03023992_m1	120
Inhibin βC	Mm00439684_m1	82
TGF β1	Mm01178820_m1	59
CTGF	Mm01192933_g1	67
Col1α1	Mm00801666_g1	89
ACTA1	Mm00808218_g1	134
Smad3	Mm01170760_m1	59
IKBKB	Mm01222247_m1	63
TNFα	Mm00443258_m1	81
CCL2	Mm00441242_m1	74
TWEAK	Mm02583406_s1	87
Fn14	Mm01302476_g1	88
CXCL1	Mm04207460_m1	111
iNOS	Mm00440502_m1	66
ACVR1	Mm01331069_m1	65

Table 2.3 continued

CKDNiB	Mm00438168_m1	81
CASP3	Mm01195085_m1	70
CASP6	Mm01321726_g1	96
GNDF	Mm00599849_m1	101
SOX4	Mm00486320_s1	94
CXCR2	Mm99999117_s1	64
IL-6	Mm00446190_m1	78
IL-1β	Mm00434228_m1	90

2.9 Blood chemistry analysis

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, and total bilirubin levels were measured using a Hitachi Modular Analyzer (Roche Diagnostics, Indianapolis, IN).

2.10 ELISA of activin A and activin B

Liver lysates were generated using 1 mL of lysis buffer per 100 mg liver tissue (Cell signaling Technologies, Dancers, MA). The protein concentration of the liver lysates was determined using bicinchoninic acid Protein Assay Kit (Thermo Scientific, Waltham, MA)[43], which is a standard method for colorimetric detection of total protein. Serially diluted bovine serum albumin (BSA) was used as a protein standard. The protein concentration was quantified at 570 nm. The same amount of total protein of liver lysate samples was treated with 10 mmol/L dithiothreitol in PBS for 1 hour at room temperature before plating. Anti-activin A antibody was coated and

reconstituted in PBS at a final concentration of 100 mg/mL (AF338; R&D Systems). Anti-activin A antibody (100 mL per well) was diluted to 1 mg/mL in coating buffer (SH30256.01; Hyclone, Waltham, MA), incubated for 1 hour at 37 °C, and blocked for 1 hour; next, the samples were incubated at room temperature for 1 hour. Poly-streptavidin horseradish peroxidase and 3-, 30-, 5-, and 50-tetramethylbenzidine block steps were performed, and plates were read at 450 to 630 nmol/L. The enzyme-linked immunosorbent assay (ELISA) was validated using purified activin A protein. Activin B proteins in the liver tissue, serum, or cell culture supernatants were quantified using ELISA (Activin B ELISA kit; Ansh Labs, Webster, TX) according to the manufacturer protocol.

2.11 Histology and immunohistochemistry

The livers were preserved in 10% neutral-buffered formalin for 24 h, embed in paraffin, cross-sectioned at 3 µm, deparaffinized at room temperature, and stained with hematoxylin and eosin (H&E). Adjacent 3 µm sections were immunolabeled using heat-induced epitope retrieval (HIER)[91] and a Dako autostainer. Briefly, sections were deparaffinized at room temperature and rehydrated in 1× TBST immediately before HIER treatment by using Biocare Decloaking Chamber Pro with DIVA, pH 6 solution (Biocare). The set point 1 was programmed for 125 °C for 30 s (20 PSI) and then cooled down to 89.5 °C for 10 s. The slides were removed and slowly rinsed with ddH_20 for 5 min, and then immediately rinsed several times in 1× TBST. Endogenous peroxidase was blocked for 10 min, and endogenous biotins were blocked for 15 min. Additional protein block was performed for 30 min before the sections were subjected to commercial anti-mouse F4/80, MPO, CK19, and Ki67 clone BM8 (Table 2.4) for 1 h. Biotinylated polyclonal rabbit anti-rat secondary antibodies (Dako) were applied for 30 min. Next, HRP-labeled streptavidin-biotin (Dako) was applied for 10 min, and 3-3-diaminobenzidine tetrahydrochloride (Dako) was

applied for 5 min; the slides were counterstained with hematoxylin. Negative controls were produced by replacing the primary antibodies with purified rat IgG2aK. Immunolabeled slides were examined along with H&E-stained slides to relate cell type identification to cytological features. Muscle images were acquired using a digital camera (Spot Digital Camera; Diagnostics Instruments, Inc.) and associated software (Spot Advanced) attached to an upright light microscope (Leica DM5000B) at 20× magnification.

Table 2.4 Antibodies used in immunohistochemistry

Antibody	**Catalogue number**	**Dilution**	**Vendor**
MPO	AF3667	1:500	R & D systems
F4/80	14-4801-82	1:500	eBioscience
CK19	ab133496	1:500	Abcam
Ki67	RM-9106	1:200	Thermo Fishers Scientific

2.12 Statistical analysis

All data are expressed as mean ± standard error of the mean. Significance of differences among group means was determined using ANOVA or Dunnett's tests, followed by two-tailed unpaired Student's *t*-test. Statistical significance is considered at $P < 0.05$. GraphPad Prism Software was used for data analysis and figure preparation.

CHAPTER 3. ACTIVIN B MEDIATES HEPATIC FIBROSIS

3.1 Introduction

Liver fibrosis is the common consequence of liver injury secondary to ALD, NASH, viral hepatitis, and autoimmune liver disease[126]. The initiation and progression of liver fibrosis are driven by complicated cellular and molecular mechanisms[11 127-129]. Damaged hepatocytes and cytokines released from inflammatory cells such as Kupffer cells can directly or indirectly activate the conversion of HSCs to myofibroblasts, leading to the accumulation of collagen I and III and other ECM components as well as liver fibrosis[62 126 130].

Activins are dimers formed by four inhibin subunits—inhibin βA, inhibin βB, inhibin βC, and inhibin βE—in mammals[84]. Widely expressed inhibin βA and inhibin βB genes are essential for inducing mesoderm formation during development and follicle stimulating hormone production in the reproductive system[86-88]. Inhibin βC and inhibin βE are expressed predominantly in the liver and are dispensable during development and for maintenance of adult homeostasis[89]. Activin A, B, AB, C, and E represent homo- or hetero-dimers of inhibin βAβA, βBβB, βAβB, βCβC, and βEβE, respectively[84]. Activin A, B, and AB signal through activin receptors/Smad2/3 pathway, whereas activin C and E might not[91]. Activin A is expressed and secreted by hepatocytes and non-parenchymal cells such as HSCs, cholangiocytes, and endothelial cells in the liver[99-101]. Several lines of evidence show that activin A is associated with liver regeneration, inflammation, fibrosis, and hepatocellular carcinoma[22 90 102-104]. Notably, activin A inhibits hepatocyte DNA synthesis and proliferation and induces hepatocyte apoptosis, which indicates that it is a negative regulator for liver homeostasis[100 101 105]. Furthermore, activin A stimulates the activation of cultured HSCs, implying its involvement in the hepatic fibrogenic response[99 102 106]. It also stimulates primary

Kupffer cells to release TNFα and TGFβ1, which activate HSCs[107]. Activin A production is induced in acute liver injury, and neutralization of activin A has been shown to reduce CCl_4-induced liver injury in mice[108].

As a structurally close protein, activin B shares 63% identity and 87% similarity to activin A[84]. Both ligands bind to the same activin receptors II and I, and multiple common AP-1 sites in the individual promoters of both inhibin βA (subunit of activin A) and inhibin βB (subunit of activin B) have been identified, suggesting that activin B might share similarities to activin A with regard to the mediation of liver pathogenesis[84 95 110 111]. Hepatocytes constitutively express abundant inhibin βA, but relatively low level of inhibin βB[99]. However, hepatic inhibin βB expression is highly upregulated in CCl_4-induced acute liver injury[109]. Recently, activin B was shown to upregulate hepcidin expression in hepatocytes via Smad1/5/8 signaling in response to several inflammatory insults in mice. This finding suggests that activin B is involved in mediating hepatic inflammatory response[112]. However, whether activin A, especially activin B, can mediate liver fibrogenesis is not yet known. This study aimed to determine the role of activin B and its mechanism in liver fibrosis.

3.2 The levels of hepatic and circulating activin B significantly increase in patients with liver fibrosis

First, we determined whether activin B and A are clinically relevant to different etiologies of liver fibrosis. With regard to mRNA expression, inhibin βA represents activin A, and inhibin βB symbolizes activin B as activin A and activin B are the homodimers of inhibin βAs and inhibin βBs, respectively. We found that, in patients with advanced liver fibrosis or cirrhosis, hepatic activin B mRNA and protein were markedly increased relative to those in healthy controls (Figures

3.1A&B). Circulating activin B did not increase in excessive alcohol users without liver disease but showed more than five-fold elevation in patients with alcoholic cirrhosis (Figure 3.1C). In patients with NASH, the hepatic and serum levels of activin B significantly increased only in those with F4 fibrosis, but not in those of the F0 and F1 groups (Figures 3.1D&E). In addition, we found that the serum level of activin A markedly increased in individuals with F1 fibrosis. Thus, we showed that the expression of activin B is correlated with advanced fibrosis/cirrhosis, irrespective of the underlying disease etiologies.

A **B**

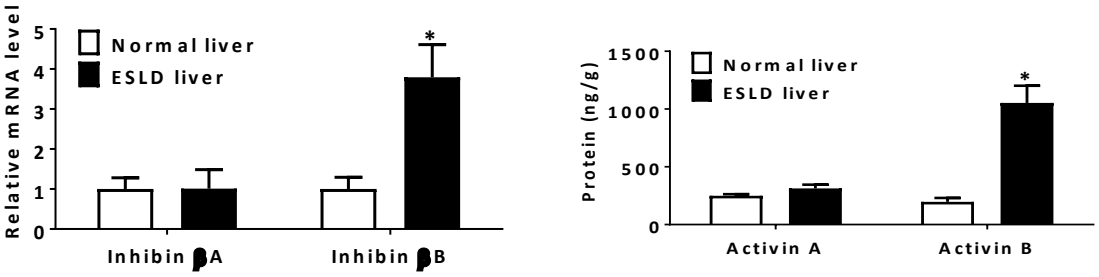
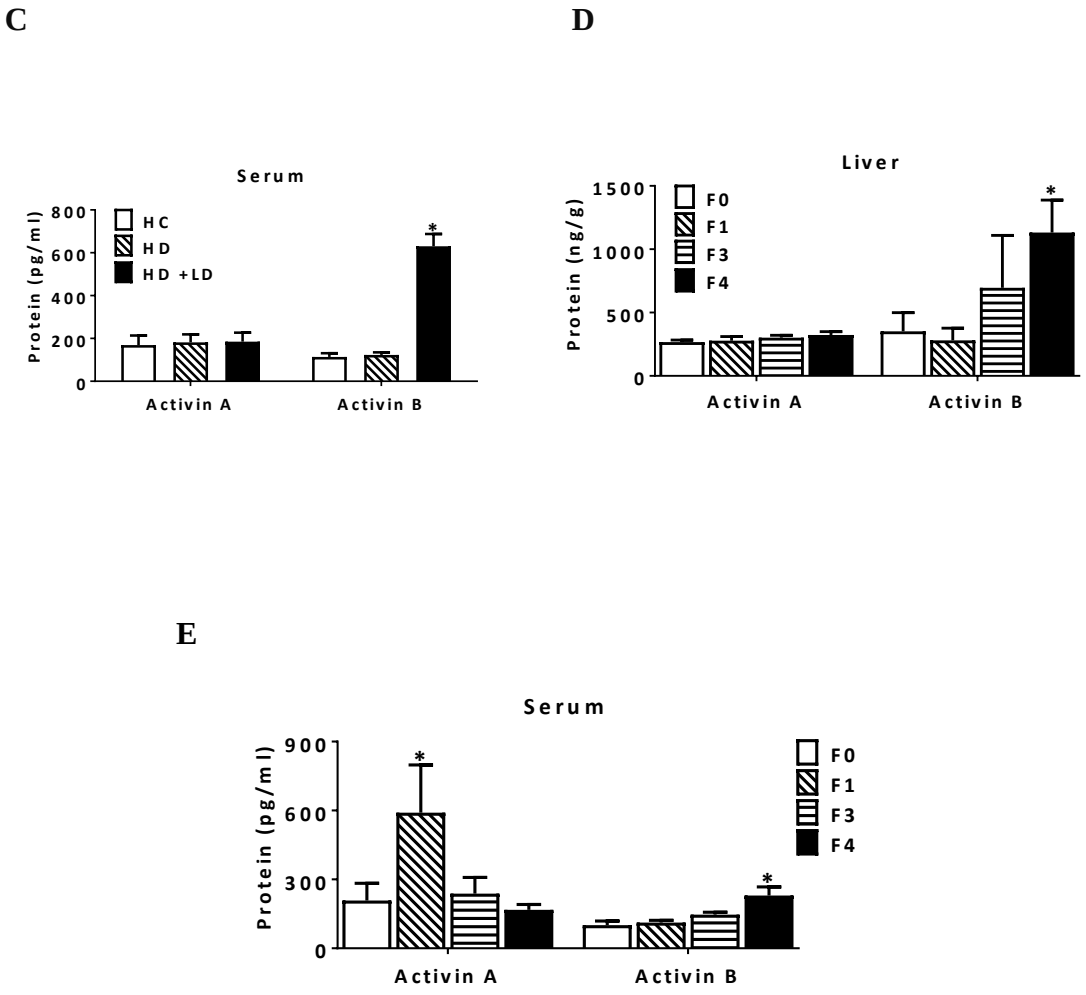


Figure 3.1 Liver and serum activin B levels increase in patients with liver fibrosis.

(A) The mRNA expression of hepatic inhibin βA and inhibin βB in patients with ESLD (n = 8) and healthy controls (n = 5) was analyzed using qRT-PCR. **(B)** Protein expression of hepatic activin A and activin B in patients with ESD (n = 8) and healthy controls (n = 5) was quantified using ELISA. **(C)** The concentrations of serum activin A and activin B proteins were determined using 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 activin B proteins were evaluated using ELISA in the livers **(D)** and serum **(E)** of patients with different stages of NASH (F0: n = 4, F1: n = 6, F3: n = 4, and F4: n = 6). For all the above assays, data are expressed as means ± S.E.M. *, $P < 0.05$ compared to healthy controls or the F0 group.

Figure 3.1 continued



3.3 Hepatic and circulating activin B levels are elevated in mouse models of CCl4-induced acute liver injury and liver fibrosis

To further investigate the expression pattern and cellular sources of activin B and A in liver injury, we performed acute and chronic liver injury studies in mice. In an acute model after the single administration of CCl_4, we found significant upregulation of hepatic inhibin βB mRNA expression up to 3 days after injection (Figure 3.2A), concomitant with the increase in hepatic activin B protein concentration (Figure 3.2B). In addition, we found an increase in serum activin B protein at 6 and 24 h after injection (Figure 3.2C). Unlike activin B, hepatic mRNA and protein

concentrations and serum levels of activin A increased only at 24 h after CCl_4 injection (Figures 3.2D–F). We used mouse liver fibrosis model with CCl_4 injection for 4 weeks and ALD model of chronic alcohol plus binge to determine the levels of activin B in hepatic fibrogenesis and chronic liver injury. In the CCl_4 model, only the mRNA expression and serum levels of activin B increased, but not of activin A (Figures 3.2G&H). Similar findings were found in mice fed chronic alcohol plus binge model (Figures 3.2J&K). The cellular sources of activin B were revealed using ISH. Activin B was mainly transcribed in the hepatocytes and biliary epithelial cells of livers in vehicle controls as well as in fibrogenic cells in the mice with fibrotic livers (Figure 3.2I). We concluded that, in CCl_4-induced liver injury, activin B is persistently associated with liver disease progression from the acute to chronic phase, whereas activin A is transiently relevant to the acute phase.

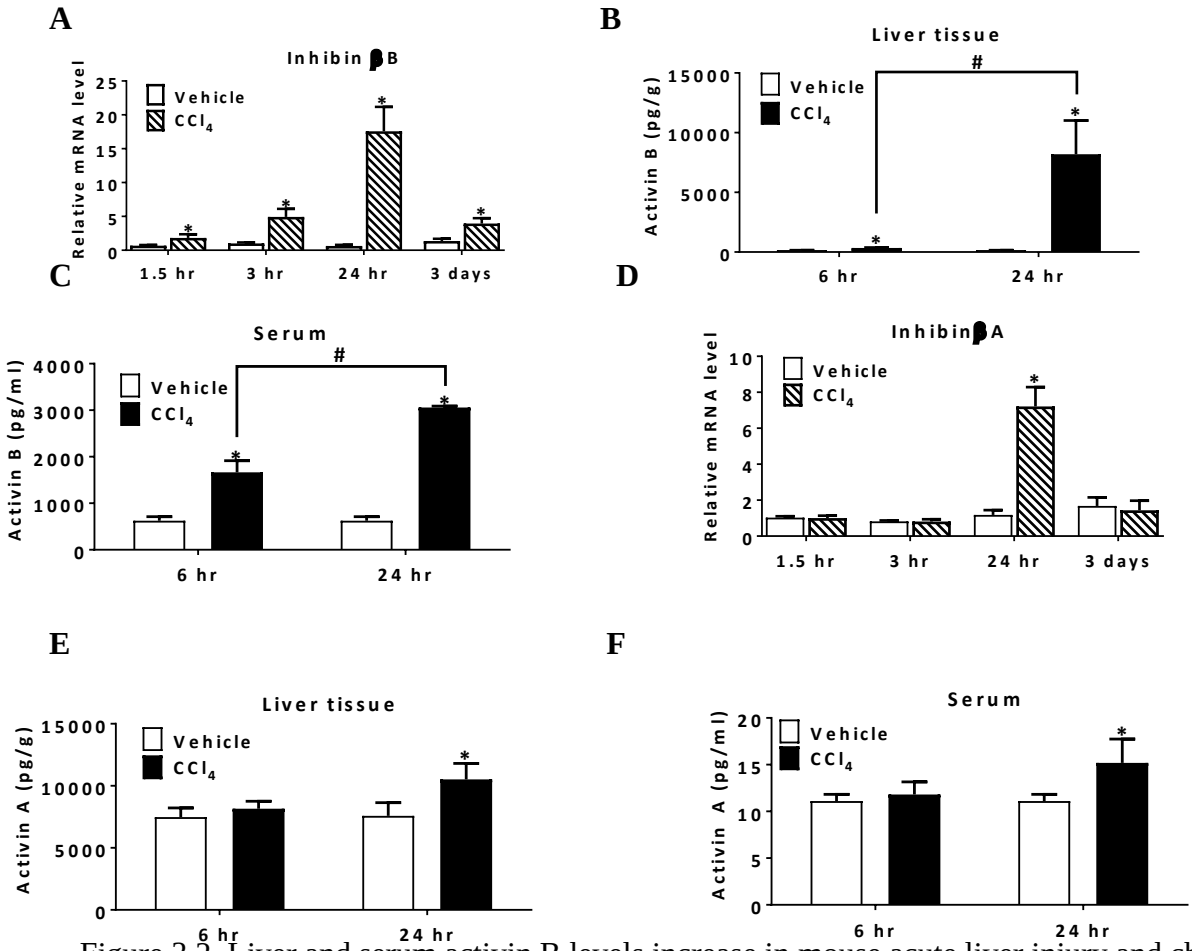
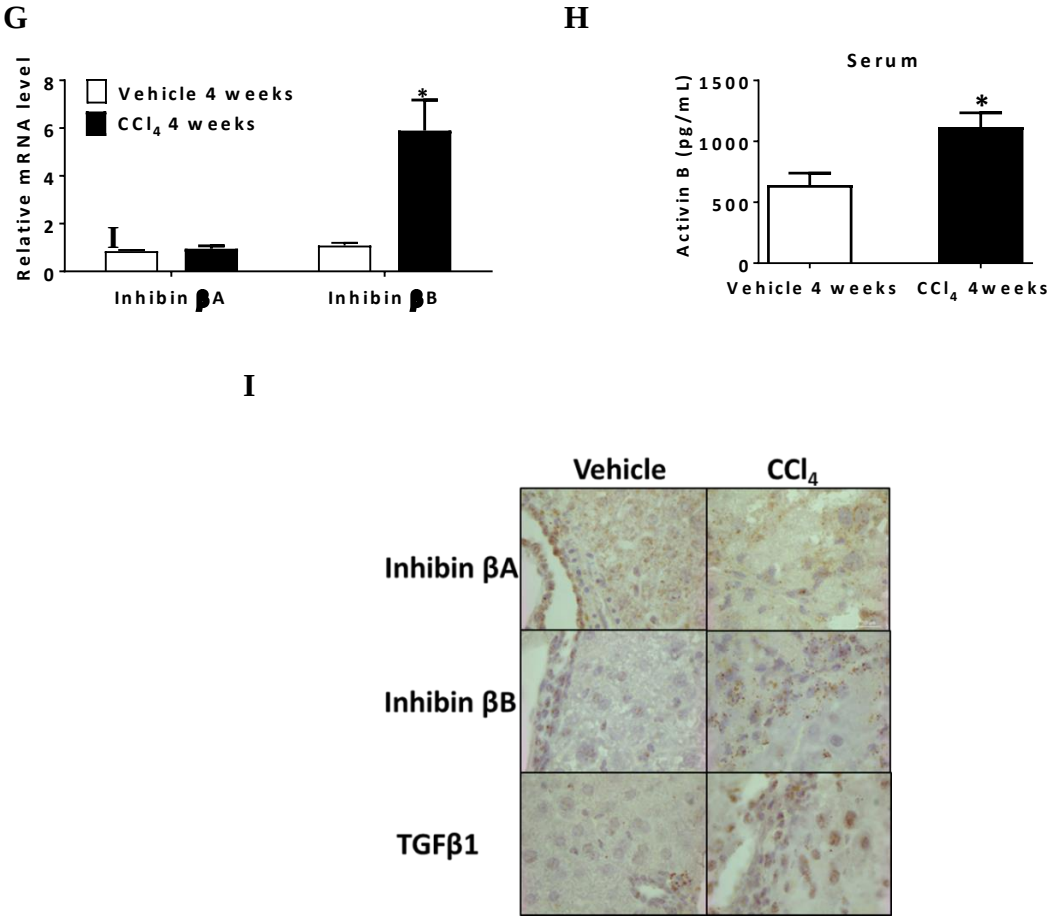


Figure 3.2 Liver and serum activin B levels increase in mouse acute liver injury and chronic liver fibrosis models induced by CCl_4 administration.

(A) The mRNA expression of hepatic inhibin βB was analyzed using qRT-PCR at the indicated time points after single CCl_4 or vehicle administration in mice (n = 6). **(B&C)** Activin B protein was quantified using ELISA in the livers **(B)** and serum **(C)** at 6 and 24 h after single CCl_4 or vehicle administration in mice (n = 8). **(D)** The mRNA expression of hepatic inhibin βA was analyzed using qRT-PCR at the indicated time points after single CCl_4 or vehicle administration in mice (n = 6). **(E&F)** Activin A protein was quantified using ELISA in the livers **(E)** and serum **(F)** at 6 and 24 h after single CCl_4 or vehicle treatment in mice (n = 8). **(G–I)** After CCl_4 or vehicle was administered twice per week for 4 weeks in mice, **(G)** mRNA expression of hepatic inhibin βA and inhibin βB was assessed using qRT-PCR (n = 10); **(H)** concentrations of serum activin B protein were quantified using ELISA (n = 10); **(I)** inhibin βA-, inhibin βB-, and TGFβ1-expressing cells were visualized using *in situ* hybridization on liver sections by using mouse inhibin A and inhibin B RNAscope probes and a 2.5 HD Assay-Brown kit. **(J&K)** Ten days after oral alcohol administration in mice, **(J)** hepatic inhibin βA and inhibin βB transcript levels were determined using qRT-PCR (n = 7), and **(K)** hepatic activin A and activin B protein contents were quantified using ELISA (n = 7). For all above quantitative assays, data are expressed as means ± S.E.M. *, $P < 0.05$ relative to vehicle controls.

Figure 3.2 continued



3.4 Hepatic and circulating activin B levels are elevated in mouse models of BDL-induced acute liver injury and liver fibrosis

To determine whether the increase in activin levels in liver fibrosis and acute live injury models is a generalized event or liver disease etiology-dependent, we measured the levels of the two activin ligands in another mouse liver injury model. Within one day (the acute phase) after BDL surgery, a surgical approach to induce cholestasis-mediated liver injury, we noted persistent increase in activin B protein and transient increase in activin A protein in the circulation (Figures 3.3A&B). Two weeks after BDL when the livers became fibrotic, activin B, but not activin A, mRNA expression was elevated in the livers and protein expression was elevated in the blood (Figures

3.3C&D). The ISH results showed that inhibin βA, inhibin βB, and TGFβ1 transcription was active mainly in the hepatocytes and biliary epithelial cells of sham controls and in the fibrogenic cells of mice with fibrotic livers (Figure 3.3E). We found that, in BDL-induced liver injury, activin B is persistently associated with liver disease progression from the acute to chronic phase, whereas activin A is transiently relevant to the acute phase.

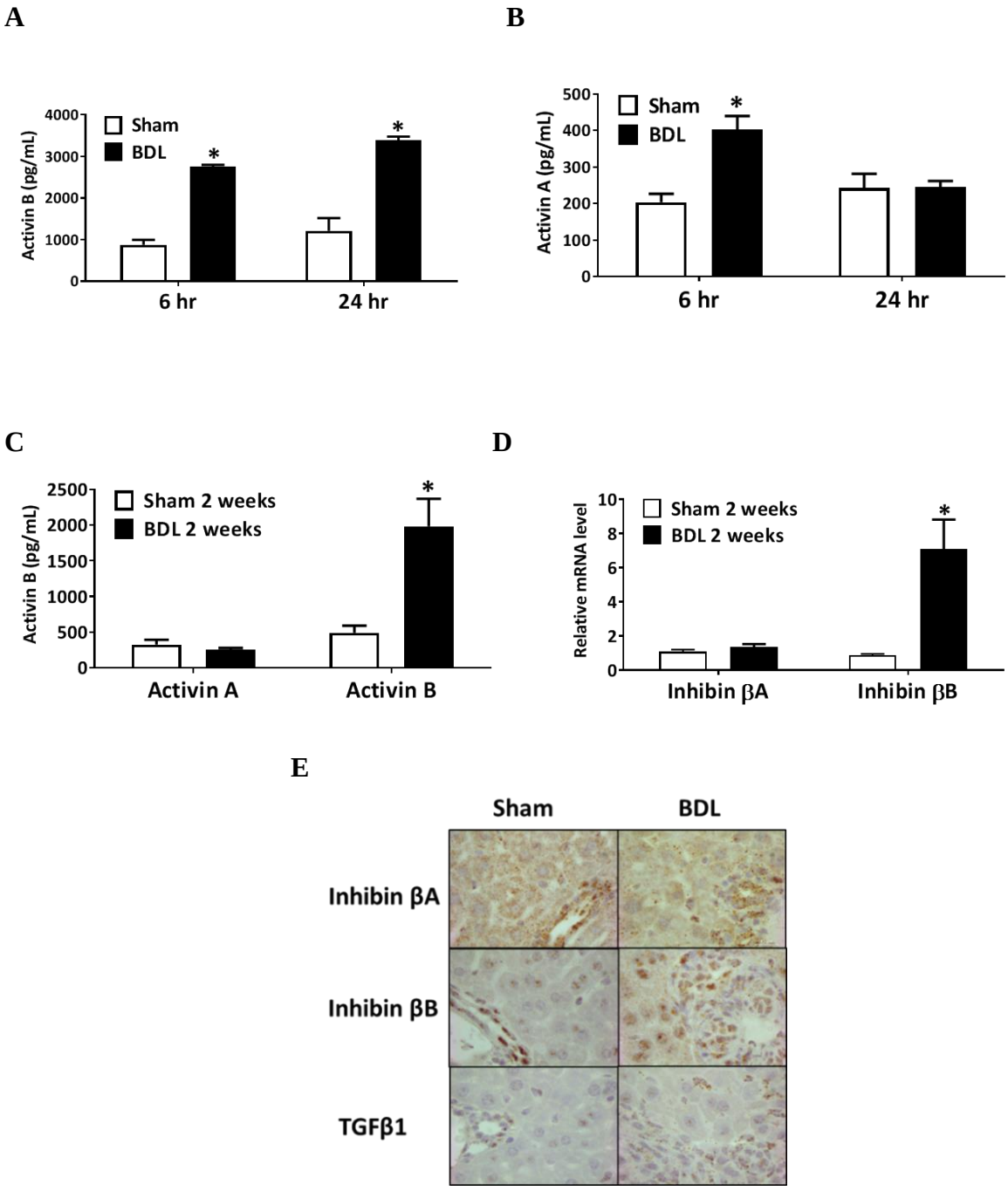


Figure 3.3 Liver and serum activin B levels increase in BDL-induced acute liver injury and chronic liver fibrosis mouse models.

(**A and B**) Protein quantification of activin A and activin B in the serum at 6 and 24 h after BDL surgery. (**C**) RT-PCR of activin A and activin B in the liver tissue at 2 weeks of BDL surgery. (**D**) Serum activin A and activin B levels in the serum at 2 weeks after BDL surgery. (**E)** Inhibin βA-, Inhibin βB-, and TGFβ1-expressing cells were visualized using *in situ* hybridization on liver sections by using mouse inhibin βA and inhibin βB RNAscope probes and a 2.5 HD Assay-Brown kit. Data are expressed as means ± S.E.M. Significance is indicated at $*P \leq 0.05$, treated group vs. vehicle group (Dunnett's one-way ANOVA).

3.5 Hepatic activin B levels are elevated in the ALD mouse model

Next, we determined activin B and activin A levels in the alcoholic liver fibrosis model. After mice were fed alcohol for 10 days, both mRNA and protein expression of activin B, but not of activin A, was upregulated in the liver (Figures 3.4A&B). Thus, we showed that, irrespective of liver injury types, activin B is persistently associated with liver disease progression from the acute to chronic phase, whereas activin A is transiently relevant to the acute phase. Moreover, the association of activin B with chronic liver injury is highly conserved between humans and mice.

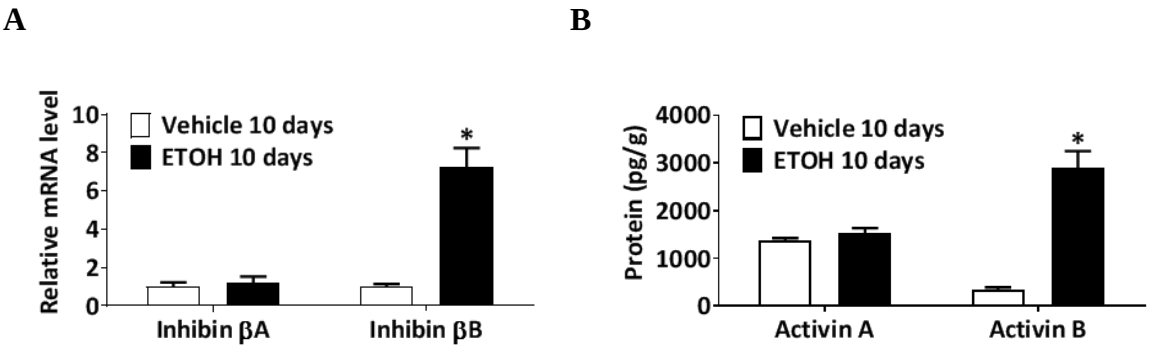


Figure 3.4 Liver activin B level increases in the alcohol-induced liver injury mouse model.

(A) Hepatic inhibin βA and inhibin βB transcript levels were determined using qRT-PCR (n = 7), and **(B)** hepatic activin A and activin B protein contents were quantified using ELISA (n = 7). For all the above quantitative assays, data are expressed as means ± S.E.M. *, $P < 0.05$ relative to vehicle controls.

3.6 Activin B and A are associated with hepatocyte injury and might induce hepatocyte differentiation

The ISH results showed that hepatocytes are the main cellular sources in the liver that express inhibin βA and inhibin βB mRNAs (Figures 3.2I&3.3E). To determine whether activin A and B proteins are secreted by hepatocytes and how these proteins respond to hepatocyte injury, we exposed PMHs to CCl_4 or LPS. We found that CCl_4 damaged these cells, induced necrosis, and elevated ALT and AST in culture supernatants (Figures 3.5A&B) as well as activin A and B protein levels (Figure 3.5C). Cell viability was marginally, significantly, and additively improved by neutralizing activin A, activin B, and their combination in PMHs, respectively (Figure 3.5D). In contrast, LPS only stimulated PMHs to increase activin A production without affecting activin B, ALT, and AST (Figure 3.5C). These data suggest that hepatocytes are one of the cellular sources responsible for the secretion of activin B, and activin A exhibits toxin-dependent responses. Moreover, activin B production in hepatocytes was accompanied by hepatocyte injury and cell death. Notably, the two activins modulate hepatocyte injury, as neutralization of these proteins improved cell viability following insults. We also found that PMHs responded to individual or

combined exogenous treatment of these two proteins by uniformly upregulating the transcription of TGFβ1, CTGF, and Col1α1, as well as by variously regulating the mRNA expression of ACTA1, Smad3, and IKBKB (Figure 3.5E). These genes are associated with myofibroblast activity, suggesting that activin A and activin B might be involved in the differentiation of hepatocytes into myofibrolast-like cells following injury. Thus, these results suggest that activin B and A have redundant, specific, and interactive actions in hepatocytes

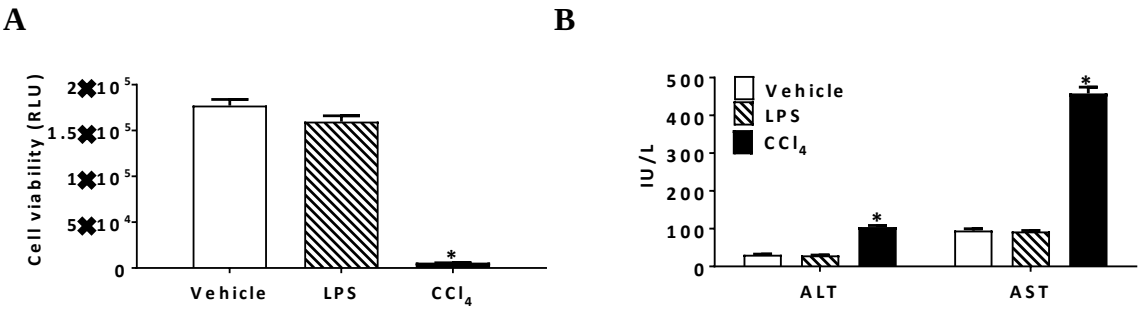


Figure 3.5 Activin A and B are produced in PMHs and induce differentiation of these cells. Primary hepatocytes were isolated from adult male mice and cultured overnight. Subsequently, the cells were treated with vehicle (corn oil), lipopolysaccharide (LPS, 10 μg/mL), or 0.5% CCl_4 for 24 h. **(A)** Cell viability, **(B)** supernatant ALT and AST, and **(C)** supernatant actin A and activin B proteins were analyzed. **(D)** Cell viability of primary hepatocytes after 24-h treatment with 0.5% CCl_4 and co-treatment with IgG, anti-activin A antibody, and anti-activin B antibody, or the combination of anti-activin A and B antibodies at 100 ng/mL each. **(E)** The mRNA levels of the indicated genes were evaluated using real-time RT-PCR in PMHs after treatment with activin A and activin B (100 ng/mL each), or their combination, for 24 h. For all the above assays, data are expressed as means ± S.E.M. *, $P < 0.05$ vs. vehicle controls.

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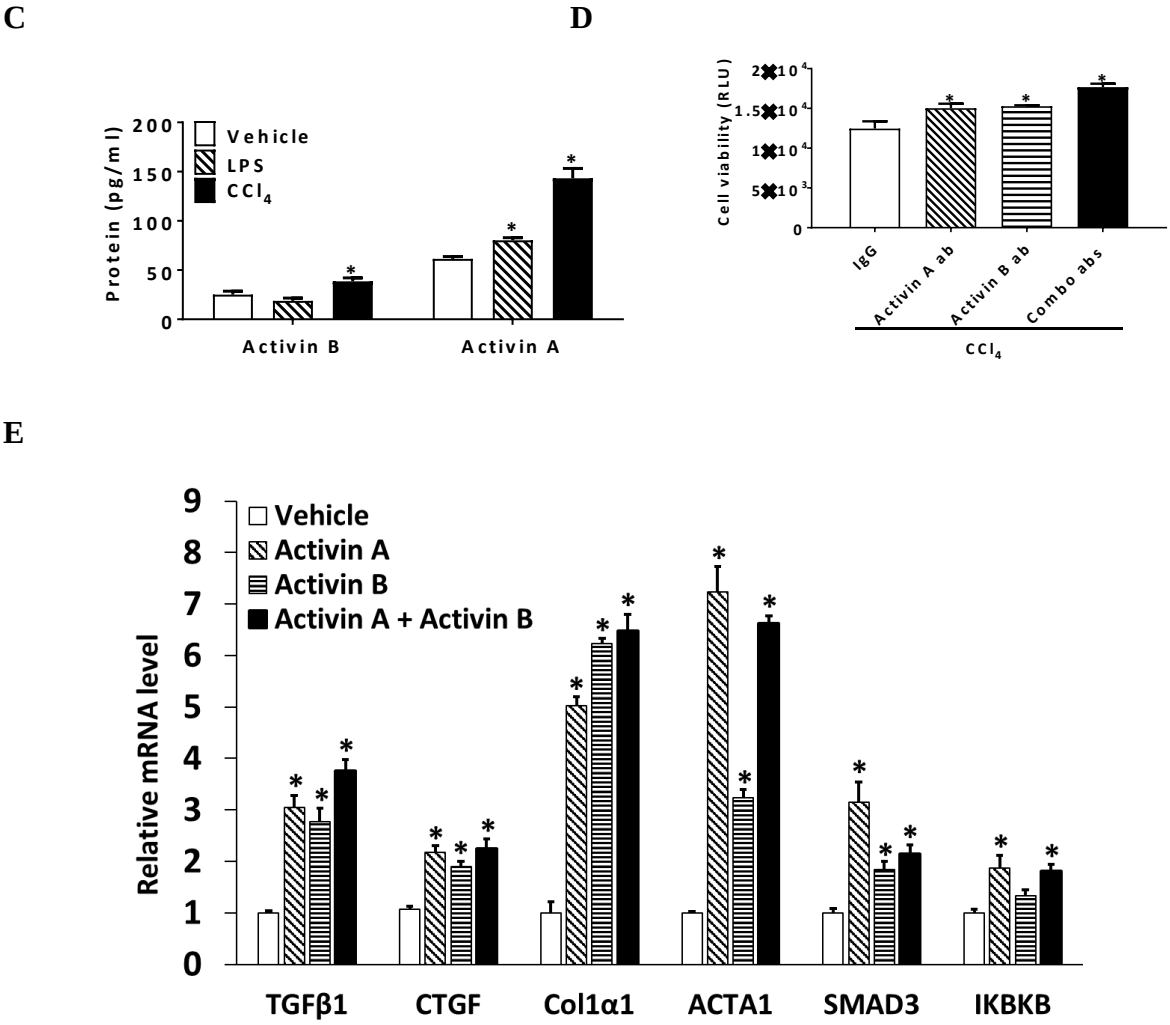
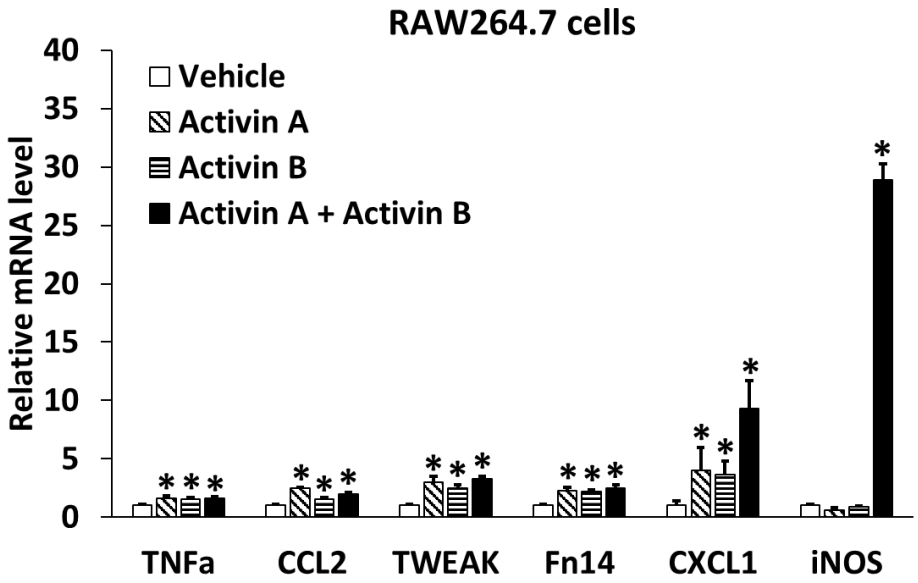


3.7 Activin B and A directly target macrophages and modulate inflammatory cytokines

Immune cells centrally mediate inflammation largely by regulating cytokine production. To understand how activin B and A regulate inflammatory responses in macrophages, we exposed RAW264.7 cells to activin B and/or activin A and evaluated the expression of inflammatory cytokines or chemokines. We found that treatment with individual or combination of both ligands exerted similar potency in upregulating TNFα, CCL2, TWEAK, and Fn14 expression (Figure

3.6A), indicating that activin B and A might have redundant actions on macrophages. Notably, treatment with individual ligands equally, whereas treatment with both ligands additively, elevated CXCL1 transcript level, coincident with inducible nitric oxide synthase (iNOS) activation only after exposure to both ligands (Figure 3.6A). This suggests that the additive increase in CXCL1 expression after exposure to both ligands, but not to either of the single ligands, was necessary to achieve iNOS activation. To test this, we treated RAW264.7 cells with CXCL1 protein and found that CXCL1 upregulated iNOS expression by 30-fold after 24 h of treatment (Figure 3.6B). Thus, activin B and A were found to directly target macrophages and additively stimulate sufficient production of autocrine CXCL1 to induce iNOS transcription. These data suggest the existence of an activin B/activin A/CXCL1/iNOS signaling pathway that modulates macrophage activity, further supporting the notion that activin B and A essentially collaborate with each other to activate the transcription of a subset of inflammatory cytokines and chemokines.

A



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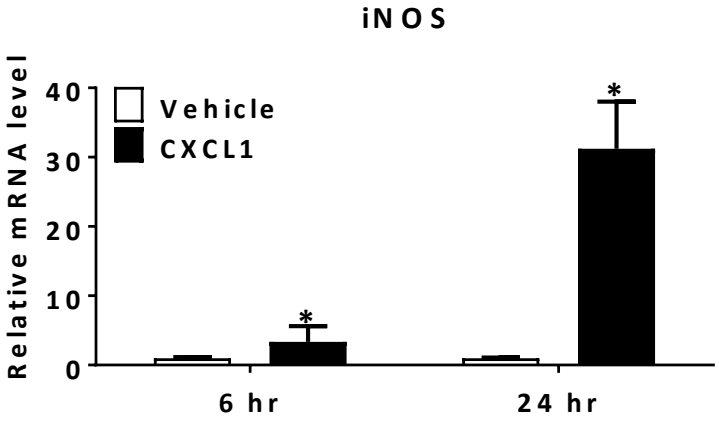

Figure 3.6 Activin A and B induce macrophages to express inflammatory cytokines or chemokines.

(A) Transcripts of the genes indicated were quantified using qRT-PCR in RAW264.7 cells after exposure to activin A (100 ng/mL), activin B (100 ng/mL), or both (100 ng/mL each) for 24 h. **(B)** The mRNA expression of iNOS was evaluated using qRT-PCR in RAW264.7 cells following vehicle or CXCL1 treatment for 6 or 24 h. For the above quantitative analyses, data are presented as means ± S.E.M. *, $P < 0.05$ vs. vehicle controls.

3.8 Activin B and A directly and coordinately promote HSC activation

Myofibroblasts centrally drive liver fibrogenesis and are primarily differentiated from activated HSCs. The human HSC cell line LX-2 has been widely used to study the function of HSCs. We assessed the behavioral response of LX-2 cells to activin A, activin B, their combination, and transforming growth factor (TGF)β1, 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 3.7A), a common behavior observed in HSCs during liver fibrogenesis. This observation suggests that activin B and A directly activate HSCs. Based on this finding, we intended to determine whether these activin ligands redundantly act on HSCs at the 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 using microarray analysis. We found that these three proteins regulate overlapping, but differential, gene networks (Figure 3.7B). The 877 overlapping genes were predominately associated with HSC activation and hepatic fibrosis, including upregulated TGFβ signaling negative feedback modulator transmembrane prostate androgen-induced protein (TMEPAI), early growth response protein 2 (EGR2), and calcium ion-binding protein matrix gla protein (MGP), and downregulated BMP4, dual specificity phosphatase 6 (DUSP6), extracellular matrix glycoprotein TNXB, IL-8, and IL-17 receptor C (Figures 3.7C–D). These data suggest that activin signaling redundantly dictates a spectrum of HSC properties via multiple ligands, including activin A and B. Conversely, each of these individual ligands has a large and unique set of genes associated with critical cellular functions. For instance, activin B exclusively decreased cell migration-associated scaffold protein Ezrin and calcium-dependent phospholipid-binding protein 3, implying its role in controlling HSC migration. These data suggest that activin B is a novel direct regulator of HSCs, and that activin ligands distinctly, but coordinately, regulate the transcriptome of HSCs.

To gain insight into how activin A and B interactively act on HSCs, we treated LX-2 cells with activin A or B alone or in combination and then determined the transcriptional response of a group of genes known to regulate HSC activity. We observed four scenarios: (1) ACVR1 and CKDNiB equivalently responded to individual ligands (Figure 3.7E); (2) CASP6 solely responded to activin A (Figure 3.7E); (3) CASP3, GNDF, and CXCL1 specifically responded to dual ligands (Figure 3.7F); and (4) CTGF equally responded to individual ligands, but interdependently to dual ligands (Figure 3.7F). These results indicate that activin B and A have redundant, unique, and interactive effects on HSCs. Taken together, these *in vitro* data show that activin B and A both redundantly and interactively modulated HSCs.

A

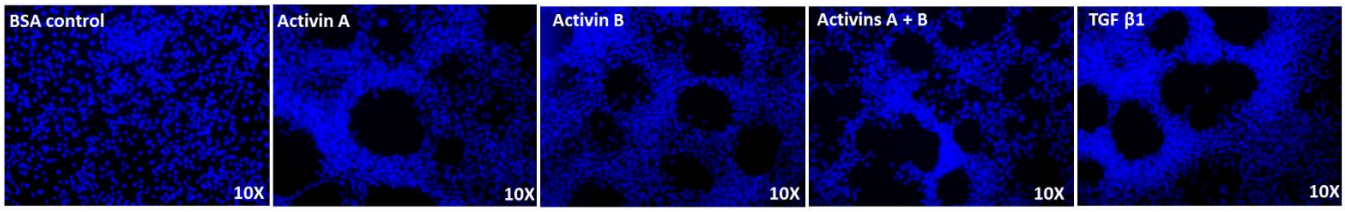

Figure 3.7 Activin A and B morphologically and molecularly activate HSCs.

(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 TGFβ1 (5 ng/mL) for 24 h and then subjected to 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 TGFβ1 (5 ng/mL) for 6 h. Total RNAs were isolated and reverse transcribed to cDNA. Microarray analysis was conducted using HG-U133 plus 2 chips (n = 6). The pie chart shows the numbers of genes commonly or uniquely regulated by individual TGFβ ligands. **(C)** The top ten signaling pathways revealed by Ingenuity canonical pathway analysis of the 877 target genes shared by these three TGFβ ligands. **(D)** Heat map of 20 genes exhibiting the highest magnitude of upregulation or downregulation in response to these three TGFβ ligands. **(E & F)** LX-2 cells were treated with vehicle, activin A (100 ng/mL), activin B (100 ng/mL), or their combination (100 ng/mL each) for 24 h. The expression of these genes was assessed using qRT-PCR. Data are shown as means of fold changes relative to vehicle controls ± S.E.M. *, $P < 0.05$.

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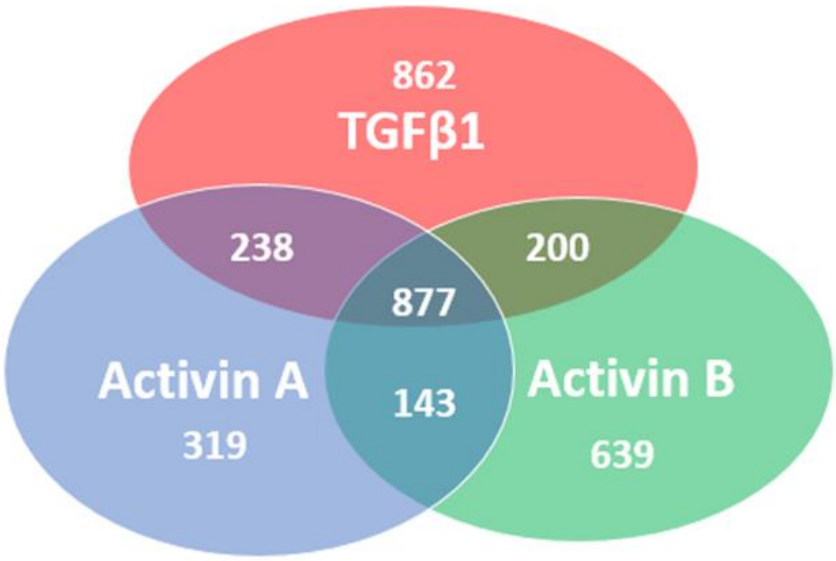

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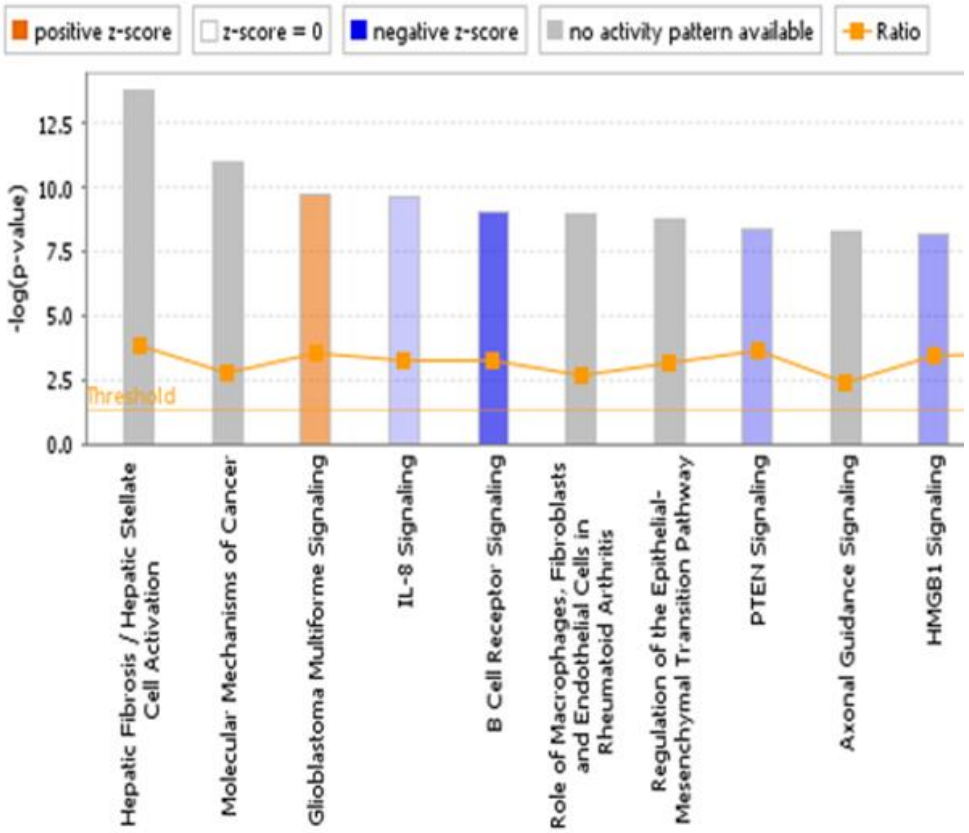

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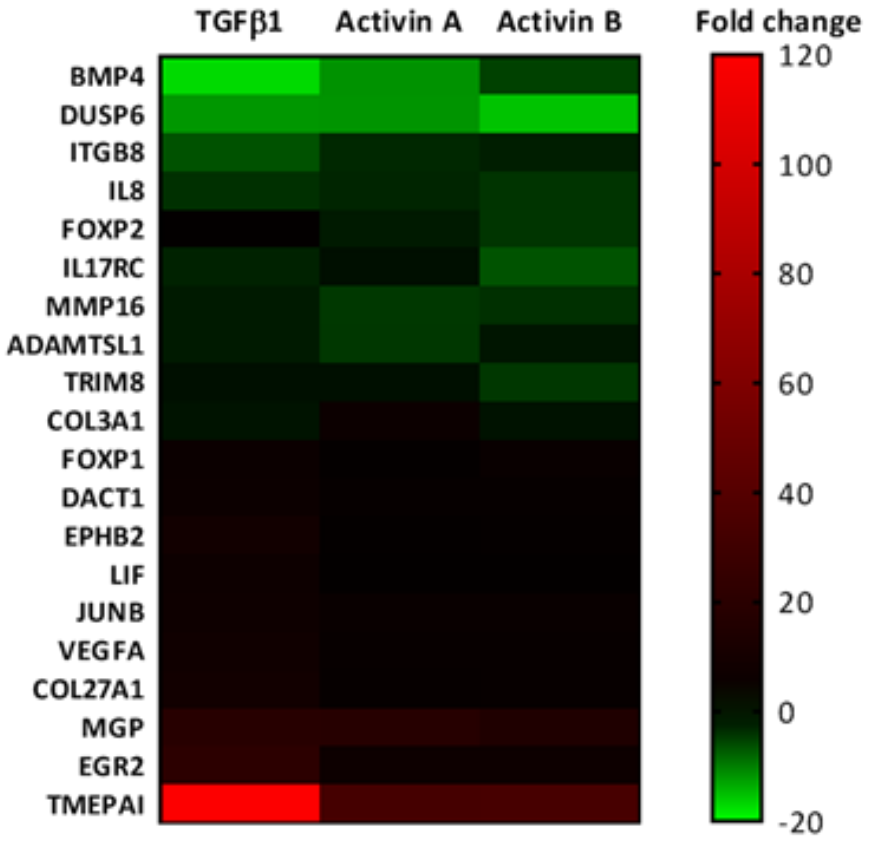

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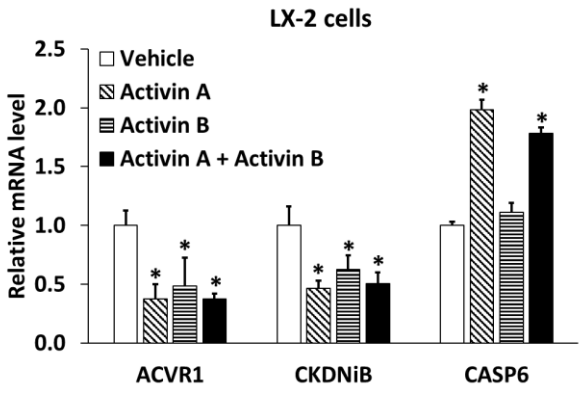

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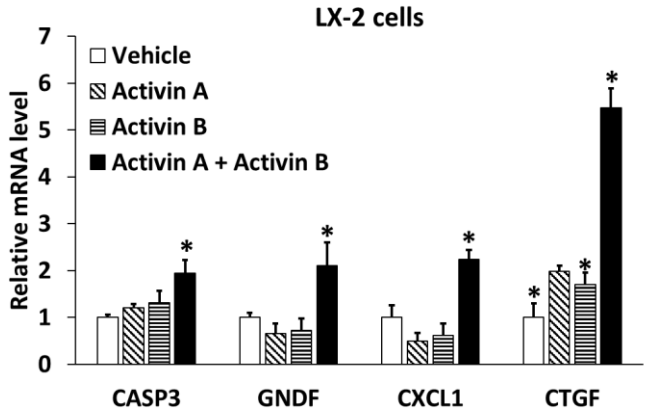

3.9 Individual or combinational neutralization of activin B and A distinctly ameliorates the progression of CCl4-induced liver fibrosis

The above association studies in humans and mice and *in vitro* studies strongly suggested that activin B and A differently participate in the regulation of liver fibrosis progression, and hence, we intended to confirm this notion. Global gene knockouts of these two widely produced activin ligands cause developmental defects, reproductive failure, or postnatal death in mice[86-88]. Therefore, we used a neutralizing antibody to systemically inactivate these two proteins and subsequently investigated their effects on the initiation of CCl_4-induced liver fibrosis. Five treatment groups were included: (1) vehicle; (2) IgG + CCl_4; (3) anti-activin A antibody + CCl_4; (4) anti-activin B antibody + CCl_4; and (5) combination of both antibodies + CCl_4. In the initial association studies, we found time windows during which both activin A and B were induced in the acute phase of liver injury (Figures 3.2A–F). This co-induction suggested a possible spatiotemporal coordination between the two activin ligands, warranting their combined application in this study. Antibodies were initially dosed half an hour before the first CCl_4 injection and were dosed weekly thereafter.

A dosage of 10 mg/kg of anti-activin A antibody weekly was used because our previous study showed the greatest efficacy of this regimen in regressing degeneration of injured skeletal muscle in mice[131]. We administered 50 mg/kg as the maximal efficacy dose of anti-activin B antibody once per week as the IC_{50} was found to be five-fold higher than that of anti-activin A antibody, as determined by using a SME promoter luciferase assay (Figures 3.8A–D)[132], and liver mass increased in a mouse homeostasis study (Figure 3.8E). Anti-activin B antibody exerted numerous beneficial effects, including reduced liver injury indicated by serum ALT and AST (Figures 3.8F&G), elevated serum glucose and decreased total bilirubin level (Figures 3.8H&I), and

decreased liver fibrosis analyzed using collagen staining and collagen 1α1 mRNA expression (Figures 3.8J–L). Anti-activin A antibody treatment reduced liver injury and improved liver functions to a lesser extent than anti-activin B antibody, but did not decrease total bilirubin and liver fibrosis, although collagen 1α1 mRNA expression was inhibited (Figures 3.9E–J). The dual antibodies showed beneficial effects equivalent to, or, in some cases, greater than that of activin B mAb alone (Figures 3.8E–J). However, only activin B mAb treatment did not increase the liver to body weight ratio (Figure 3.8M). In livers chronically damaged by CCl_4, activin B and A are essential collaborators to induce CXCL1, because neutralizing either one of them prevented CXCL1 upregulation, resulting in prohibited iNOS elevation (Figure 3.8P). In addition, hepatic CTGF and TGFβ1 upregulation was completely suppressed by neutralizing either one of the two activin ligands in mice chronically treated with CCl_4 (Figure 3.8P). CXCL1 has multiple functions, one of which is to attract neutrophils to infiltrate the liver injured by alcohol[133]. However, we observed that, in livers chronically damaged by CCl_4, myeloperoxidase (MOP)-positive neutrophils were concentrated in the septa (Figure 3.8Q). When anti-activin A antibody treatment prevented hepatic CXCL1 induction without affecting septa formation, MOP-positive neutrophils were still largely located in the septa (Figure 3.8Q). These observations suggest that neutrophils are closely associated with liver fibrogenesis, and their infiltration might not be regulated by CXCL1 in this setting. When liver fibrosis was largely prevented, neutrophils were overtly reduced and diffused (Figure 3.8Q). The distribution of F4/80-positive hepatic macrophages (Kupffer cells) was similar to that of neutrophils in all experimental groups (Figure 3.8Q). These data suggest that (1) activin B, and to a lesser extent activin A, mediate the initiation of liver fibrosis by promoting inflammatory response and fibrogenesis, and (2) activin B inhibition or, even better, both activin B and A inhibition prevents liver fibrosis.

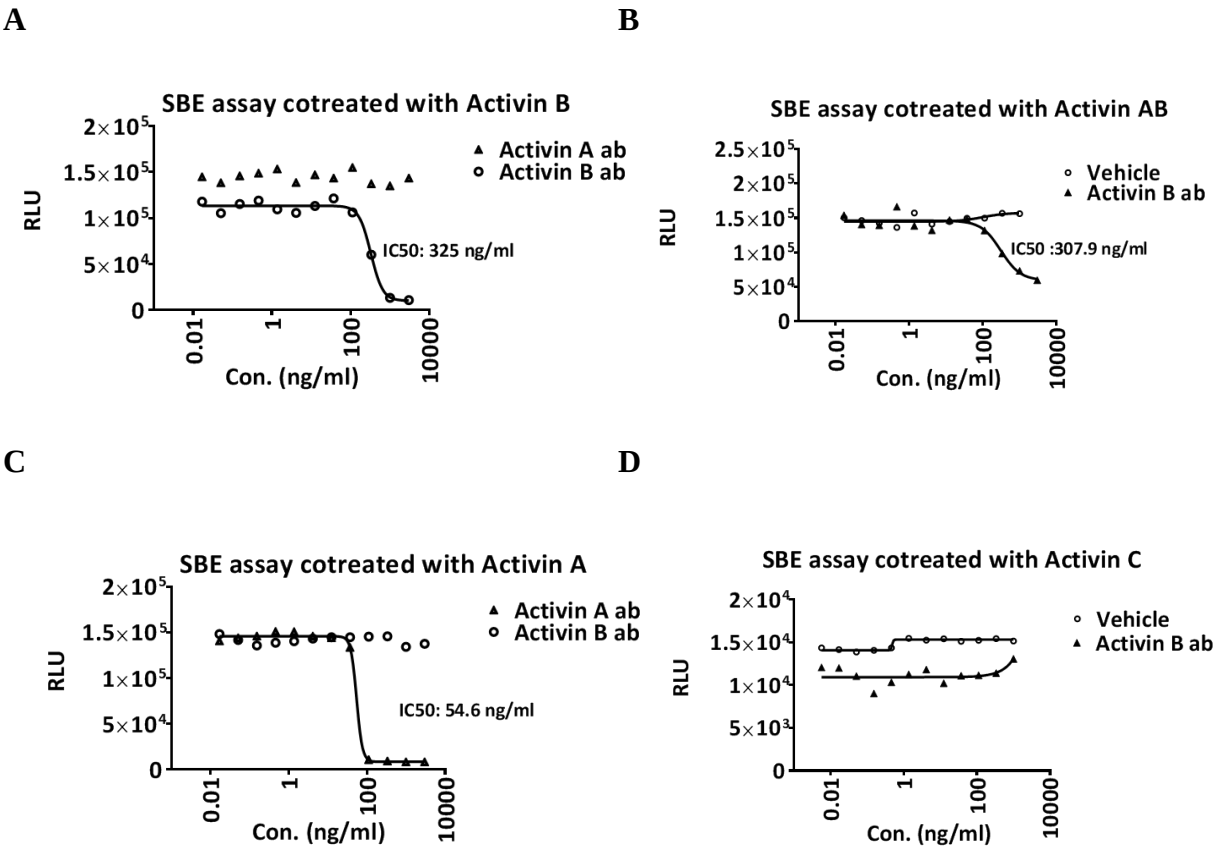

Figure 3.8 Anti-activin A antibody, anti-activin B antibody, and their combination show distinct effects in preventing liver fibrosis induced by CCl_4 in mice.

The Smad2/3 Binding Element (SBE) luciferase assay in SBE-transfected HEK 293 cells to determine activin antibody specificity. SBE-transfected HEK293 cells co-treated with activin A and activin B antibodies **(A)**, or activin AB **(B)**, activin A **(C)**, or activin C **(D)** proteins for 24 h. Adult female mice were subjected to CCl_4 or vehicle injection (*i.p.*) twice per week for 4 weeks. Half an hour before the first CCl_4 injection, mice were treated (*s.c.*) with IgG (60 mg/kg), anti-activin A antibody (10 mg/kg of anti-activin A antibody + 50 mg/kg of IgG), anti-activin B antibody (50 mg/kg of anti-activin B antibody + 10 mg/kg of IgG), or combination of activin A and activin B antibodies (10 mg/kg of anti-activin A antibody + 50 mg/kg of anti-activin B antibody). Subsequently, antibody treatments were performed once per week. Four weeks after the initial CCl_4 injection, **(E)** ALT, **(F)** AST, **(G)** glucose, and **(H)** total bilirubin in the blood were analyzed. **(I)** Representative liver sections stained with Masson trichrome. **(J)** Quantification of the percentage of Masson trichrome staining areas**. (K)** The mRNA expression of hepatic Col1α1 was evaluated using qRT-PCR. **(L)** Liver-to-body weight ratios. **(M–O)** Total liver RNA samples generated from the experiment described in Figure 3 were subjected to qRT-PCR analysis for determining the expression of the genes indicated. **(P)** Immunohistochemical analysis of MOP-positive cells (neutrophils), F4/80-positive cells (Kupffer cells), and Ki67-positive cells (proliferating hepatocytes) on liver sections prepared from the experiment described in Figure 3. Data are expressed as means ± S.E.M. (n = 10). *, $P < 0.05$ compared to vehicle controls. #, $P < 0.05$, compared to IgG controls.

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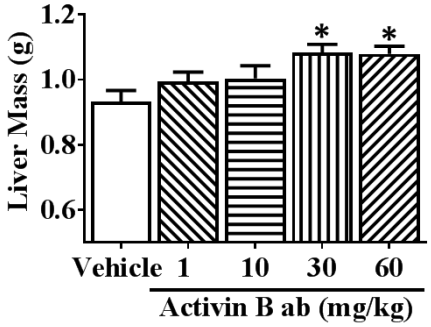

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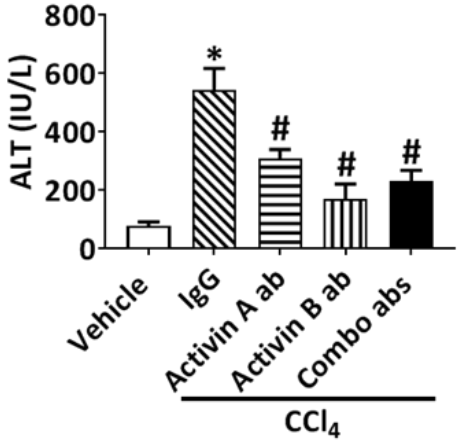

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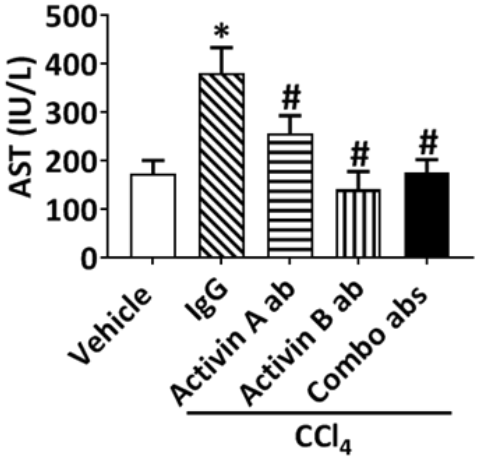

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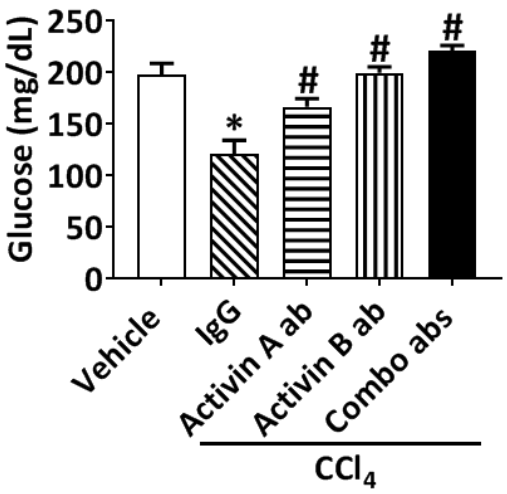

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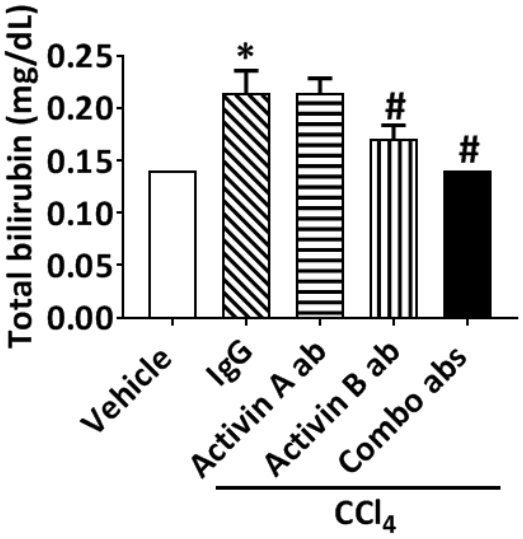

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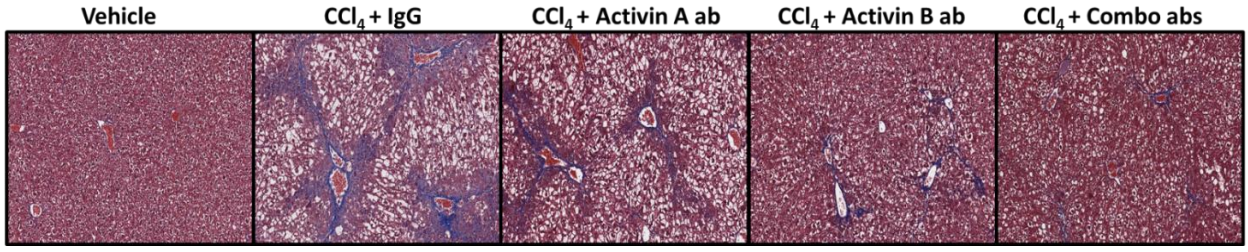

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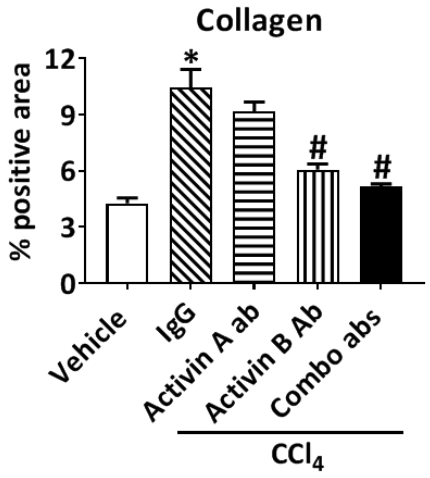

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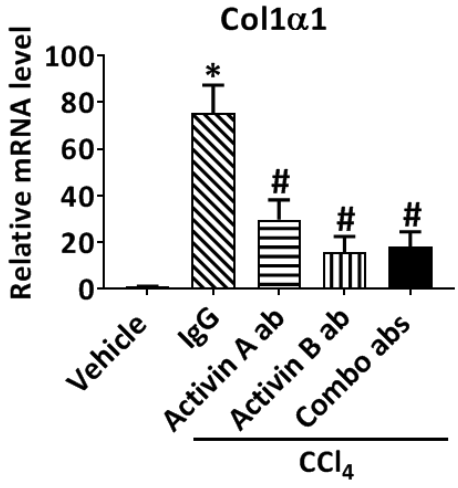

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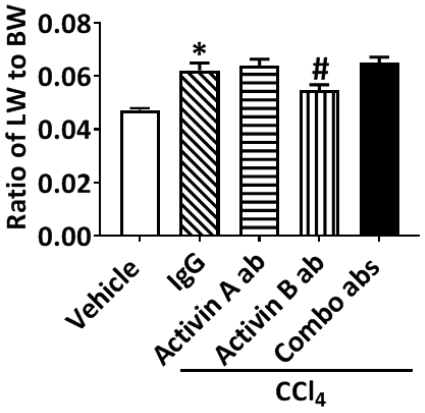

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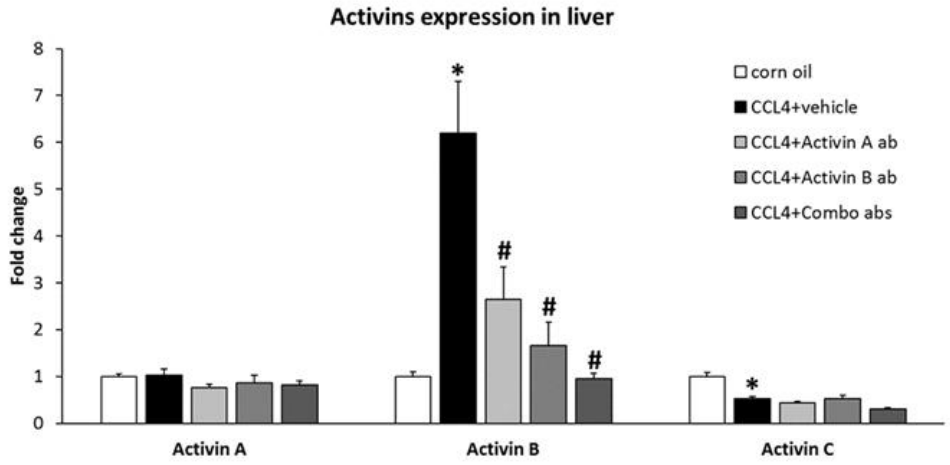

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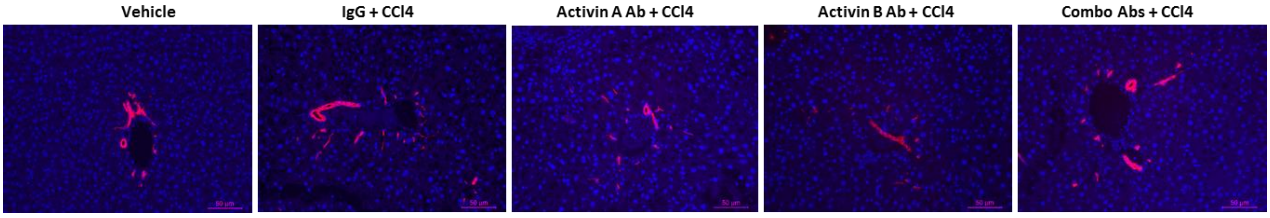

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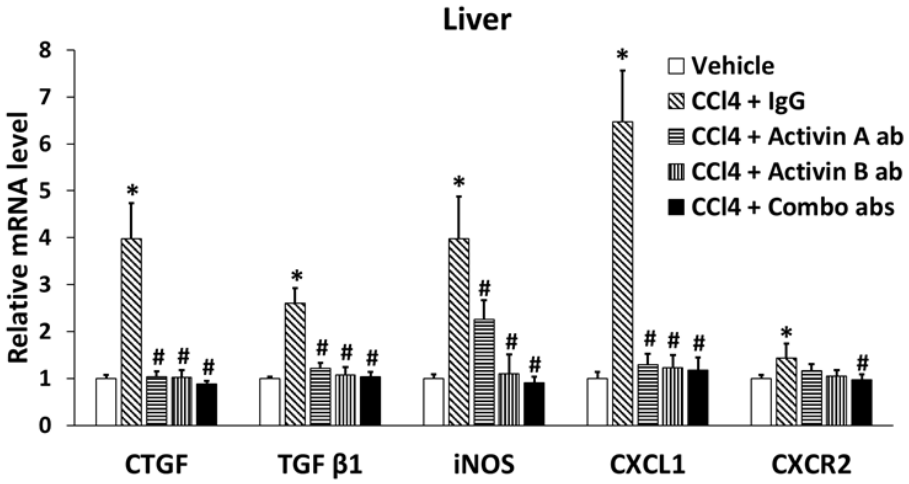

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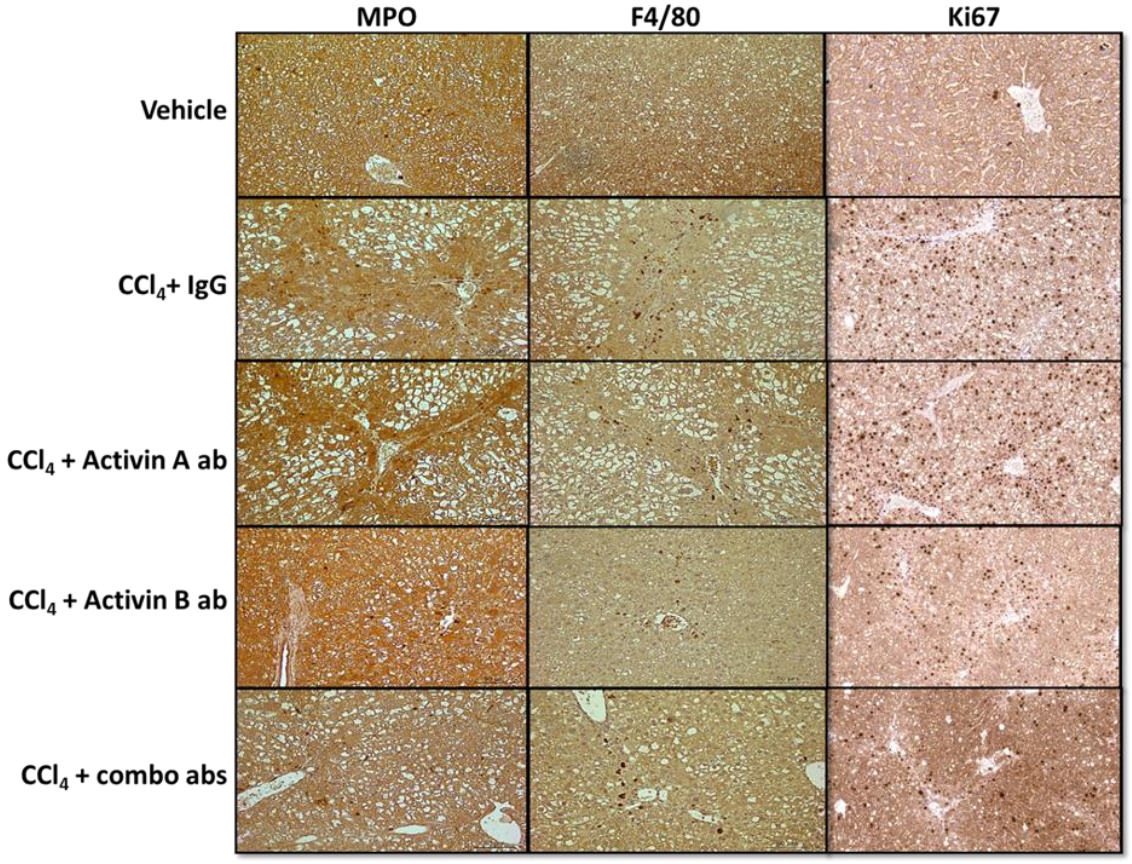

3.10 Neutralization of activin B and A regresses CCl4-induced liver fibrosis

Because of the superior effects of antibody-mediated inactivation of activin B or both activin B and A in preventing liver fibrosis, we tested the same strategy to reverse fibrosis in the CCl_4 mouse model after the disease has been established. Following the same study design as the preventive liver fibrosis study, CCl_4 was injected twice per week for 10 continuous weeks. Starting at the seventh week when liver fibrosis was completely established, antibodies were dosed weekly for the remaining 4 weeks. Consequently, we found distinct reversal effects in both the anti-activin B and A antibody treatment groups. The reversal effects followed the sequence of the 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. Combinational inactivation exerted the most beneficial effects across all assessments, including reduced liver injury (as measured by serum ALT and AST), increased serum glucose and total bilirubin level, decreased collagen deposition, and less macrophage infiltration (Figures 3.9A–K). Inactivating activin B alone generated a stronger anti-fibrotic effect, but nearly equal effects in other assessments, compared with those noted after inactivating activin A alone (Figures 3.9A–K). Notably, inactivating activin B alone and inactivating both activin B and A equivalently regressed liver fibrosis (Figure 3.9F). Neutrophils and Kupffer cells were similarly distributed in fibrotic livers and were concentrated in the septa. Neutralizing activin A, activin B, or both did not alter the total number of neutrophil infiltrations, but almost equally reduced the total number of Kupffer cells (Figures 3.9H–K). This suggests that activin B and activin A essentially cooperate to modulate the functional state of Kupffer cells. Taken together, these results suggest that activin B is a stronger driver of liver fibrogenesis than activin A, and that these two activin ligands might act cooperatively during the progression of chronic liver injury. Moreover, neutralization of either or both ligands might largely reverse the already established liver fibrosis, in addition to preventing the onset of disease.

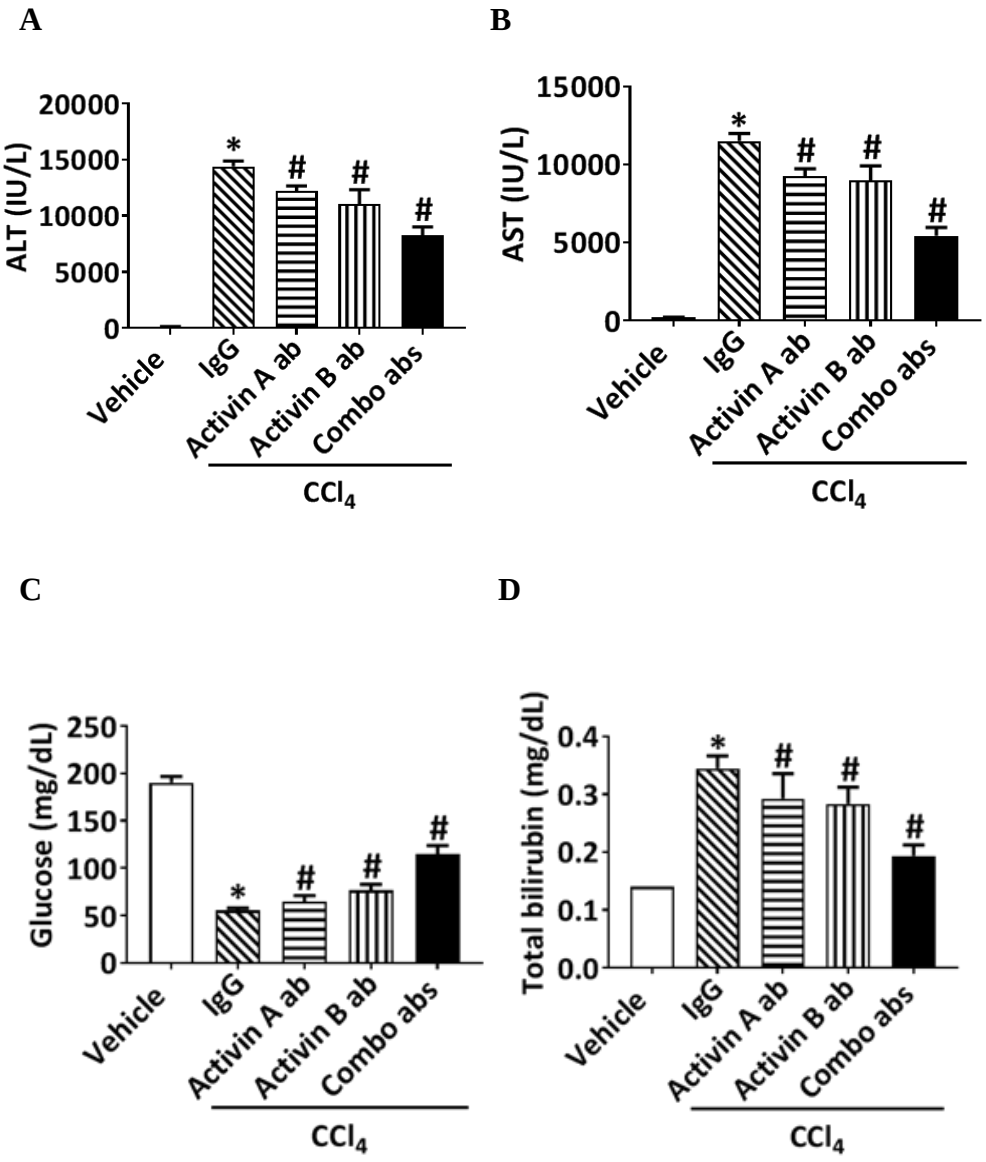

Figure 3.9 Anti-activin B antibody, anti-activin A antibody, and their combination show different effects in regressing liver fibrosis induced by CCl_4 in mice.

Adult female mice were subjected to CCl_4 or vehicle injection (*i.p.*) twice per week for 10 weeks. Starting from the seventh week, the mice were treated (*s.c.*) with IgG (60 mg/kg), activin A mAb (10 mg/kg of activin A mAb + 50 mg/kg of IgG), activin B mAb (50 mg/kg of activin B mAb + 10 mg/kg of IgG), or the combination of activin A and activin B antibodies (10 mg/kg of activin A mAb + 50 mg/kg of activin B mAb) once per week. Ten weeks after the initial CCl_4 injection, **(A)** ALT, **(B)** AST, **(C)** glucose, and **(D)** total bilirubin in the blood were analyzed. **(E)** Representative liver sections stained with Masson trichrome. **(F)** Quantification of the percentage of Masson trichrome staining areas. **(G)** Liver-to-body weight ratios. **(H)** Immunohistochemical analysis of MOP-, F4/80-, and Ki67-positive cells on liver sections. Quantification of the percentage of MOP- **(I)**, F4/80- **(J)**, and Ki67-positive cells **(K)**. Data are expressed as means ± S.E.M. (n = 10). *, $P < 0.05$ compared to vehicle controls. #, $P < 0.05$, compared to IgG controls.

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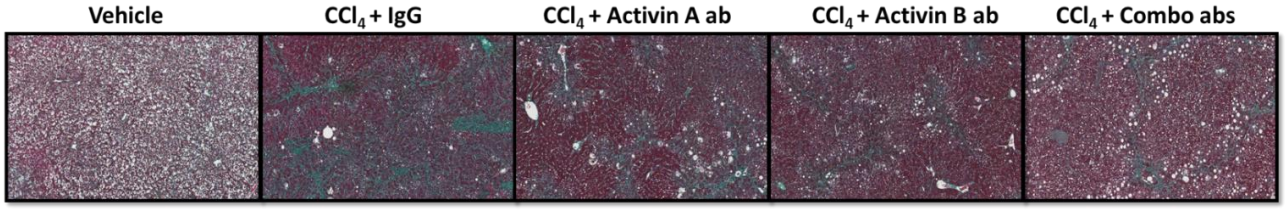

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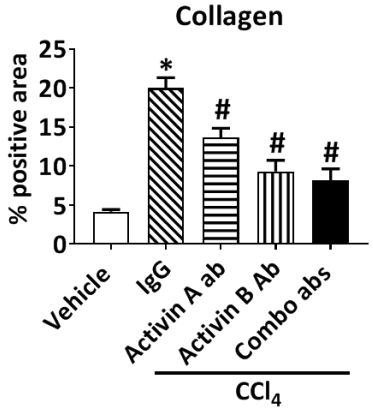

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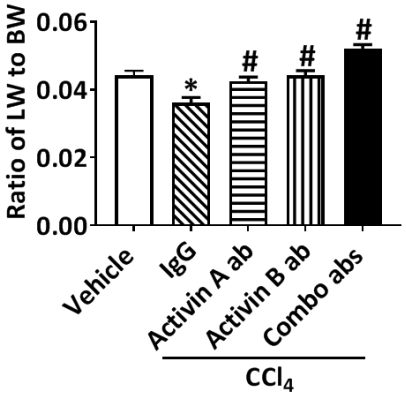

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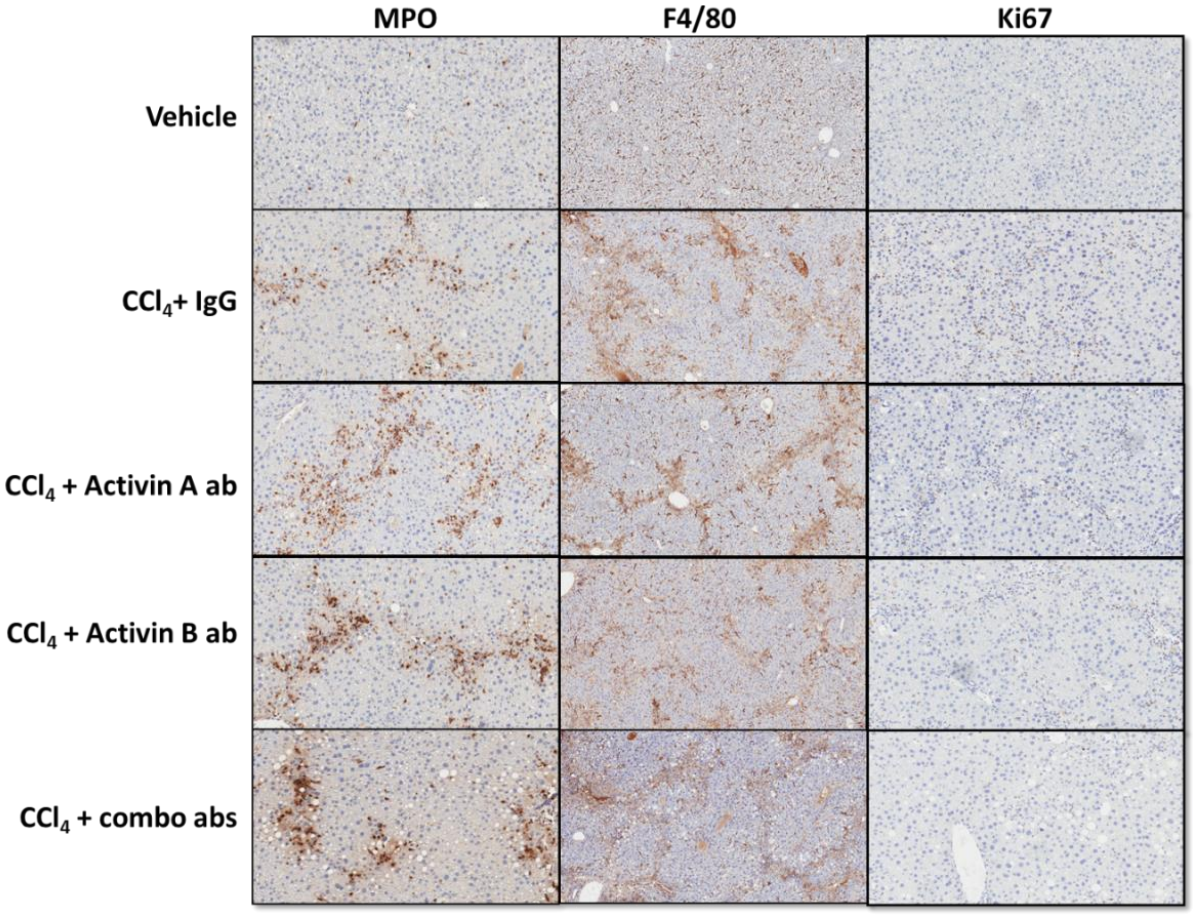

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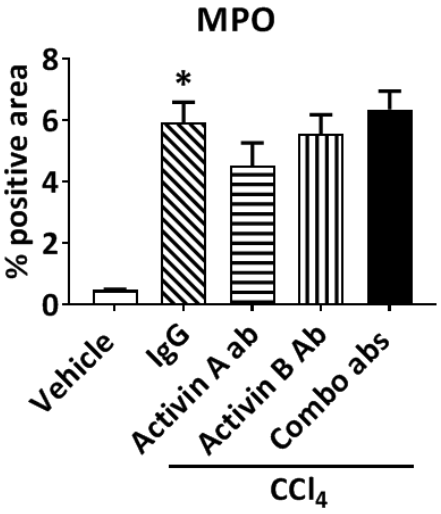

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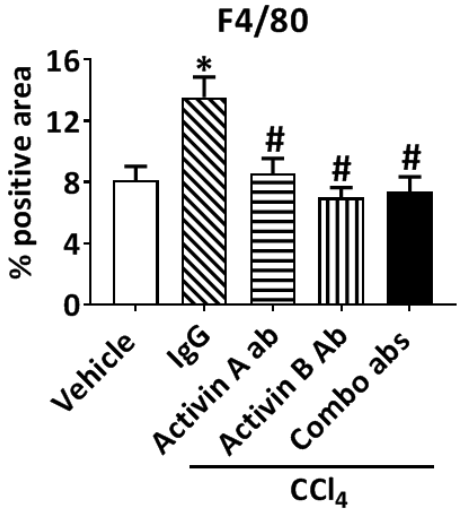

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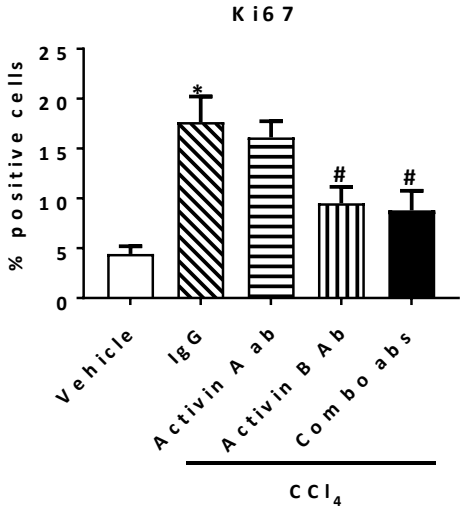

3.11 Individual or combinational neutralization of activin B and A distinctly prevents the progression of BDL-induced liver fibrosis

To further validate the above *in vivo* findings, we conducted a similar study in mice with BDL-induced chronic liver injury. Experimental groups included (1) sham control; (2) IgG + BDL; (3) activin A mAb + BDL; (4) activin B mAb + BDL; and (5) activin A mAb + activin B mAb + BDL. We dosed the first antibody one day before BDL surgery and the second one a week after BDL. Endpoint analyses were conducted 2 weeks after BDL. We found that BDL induced the upregulation of activin B mRNA expression without affecting activin A and activin C in the liver (Figure 3.10A). Individual antibody mildly, but dual antibodies almost entirely, inhibited activin B mRNA induction in bile duct-ligated livers (Figure 3.10A). Anti-activin B antibody treatment reduced liver injury, improved liver function, and decreased liver fibrosis (Figures 3.10B–G). Surprisingly, anti-activin A antibody did not show beneficial effects in nearly all the endpoints analyzed except that it ameliorated the total bilirubin index (Figure 3.10E). Remarkably, the dual antibodies exerted the most prominent efficacy, manifested by reduced liver injury, improved liver functions, and decreased liver fibrosis, but further enlarged the livers compared to those of IgG controls (Figures 3.10B–H). BDL typically induces biliary ductal reaction or new bile duct formation, which was not overtly affected by these antibodies, as revealed by CK19 immunostaining (Figure 3.10J). Activin B mAb and combination of both antibodies decreased hepatic inflammatory cytokines, CXCL1, IL-6, and IL-1β in the liver, and IL-6, TNFα, and IL-1β in the blood, whereas activin A mAb only inhibited serum IL-2 (Figures 3.10K–P). These results suggest that, in this model, (1) activin B strongly, but activin A minimally, promotes the progression of chronic liver injury; (2) activin B profoundly, but activin A slightly, induces the inflammatory response of liver fibrosis; and (3) the presence of activin A enhances the promoting actions of activin B, indicating why the dual targeting approach is the most beneficial.

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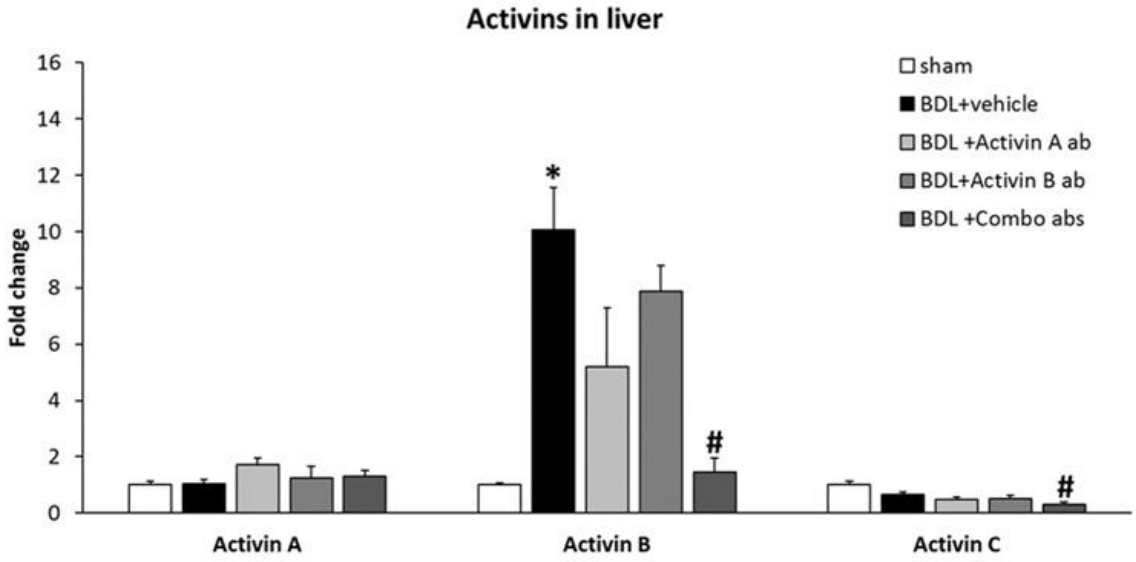

Figure 3.10 Anti-activin A antibody, anti-activin B antibody, and their combination exhibit anti-inflammatory and anti-fibrotic effects in the BDL liver fibrosis model.

Liver injury marker ALT **(A)** and AST **(B)**, liver functional marker glucose **(C)**, and total bilirubin **(D)** levels were determined in the serum of all the groups. **(E)** Representative histological images of trichrome staining, and **(F)** quantitative graph for collagen in all groups. **(G)** Liver index (ratio of liver weight to body weight) and **(H)** RT-PCR used to measure the levels of Col1a1, CTGF and TGFβ1, iNOS, CXCL1, SOX4, CXCR2, IL-6 and IL-1β at 2 weeks after BDL surgery after co-treatment with vehicle (mIgG), anti-activin A antibody, anti-activin B antibody, and combination of both (n = 8). **(I)** RT-PCR used to measure the level of activin A, activin B, and activin C in all groups. **(J)** Representative histological images of immunohistochemistry and CK19 fluorescence staining. **(L and M)** Quantification of inflammatory cytokines, TNFα, IL-2, and IL-6 in the serum and (**N**) CXCL1, IL-6, and IL-1β in the liver lysates of mice at 2 weeks after BDL surgery. Data are expressed as means ± S.E.M. Significance is indicated as $*P \leq 0.05$, treated group versus vehicle group (Dunnett's one-way ANOVA).

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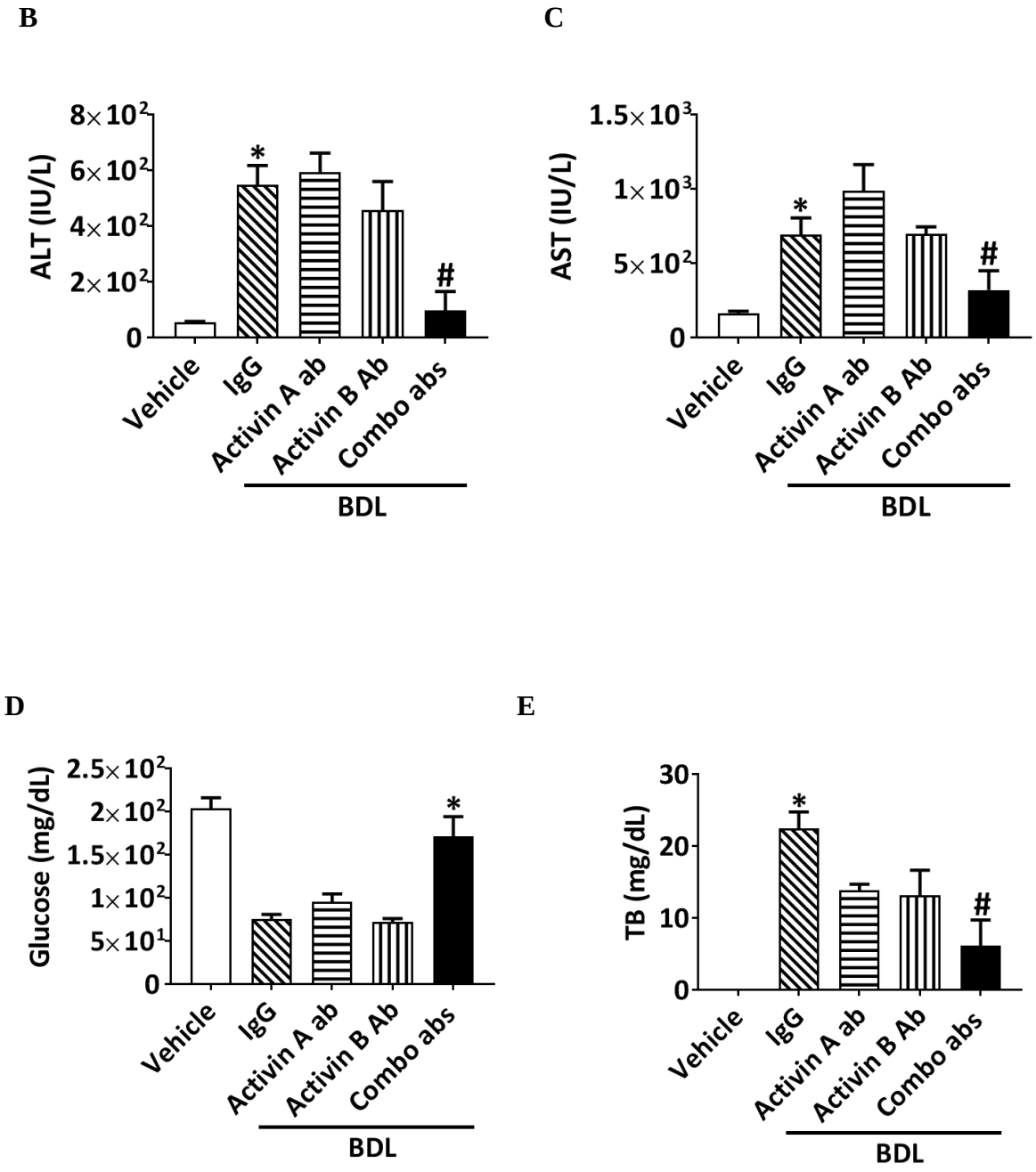

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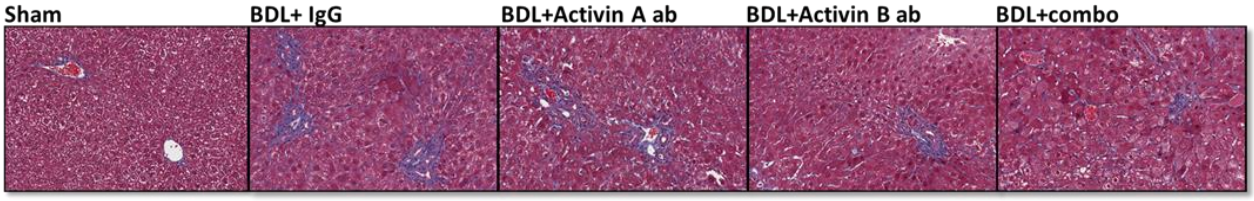

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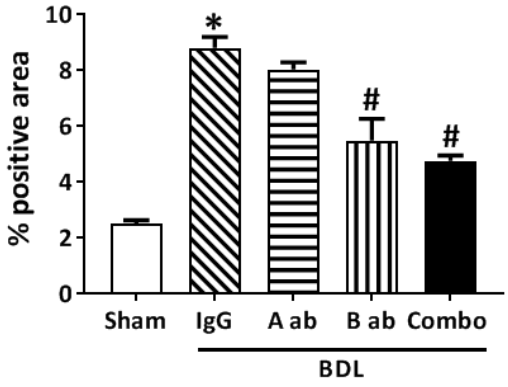

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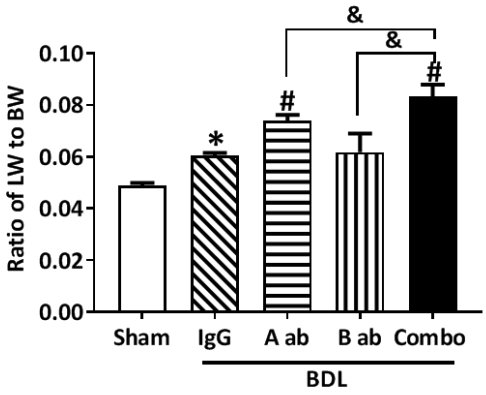

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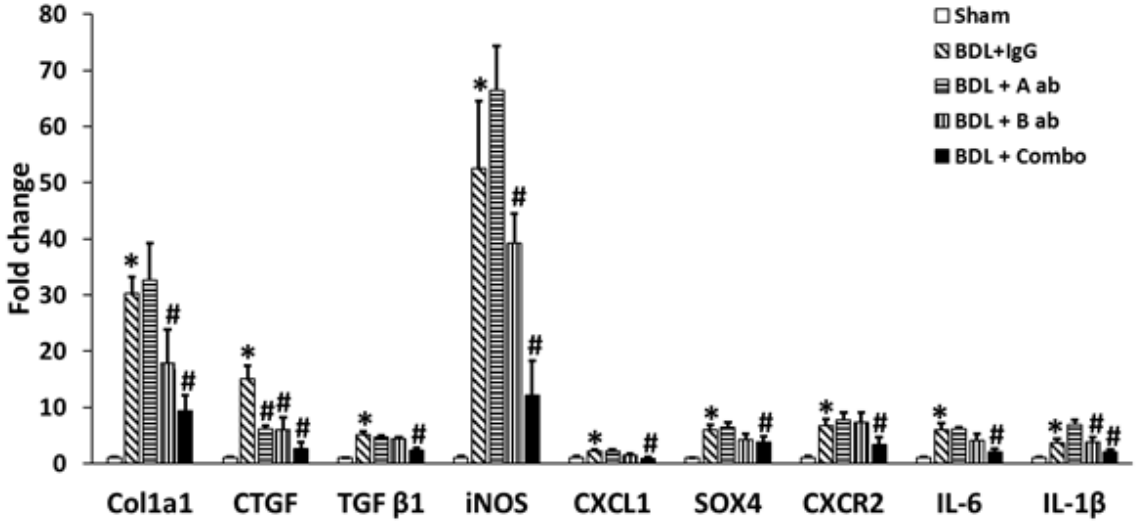

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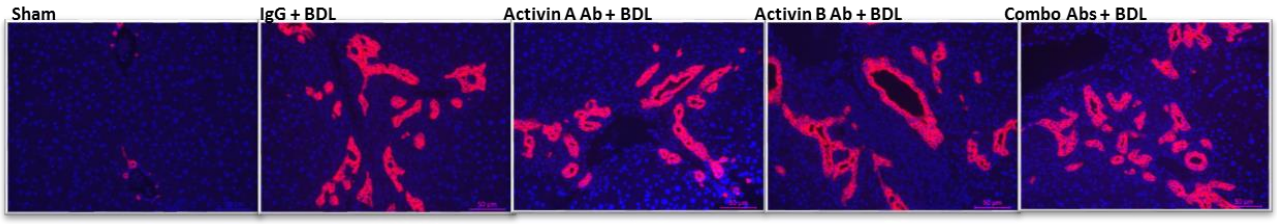

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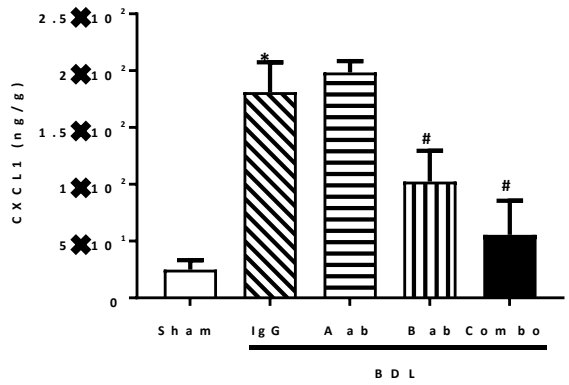

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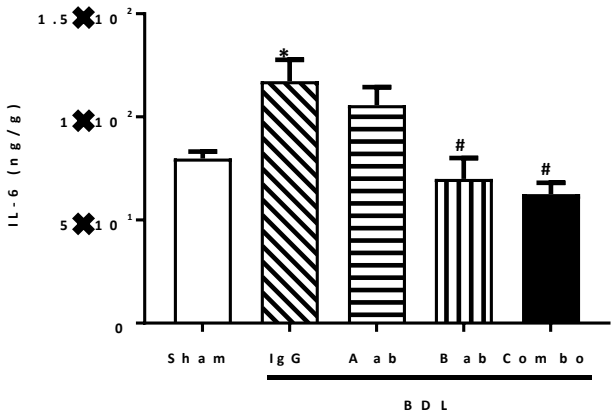

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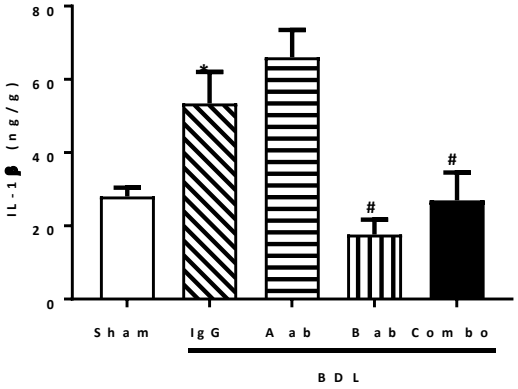

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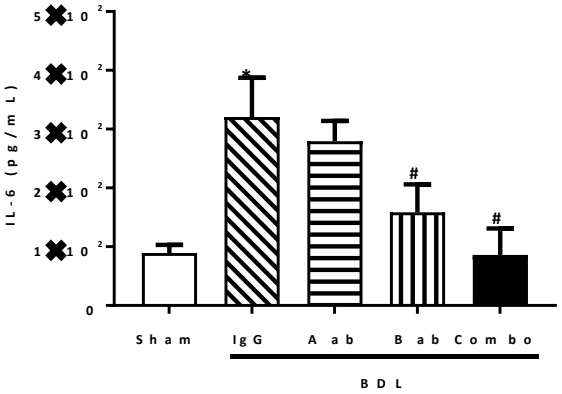

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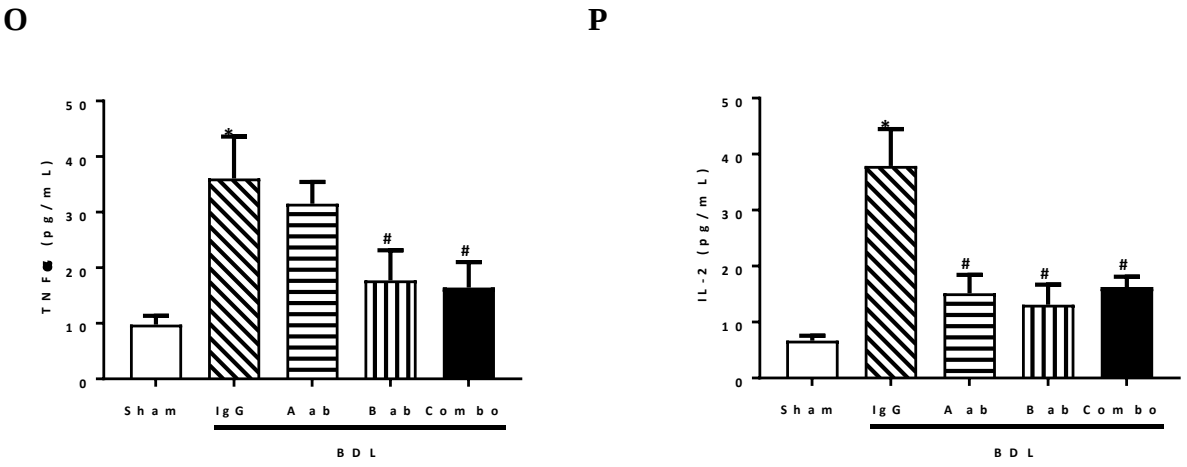

3.12 Discussion

This study mainly aimed to investigate the role of activin B in hepatic fibrogenesis. Our clinical and animal studies revealed a correlation between circulating activin B and liver fibrosis severity, which indicates that activin B is a potential biomarker and therapeutic target of liver fibrosis. In the liver, activin B acts directly on multiple cell populations to induce hepatic fibrogenesis either by itself or cooperatively with activin A, especially in HSCs and macrophages. Our study suggests that activin B promotes hepatocyte damage and macrophage and HSC activation to induce hepatic fibrogenesis by regulating TWEAK/Fn14, CXCL1/iNOS, CTGF, and TGFβ1 multiple signaling pathways. Moreover, the inhibition of activin B action remarkably decreased liver injury, inflammation, and fibrosis and improved liver function in liver fibrosis mouse models; these effects were enhanced when combined with activin A inhibition. The effects of activin B and A inhibition on liver fibrosis in animal models open potential clinical therapeutic possibilities for patients with liver fibrosis. The primary function of activin A and B is to regulate the reproductive system, developmental processes, inflammation, immunity, hematopoiesis, and tumorigenesis. In addition to these functions, our findings suggest that activins play a pivotal role in liver fibrosis.

The novel roles of activin B in hepatic fibrogenesis other than the reproductive function

Activins were initially isolated and identified for their roles in regulating developmental and reproductive processes. Activin A, a 28 kDa protein, was isolated in 1986 and activin B, a 25 kDa protein, was purified in 1992 from porcine ovarian follicular fluid[134 135]. Both activin A and B possess the ability to release follicle stimulating hormone from rat anterior pituitary cells *in vitro*. In developmental processes, activin A plays an important role in embryonic induction, limb and nervous system development, and morphogenesis of branched glandular organs and Wolffian duct. The involvement of activin A and B in development is also highlighted by the phenotype changes in corresponding genetically modulated mice. The phenotype of mice with inhibin βA gene knockout is lethal because of the failure in sucking due to mandibular and palate defects and absence of coiling in the epididymis[86 136 137]. Even though mice with inhibin βB gene knockout remain alive and fertile, their eye lids are dysfunctional and hence the eyes remain open, resulting in permanent ocular damage, and females show prolonged gestation and poor nursing behavior[87 88]. In addition, double knockout of inhibin βA and inhibin βB leads to the same defects as those noted after individual knockout, without any additional abnormalities[86]. Knockout of either inhibin βA or inhibin βB gene exhibits a distinct phenotype, indicating that each of them has a distinct and irredundant function in development. Furthermore, even though activin A and activin B have similar actions, their potency varies in developmental and reproductive processes. Activin A is a more effective regulatory protein of ovarian and testicular development, whereas activin B is more potent to induce embryonic mesoderm formation[135 138]. In addition, only activin B is involved in central nervous system development[77 139]. All these findings suggest that activin A and activin B share certain redundant functions and have some specific functions in developmental and reproductive processes, which might extend to other process such as liver fibrosis.

The complicated dual roles of activin A in mediating inflammation were shown by *in vitro* monocyte and macrophage studies. At low concentration, activin A induced inflammatory cytokines, including IL-1β, IL-6, and TNFα, via the p38 MAPK and ERK1/2 pathways in resting monocytes/macrophages, whereas it had inhibitory inflammatory effects in activated macrophages. Recently, accumulating evidence shows that activin B is involved in inflammation in acute liver injury[112 140 141]. In mice, activin B mRNA and protein are induced in the liver at 6 hours after the administration of LPS, and Kupffer cells and vascular endothelial cells are the main cellular sources of activin B[140]. In cell culture, activin A induced fibroblast proliferation and differentiation into myofibroblasts[142]. It also stimulated TGFβ1 production in renal and lung fibroblasts as well as pancreatic stellate cells, indicating that activin A might be a generalized pro-fibrotic regulator in multiple tissues[143-145]. The results of these *in vitro* and *in vivo* studies indicate that activin A and B might participate in the regulation of inflammation and pathogenesis of liver fibrosis.

Liver fibrosis is the common consequence of liver injury secondary to various chronic liver diseases[126]. The pathogenesis of liver fibrosis is mediated by complex cellular and molecular mechanisms. Herein, we provide the first clinical and experimental evidence suggesting that activin B has a functional role in liver fibrosis in initiating liver inflammation and fibrogenesis by inducing CXCL1/iNOS in macrophages and CTGF/TGFβ1 in HSCs. In this study, we also showed that activin B has substantially similar effects on hepatocytes, macrophages, and HSCs as those of activin A, whereas activin B specifically induced a group of genes in HSCs. Moreover, the *in vivo* efficacy results from multiple liver fibrosis mouse models showed that the administration of activin B mAb remarkably prevents liver injury and fibrosis induced by CCl_4 and BDL, and activin A mAb enhanced these preventive effects. The results of our study provide evidence that, in addition

to their role in reproductive and developmental processes, activin B and A are involved in liver fibrosis.

Activin B is a potential biomarker of liver fibrosis

Our study identified activin B as a factor closely associated with liver injury, irrespective of etiology and species. This reveals a highly conserved, activin B-mediated machinery, which fundamentally supports the liver's ability to respond to various insults in mammals. This machinery is activated rapidly following liver injury, operates stably throughout the pathogenesis, and predominates until the injured liver becomes fibrotic. We found that activin B mRNA was abundantly transcribed in fibrotic cells in chronically injured livers, and hepatic activin B transcript expression and protein level were always concomitantly elevated and were correlated with enriched circulating activin B. Therefore, increased production of hepatic activin B could largely contribute to its systemic enrichment during liver fibrosis development. As activin B is elevated both locally as well as systemically in multiple liver fibrosis rodent models and in patients with NASH, it is considered a promising biomarker. Further investigations are warranted to potentially develop activin B as a reliable and sensitive serum marker for monitoring liver fibrosis progression, especially for the diagnosis, staging, and prognosis of liver fibrosis and cirrhosis. The diagnosis of liver fibrosis is critical because hepatic fibrosis might progress and ultimately lead to cirrhosis with severe complications or hepatocellular carcinoma if the underlying insults persist, and management of patients with different stages of liver fibrosis relies on reliable diagnosis. At present, liver biopsy remains the gold standard for diagnosis and staging purposes[146]. Although it allows the histological assessment and quantification of liver fibrosis, it has several shortcomings, including patient's unwillingness because of its invasiveness, subsequent pain, and potential complications; sampling error; and high cost. These disadvantages limit its repeated application in

patients, requiring the exploration of non-invasive and reliable biomarkers of liver fibrosis. In addition to liver biopsy, the two other options for diagnosing liver fibrosis are image-based assessments and blood tests. Ultrasound-based elastography and magnetic resonance imaging are two representative imaging methods for liver fibrosis even though their diagnostic accuracy for fibrosis is low[147 148]. Blood tests might be divided into two classes: class I markers are direct serum markers that can be used to measure liver ECM turnover, whereas class II markers are indirect serum markers calculated from a mathematical model of liver function change. MMPs, TIMPs, collagen IV and VI, hyaluronic acid (HA), and laminin belong to class I biomarkers[149 150]. Collagens and HA are the most widely used markers for evaluating liver fibrosis development. The limitations of class I biomarkers are not always correlated with whole tissue function and are affected by other factors such as inflammation. Class II biomarkers are cost-effective and include ALT, AST, ALP, platelet count, and bilirubin, which reflect liver function, but are not necessary for monitoring liver fibrosis[151 152]. The sensitivity and specificity of these markers can be improved by combining diverse serological biomarkers in various degree of complexity. Two representatives of combined biomarkers are ALT/AST ratio and AST to platelet ratio index (APRI). Both are effective in diagnosing liver fibrosis with relatively high sensitivity in patients with HCV, although APRI might not be used in ALD due to the significant platelet suppression of alcohol[153 154]. Nevertheless, these investigations need further exploration, particularly for the detection of intermediate fibrosis grades. An ideal non-invasive biomarker is correlated with liver fibrosis severity. Activin B is a strong candidate because it is increased in the liver and blood in animal models and human patients with liver fibrosis. In particular, hepatic and serum activin B protein levels are significantly increased at the F4 stage of NASH (Figure 3.2 D&E). Because of the possible direct participation of activin B in hepatic fibrogenesis, this finding provides a new way

to explore liver fibrosis biomarkers other than ECM turnover components or liver functional enzymes. Future studies need to focus on identifying liver- and serum-correlated soluble proteins that are associated with the severity and staging of liver fibrosis. At present, no Food and Drug Administration-approved standard medicine is available for liver fibrosis treatment[155]. The promising targets revealed in animal studies encourage scientists to evaluate them in clinical trials. However, clinical trials of liver fibrosis are costly, and recruiting patients is difficult as they are usually long-term studies that require serial liver biopsies to assess fibrosis progression. Therefore, reliable noninvasive markers of liver fibrosis become a key factor to design and monitor clinical trials.

Activin B is a novel driver of hepatic fibrogenesis

Liver fibrosis is a severe health problem as the destruction of the normal liver architecture by ECM accumulation and fibrous scar formation along with the loss of functional hepatocytes eventually leads to liver failure. At present, no clinically effective therapies are available for liver fibrosis except liver transplantation. The potential targets of hepatic fibrogenesis are explored using activated HSCs and Kupffer cells as the key fibrogenic effector cells to determine ways to prevent its progression and/or induce its resolution. The majority of myofibroblasts are differentiated from activated HSCs, and HSC activation is a key step during hepatic fibrogenesis. The molecules and pathways required for HSC activation are attracting attention and are investigated as potential therapeutic targets. The therapeutic strategy involves suppression of HSC activation, induction of activated HSC apoptosis, or manipulation of macrophage activity. In experimental studies, some molecules have been shown to be the targets for liver fibrosis. TGFβ1 and hepatocyte growth factor (HGF) are representatives of these targets. TGF-β1 promotes HSC activation and proliferation, as

well as induces EMT and EndoMT to contribute to myofibroblast populations. However, the inhibition of TGFβ1 is ineffective in long-term treatment because of the serious adverse effects and complications because of its pleiotropic roles in homeostasis. HGF is a multifunctional cytokine involved in hepatic fibrogenesis. In animal models, blocking HGF activity is effective, but it increases the risk of carcinogenesis. Therefore, identifying a general target of liver fibrosis that is associated with liver fibrosis severity and its blocking has anti-fibrotic effects in multiple models is urgently required.

By using both *in vitro* and *in vivo* approaches, we showed that activin B is a potent driver of the complications (hepatocyte injury, inflammation, and fibrosis) of chronic liver injury. In the liver, the initiation and perpetuation of liver fibrosis are controlled by multiple cell populations that mainly include hepatocytes, macrophages, and HSCs. We found that activin B mediates hepatocyte injury. This is manifested by improved hepatocyte viability and reduced ALT after activin B is neutralized *in vitro* and *in vivo*. In addition, activin B and A stimulate TGFβ1, CTGF, Col1α1, and ACTA1 gene expression in hepatocytes. These activin A- and activin B-induced fibrotic transcripts facilitate the trans-differentiation of to a myofibroblast-like phenotype, which provides evidence that EMT could be one of the possible sources of myofibroblasts. At the molecular level, we revealed some important clues for further mechanistic investigations to understand how activin B regulates the activities of macrophages and other immune cells. For example, activin B upregulated TWEAK and its receptor Fn14 in macrophages. The TWEAK/Fn14 pathway was shown to promote ROS production and oxidative stress in these cells[156]. TWEAK is known to be primarily produced by macrophages and natural killer cells; it induces the expansion of liver progenitor cells, mediates the cross-talk among liver progenitor cells/immune cells/HSCs, and eventually augments

inflammatory and fibrotic responses in chronically injured livers[157-159]. This reveals an activin B/TWEAK/Fn14 axis operating in multiple liver cell populations in injured livers. Strikingly, we showed that activin B is a potent pro-fibrotic factor. It massively altered the transcriptome of HSCs *in vitro* and forced them to form a septa-like structure. Neutralizing activin B alone largely repressed septa formation, collagen deposition, and fibrotic gene expression, such as CTGF and TGFβ1, in chronically injured liver *in vivo*. The microarray data provided a list of activin B target genes of interest for further studies to elucidate how activin B controls the activities of HSCs. Based on these findings and given the persistent increases of hepatic and systemic activin B with the progression of liver injuries regardless of etiology and species, we propose activin B as a primary and critical factor to sustain the activation of immune cells and HSCs during various chronic liver diseases.

Activin B and A are novel and direct regulators of HSCs

Previous studies have shown that TGFβ signaling might modulate liver fibrogenesis, for which HSCs are central. However, the subset of TGFβ superfamily ligands that directly target HSCs has not been well defined, and whether the ligands activate redundant or distinct TGFβ signaling in these cells remains unclear. We found that TGFβ ligand, activin B was persistently induced in the liver and blood during both CCl_4- and BDL-induced chronic liver injury in mice. More remarkably, both activin B and activin A proteins stimulated LX-2 cells to form a septa-like structure *in vitro*, similar to TGFβ1, a mostly studied TGFβ ligand in liver fibrosis. These findings suggest that activin A and B are involved in liver injury progression at least by directly regulating the activities of HSCs. To further confirm this at the molecular level, we treated LX-2 cells with activin A, activin B, or TGFβ1 for 6 h and subsequently profiled their early responsive genes by using

microarray analysis. We found that these three TGFβ ligands regulate overlapping, but differential gene networks, which are associated predominately with HSC activation and hepatic fibrosis and with many other HSC activities. Notably, with equivalently high magnitudes, these three TGFβ ligands upregulated TMEPAI, apoptosis regulator SOX4, calcium ion-binding protein MGP, and EGR2 and down-regulated dual specificity phosphatase 6, BMP4, extracellular matrix glycoprotein TNXB, IL-8, and IL-17 receptor C. These data suggest that TGFβ signaling dictates a spectrum of HSC properties independent of its ligands. However, each of these three TGFβ ligands targets a unique and large set of genes associated with critical cellular functions. In particular, activin A specifically down-regulates solute carrier family 25 member 29, a mitochondrial transporter of basic amino acids, suggesting its role in mitochondrial amino acid metabolism. Activin B exclusively decreased cell migration-associated scaffold protein Ezrin and calcium-dependent phospholipid-binding protein 3, implying that activin B plays a role in HSC migration. TGFβ1 exceptionally suppressed myostatin, a well-established potent inhibitor of myogenesis, indicating its possible role in liver–muscle cross-talk. Thus, we showed that (1) activin A and B are new regulators of HSCs and thus potentially participate in mediating liver fibrogenic responses, and (2) TGFβ signaling exhibits ligand-independent and ligand-dependent actions in HSCs, warranting future studies of individual TGFβ ligands in liver fibrogenesis.

Activin A and B additively and interdependently act on HSCs and macrophages

Our study showed that the presence of both activin B and activin A is required to optimally promote liver injury progression. We showed that hepatic and systemic activin A was transiently increased at the acute phase of liver injury and was maintained at the pre-injury level during the long-lasting chronic phase. Although the neutralization of activin A alone produced beneficial

effects, they were weaker than those noted after the neutralization of activin B alone in both the prevention and reversal studies. Furthermore, we observed many additive or synergistic effects between activin B and A *in vitro* in macrophages and HSCs as well as *in vivo* in chronically injured livers, as described in the result section. For example, we showed a novel activin B and A/CXCL1/iNOS pathway that modulates macrophages. In response to immunological stimuli, iNOS is highly induced and generates a large amount of nitric oxide and hence promotes many pathological processes, including liver fibrosis of diverse etiology[160-162]. Identification of this pathway enabled us to gain important mechanistic insights into the actions of activin B and A. Another example is that the upregulation of CTGF and TGFβ1 gene expression requires the collaboration of activin B and A in chronically damaged livers. Most strikingly, inactivating both activin B and A yielded the most profound beneficial effects across our structural and functional assessments compared to those noted after inactivating activin B or A alone. These observations indicate that, as liver injury progresses, elevated activin B needs constitutive activin A for the activation of certain cellular programs that otherwise would not be initiated by a single ligand. Thus, unchanged activin A is not only an auxiliary factor, but also an essential collaborator, of increased activin B, on which individual cellular programs depend, in persistently injured livers. This represents a novel mode of action of TGFβ ligands in general and a new mechanism governing the actions of activins B and A in specific.

Neutralization of both activin A and B is highly efficient in preventing and regressing liver fibrosis

Our pre-clinical studies showed that targeting activin B or ideally both activin B and A is a promising strategy to prevent and even reverse liver fibrosis. In addition to blocking activin A and B, neutralization of both activin A and B produced additive or synergistic effect that can be attributed to the blocking of activin AB; partial inactivation of activin AC, activin AE, activin BC, inhibin A, and inhibin B; and potential interactions between activin A/Smad2/3 signaling and activin B/Smad1/5/8 pathway. Anti-activin A antibody neutralizes activin A and any of the heterodimers having inhibin βA subunit, whereas anti-activin B antibody blocks activin B and partially blocks any of the heterodimers having inhibin βB subunit. This broad-spectrum neutralization of multiple activins and inhibins provides enhanced anti-fibrotic and anti-inflammatory effects. Furthermore, the inhibition of both of Smad2/3 and Smad1/5/8 pathways supports the profound anti-fibrotic efficacy. Both pathways share Smad4, the common transcription factor, which might explain the additive or synergistic effects after combined activation or inhibition of activin A and B. Improved understanding of TGFβ signaling in homeostasis and pathophysiology has accelerated continuous preclinical and clinical efforts targeting its ligands, receptors, or Smads for therapeutic benefits, including reversing organ fibrosis. However, few studies have shown positive patient outcomes largely because of off-target complications[3 64]. Thus far, Pirfenidone is the only small-molecule TGFβ signaling inhibitor approved for the treatment of human idiopathic pulmonary fibrosis. It has significant adverse effects in the gastrointestinal tract and skin[132]. A soluble ActRIIB, ACE-031 has been tested in clinical trials in healthy volunteers and patients with Duchene Muscular Dystrophy[163]. Soluble ActRIIB binds to various TGFβ superfamily ligands, including GDF5, GDF8, GDF11, activin A, activin B, activin C, activin E, Nodal, BMP2, BMP4, BMP6/7, BMP9, and BMP10 and other

negative regulators of muscle mass. Clinically, ActRIIB showed pronounced effect on increasing skeletal muscle growth, but caused bleeding, which might be attributed to its non-specific binding to TGFβ superfamily ligands, limited its clinical application[164]. Although our study has some limitations in that the PK/PD of the antibodies was not determined and thus the dosing regimens used might not be optimal, once per week administration of activin B antibody alone or activin B and A dual antibodies showed high therapeutic efficacy. Our results might form a basis for further translational development of this strategy.

Liver fibrosis is mediated by multiple cell populations

The development of hepatic fibrogenesis is orchestrated by many cell populations in the liver, such as hepatocytes, HSCs, and macrophages. Other cell types also participate in liver fibrosis, including endothelial cells, progenitor cells, and natural killer cells. At present, therapeutic targets of liver fibrosis only focus on myofibroblast inactivation and apoptosis and/or macrophage phenotype switch. Other cell types might need to be considered in liver pathogenesis. Recently, accumulating evidence implies that epigenetic regulation might affect liver fibrosis development, which is represented by DNA methylation and histone modification[165-167]. The proteins and non-coding regulatory RNA molecules involved in epigenetic mechanism might reveal new biomarkers and therapeutic targets for liver fibrosis. In the present study, we identified activin B as a novel biomarker and therapeutic target of liver fibrosis. Interestingly, serum activin B was also elevated in idiopathic pulmonary fibrosis, indicating that it might be involved in fibrogenesis in other organs[168 169].

3.13 Conclusion

In summary, we identified activin B as a potent driver, potential clinical biomarker, and a promising therapeutic target of liver fibrosis. Based on our findings, we propose the following theory (Figure 3.13). Irrespective of the etiology, chronically injured livers constantly produce increased activin B. Synergistically with constitutive activin A, it promotes hepatocyte injury and possibly trans-differentiation; modulates macrophages and other immune cells to secrete inflammatory cytokines through CXCL1/iNOS, TWEAK/Fn14, and other signaling pathways; and, most importantly, initiates and maintains the activation of HSCs by increasing the expression of pro-fibrotic genes, including *CTGF* and *TGFβ1*. Thus, activin B potently promotes liver fibrogenesis. This theory directs our future investigations to elucidate activin B- and A-triggered signaling pathways and their functions in each liver cell population during the progression of liver fibrosis.

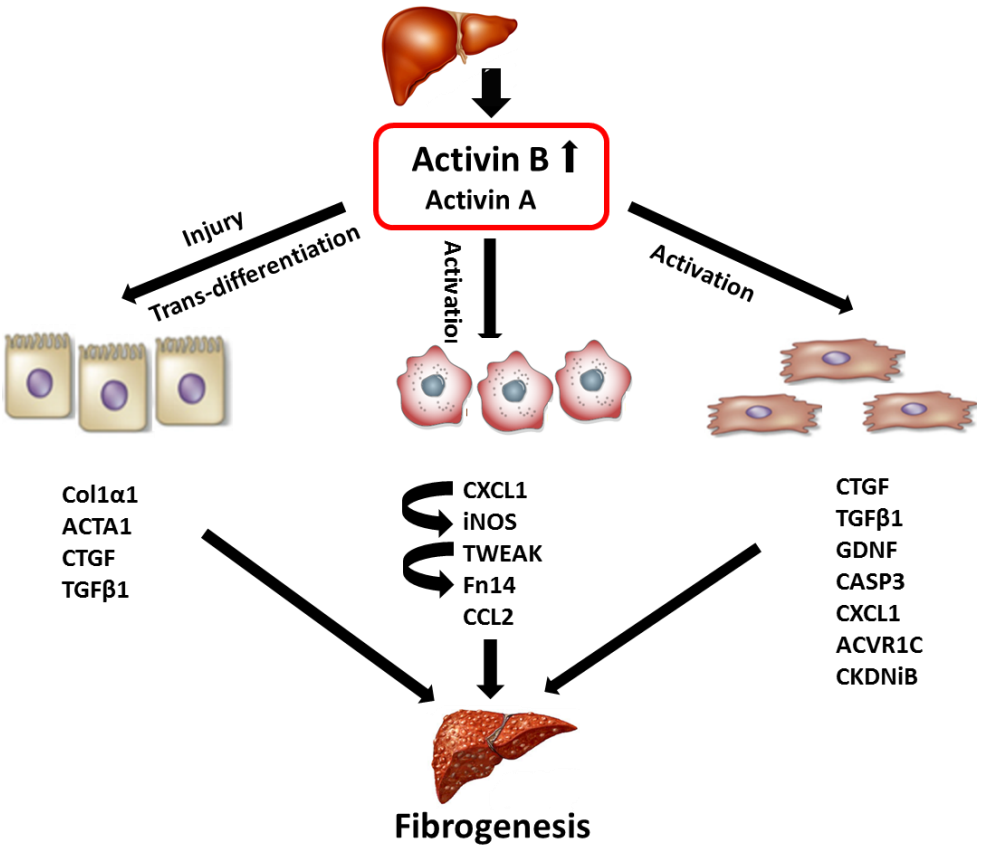

Figure 3.11 Scheme of the proposed role of activin B and activin A in promoting hepatic fibrogenesis

Hepatic fibrogenesis mediated by activin B and activin A is orchestrated by multiple cell types, including hepatocytes, HSCs, and KCs. (1) Liver injury (for example by CCl_4 and alcohol) causes parenchymal cell (hepatocytes and cholangiocytes) necrosis and/or apoptosis. (2) The injured hepatocytes release various growth factors, including activin A and activin B, which in turn activate hepatic stellate cells, attract and activate Kupffer cells, and trans-differentiate hepatocytes to myofibroblasts as autocrine cytokines. (3) The sustained elevation of activin B and constitutive activin A, in particular, lead to the trans-differentiation of hepatic stellate cells and hepatocytes into myofibroblasts, which express profibrotic genes, including ACTA1, collagen I, CTGF, and TGFβ1. Activin A and activin B activate macrophages that highly express inflammatory genes such as *TWEAK*, *Fn14*, and *CCL2*. TWEAK/Fn14 and CXCL1/iNOS signaling promotes a hepatic inflammatory response. (4) Synergistic induction by activin A and activin B of CXCL1 and iNOS in macrophages and CTGF, GNDF, IL-6, IL-1β, caspase 3, and CXCL1 in HSCs promote hepatocytes apoptosis, hepatic inflammation, and liver fibrogenesis.

3.14 Future directions

Our results reveal that activin B is a novel biomarker and mediator of liver fibrosis. Circulating activin B level is associated with fibrosis progression in preclinical liver fibrosis induced by hepatotoxins such as CCl_4 or ethanol and cholestasis and in human NASH and alcoholic liver diseases. Further clinical evidence from liver and serum samples from patients with NASH and ALD as well as other chronic liver diseases, including chronic virus hepatitis or autoimmune hepatitis, is required. Neutralization of activin A or activin B alone prevents and even reverses hepatotoxin-induced liver fibrosis, whereas neutralization of both enhances the anti-fibrotic effect and optimally improves liver function. Antagonizing both activin A and activin B signaling seems to be a promising target for preventing and reversing liver fibrosis. Thus, a conjugate antibody needs to be developed to block both activin A and B. Dual antibody generation and its PK/PD results might allow its evaluation in preclinical animal models and even in clinical trials of liver fibrosis or NASH.

In the microarray study, we found a novel transcription factor, the SRY-related High Mobility Group box transcription factor 4 (SOX4), which was upregulated with activin A, activin B, and TGFβ1 in HSCs. In developmental biology, gene knockout studies revealed that SOX4 cooperatively with SOX9 acts as a pivotal transcription factor and regulator of biliary development[170]. The role of SOX4 in hepatic fibrogenesis has not yet been determined. The mechanism of SOX4 in hepatic fibrogenesis, especially in HSC activation and differentiation, needs to be further investigated. Liver-specific or stellate cell-specific SOX4 knockout or overexpression studies might enable the elucidation of target genes and their effects on septa structure formation in liver fibrosis development. Further studies are required to investigate whether activin A and B mediate hepatic fibrogenesis through SOX4 in HSCs.

In conclusion, future translational studies are required to develop activin B as a potential biomarker, generate dual antibodies, and conduct evaluations in rodent models and clinical patients, as well as to determine the mechanism of SOX4 in activin-mediated liver fibrosis.

REFERENCES

1. Altamirano-Barrera A, Barranco-Fragoso B, Mendez-Sanchez N. Management strategies for liver fibrosis. *Ann Hepatol* 2017;16(1):48-56. doi: 10.5604/16652681.1226814 [published Online First: 2017/01/05]

2. Fuchs BC, Wang H, Yang Y, et al. Molecular MRI of collagen to diagnose and stage liver fibrosis. *J Hepatol* 2013;59(5):992-8. doi: 10.1016/j.jhep.2013.06.026 [published Online First: 2013/07/11]

3. Akhurst RJ. Targeting TGF-beta Signaling for Therapeutic Gain. *Cold Spring Harb Perspect Biol* 2017;9(10) doi: 10.1101/cshperspect.a022301 [published Online First: 2017/03/02]

4. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet* 2008;371(9615):838-51. doi: 10.1016/S0140-6736(08)60383-9 [published Online First: 2008/03/11]

5. Herrmann E, de Ledinghen V, Cassinotto C, et al. Assessment of biopsy-proven liver fibrosis by two-dimensional shear wave elastography: An individual patient data-based meta-analysis. *Hepatology* 2018;67(1):260-72. doi: 10.1002/hep.29179 [published Online First: 2017/04/04]

6. Goodman ZD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *J Hepatol* 2007;47(4):598-607. doi: 10.1016/j.jhep.2007.07.006 [published Online First: 2007/08/19]

7. Ramadori G, Saile B. Portal tract fibrogenesis in the liver. *Lab Invest* 2004;84(2):153-9. doi: 10.1038/labinvest.3700030 [published Online First: 2003/12/23]

8. Lo RC, Kim H. Histopathological evaluation of liver fibrosis and cirrhosis regression. *Clin Mol Hepatol* 2017;23(4):302-07. doi: 10.3350/cmh.2017.0078 [published Online First: 2017/12/29]

9. Lichtinghagen R, Pietsch D, Bantel H, et al. The Enhanced Liver Fibrosis (ELF) score: normal values, influence factors and proposed cut-off values. *J Hepatol* 2013;59(2):236-42. doi: 10.1016/j.jhep.2013.03.016 [published Online First: 2013/03/26]

10. Younossi ZM, Loomba R, Anstee QM, et al. Diagnostic modalities for nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, and associated fibrosis. *Hepatology* 2018;68(1):349-60. doi: 10.1002/hep.29721 [published Online First: 2017/12/10]

11. Schuppan D, Ashfaq-Khan M, Yang AT, et al. Liver fibrosis: Direct antifibrotic agents and targeted therapies. *Matrix Biol* 2018;68-69:435-51. doi: 10.1016/j.matbio.2018.04.006 [published Online First: 2018/04/16]

12. Sun M, Kisseleva T. Reversibility of liver fibrosis. *Clin Res Hepatol Gastroenterol* 2015;39 Suppl 1:S60-3. doi: 10.1016/j.clinre.2015.06.015 [published Online First: 2015/07/25]

13. Giannelli G, Mikulits W, Dooley S, et al. The rationale for targeting TGF-beta in chronic liver diseases. *Eur J Clin Invest* 2016;46(4):349-61. doi: 10.1111/eci.12596 [published Online First: 2016/01/30]

14. Caja L, Dituri F, Mancarella S, et al. TGF-beta and the Tissue Microenvironment: Relevance in Fibrosis and Cancer. *Int J Mol Sci* 2018;19(5) doi: 10.3390/ijms19051294 [published Online First: 2018/04/28]

15. Elpek GO. Cellular and molecular mechanisms in the pathogenesis of liver fibrosis: An update. *World J Gastroenterol* 2014;20(23):7260-76. doi: 10.3748/wjg.v20.i23.7260 [published Online First: 2014/06/27]

16. Gao B, Ahmad MF, Nagy LE, et al. Inflammatory pathways in alcoholic steatohepatitis. *J Hepatol* 2019;70(2):249-59. doi: 10.1016/j.jhep.2018.10.023 [published Online First: 2019/01/20]

17. Seki E, Brenner DA. Recent advancement of molecular mechanisms of liver fibrosis. *J Hepatobiliary Pancreat Sci* 2015;22(7):512-8. doi: 10.1002/jhbp.245 [published Online First: 2015/04/15]

18. Weiskirchen R, Weiskirchen S, Tacke F. Recent advances in understanding liver fibrosis: bridging basic science and individualized treatment concepts. *F1000Res* 2018;7 doi: 10.12688/f1000research.14841.1 [published Online First: 2018/07/14]

19. Karin D, Koyama Y, Brenner D, et al. The characteristics of activated portal fibroblasts/myofibroblasts in liver fibrosis. *Differentiation* 2016;92(3):84-92. doi: 10.1016/j.diff.2016.07.001 [published Online First: 2016/10/26]

20. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol* 2017;66(6):1300-12. doi: 10.1016/j.jhep.2017.02.026 [published Online First: 2017/03/08]

21. Roderfeld M. Matrix metalloproteinase functions in hepatic injury and fibrosis. *Matrix Biol* 2018;68-69:452-62. doi: 10.1016/j.matbio.2017.11.011 [published Online First: 2017/12/10]

22. Rodgarkia-Dara C, Vejda S, Erlach N, et al. The activin axis in liver biology and disease. *Mutat Res* 2006;613(2-3):123-37. doi: 10.1016/j.mrrev.2006.07.002 [published Online First: 2006/09/26]

23. Stanger BZ. Cellular homeostasis and repair in the mammalian liver. *Annu Rev Physiol* 2015;77:179-200. doi: 10.1146/annurev-physiol-021113-170255 [published Online First: 2015/02/11]

24. Tu T, Calabro SR, Lee A, et al. Hepatocytes in liver injury: Victim, bystander, or accomplice in progressive fibrosis? *J Gastroenterol Hepatol* 2015;30(12):1696-704. doi: 10.1111/jgh.13065 [published Online First: 2015/08/05]

25. Conde de la Rosa L, Schoemaker MH, Vrenken TE, et al. Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. *J Hepatol* 2006;44(5):918-29. doi: 10.1016/j.jhep.2005.07.034 [published Online First: 2005/11/29]

26. Sanchez-Rodriguez R, Torres-Mena JE, Quintanar-Jurado V, et al. Ptgr1 expression is regulated by NRF2 in rat hepatocarcinogenesis and promotes cell proliferation and resistance to oxidative stress. *Free Radic Biol Med* 2017;102:87-99. doi: 10.1016/j.freeradbiomed.2016.11.027 [published Online First: 2016/11/22]

27. Koyama Y, Brenner DA. Liver inflammation and fibrosis. *J Clin Invest* 2017;127(1):55-64. doi: 10.1172/JCI88881 [published Online First: 2017/01/04]

28. Cazanave SC, Mott JL, Bronk SF, et al. Death receptor 5 signaling promotes hepatocyte lipoapoptosis. *J Biol Chem* 2011;286(45):39336-48. doi: 10.1074/jbc.M111.280420 [published Online First: 2011/09/24]

29. Feldstein AE, Canbay A, Angulo P, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 2003;125(2):437-43. [published Online First: 2003/08/02]

30. Idrissova L, Malhi H, Werneburg NW, et al. TRAIL receptor deletion in mice suppresses the inflammation of nutrient excess. *J Hepatol* 2015;62(5):1156-63. doi: 10.1016/j.jhep.2014.11.033 [published Online First: 2014/12/03]

31. Guicciardi ME, Malhi H, Mott JL, et al. Apoptosis and necrosis in the liver. *Compr Physiol* 2013;3(2):977-1010. doi: 10.1002/cphy.c120020 [published Online First: 2013/05/31]

32. Rutherford A, Chung RT. Acute liver failure: mechanisms of hepatocyte injury and regeneration. *Semin Liver Dis* 2008;28(2):167-74. doi: 10.1055/s-2008-1073116 [published Online First: 2008/05/03]

33. Gautheron J, Vucur M, Reisinger F, et al. A positive feedback loop between RIP3 and JNK controls non-alcoholic steatohepatitis. *EMBO Mol Med* 2014;6(8):1062-74. doi: 10.15252/emmm.201403856 [published Online First: 2014/06/26]

34. Kubes P, Mehal WZ. Sterile inflammation in the liver. *Gastroenterology* 2012;143(5):1158-72. doi: 10.1053/j.gastro.2012.09.008 [published Online First: 2012/09/18]

35. Szabo G, Csak T. Inflammasomes in liver diseases. *J Hepatol* 2012;57(3):642-54. doi: 10.1016/j.jhep.2012.03.035 [published Online First: 2012/05/29]

36. Zimmermann HW, Tacke F. Modification of chemokine pathways and immune cell infiltration as a novel therapeutic approach in liver inflammation and fibrosis. *Inflamm Allergy Drug Targets* 2011;10(6):509-36. [published Online First: 2011/12/14]

37. Miyazaki S, Takasaki K, Yamamoto M, et al. Liver regeneration and restoration of liver function after partial hepatectomy: the relation of fibrosis of the liver parenchyma. *Hepatogastroenterology* 1999;46(29):2919-24. [published Online First: 1999/11/27]

38. Tamori Y, Deng WM. Compensatory cellular hypertrophy: the other strategy for tissue homeostasis. *Trends Cell Biol* 2014;24(4):230-7. doi: 10.1016/j.tcb.2013.10.005 [published Online First: 2013/11/19]

39. Yanger K, Knigin D, Zong Y, et al. Adult hepatocytes are generated by self-duplication rather than stem cell differentiation. *Cell Stem Cell* 2014;15(3):340-49. doi: 10.1016/j.stem.2014.06.003 [published Online First: 2014/08/19]

40. Fabris L, Spirli C, Cadamuro M, et al. Emerging concepts in biliary repair and fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2017;313(2):G102-G16. doi: 10.1152/ajpgi.00452.2016 [published Online First: 2017/05/21]

41. Sato K, Meng F, Giang T, et al. Mechanisms of cholangiocyte responses to injury. *Biochim Biophys Acta Mol Basis Dis* 2018;1864(4 Pt B):1262-69. doi: 10.1016/j.bbadis.2017.06.017 [published Online First: 2017/06/27]

42. Wang J, Kubes P. A Reservoir of Mature Cavity Macrophages that Can Rapidly Invade Visceral Organs to Affect Tissue Repair. *Cell* 2016;165(3):668-78. doi: 10.1016/j.cell.2016.03.009 [published Online First: 2016/04/12]

43. Klein I, Cornejo JC, Polakos NK, et al. Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. *Blood* 2007;110(12):4077-85. doi: 10.1182/blood-2007-02-073841 [published Online First: 2007/08/11]

44. Ramachandran P, Pellicoro A, Vernon MA, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A* 2012;109(46):E3186-95. doi: 10.1073/pnas.1119964109 [published Online First: 2012/10/27]

45. Beattie L, Sawtell A, Mann J, et al. Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions. *J Hepatol* 2016;65(4):758-68. doi: 10.1016/j.jhep.2016.05.037 [published Online First: 2016/06/06]

46. Sato K, Hall C, Glaser S, et al. Pathogenesis of Kupffer Cells in Cholestatic Liver Injury. *Am J Pathol* 2016;186(9):2238-47. doi: 10.1016/j.ajpath.2016.06.003 [published Online First: 2016/07/28]

47. Wan J, Benkdane M, Teixeira-Clerc F, et al. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. *Hepatology* 2014;59(1):130-42. doi: 10.1002/hep.26607 [published Online First: 2013/07/09]

48. Fischer R, Cariers A, Reinehr R, et al. Caspase 9-dependent killing of hepatic stellate cells by activated Kupffer cells. *Gastroenterology* 2002;123(3):845-61. [published Online First: 2002/08/29]

49. Mossanen JC, Krenkel O, Ergen C, et al. Chemokine (C-C motif) receptor 2-positive monocytes aggravate the early phase of acetaminophen-induced acute liver injury. *Hepatology* 2016;64(5):1667-82. doi: 10.1002/hep.28682 [published Online First: 2016/10/22]

50. Martinez-Nunez RT, Louafi F, Sanchez-Elsner T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem* 2011;286(3):1786-94. doi: 10.1074/jbc.M110.169367 [published Online First: 2010/11/26]

51. Taura K, Iwaisako K, Hatano E, et al. Controversies over the Epithelial-to-Mesenchymal Transition in Liver Fibrosis. *J Clin Med* 2016;5(1) doi: 10.3390/jcm5010009 [published Online First: 2016/01/20]

52. Iwaisako K, Jiang C, Zhang M, et al. Origin of myofibroblasts in the fibrotic liver in mice. *Proc Natl Acad Sci U S A* 2014;111(32):E3297-305. doi: 10.1073/pnas.1400062111 [published Online First: 2014/07/31]

53. Friedman SL, Roll FJ, Boyles J, et al. Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. *Proc Natl Acad Sci U S A* 1985;82(24):8681-5. doi: 10.1073/pnas.82.24.8681 [published Online First: 1985/12/01]

54. Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis* 2001;21(3):311-35. doi: 10.1055/s-2001-17550 [published Online First: 2001/10/05]

55. Soon RK, Jr., Yee HF, Jr. Stellate cell contraction: role, regulation, and potential therapeutic target. *Clin Liver Dis* 2008;12(4):791-803, viii. doi: 10.1016/j.cld.2008.07.004 [published Online First: 2008/11/06]

56. Mallat A, Lotersztajn S. Cellular mechanisms of tissue fibrosis. 5. Novel insights into liver fibrosis. *Am J Physiol Cell Physiol* 2013;305(8):C789-99. doi: 10.1152/ajpcell.00230.2013 [published Online First: 2013/08/02]

57. Zeisberg M, Yang C, Martino M, et al. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem* 2007;282(32):23337-47. doi: 10.1074/jbc.M700194200 [published Online First: 2007/06/15]

58. Scholten D, Osterreicher CH, Scholten A, et al. Genetic labeling does not detect epithelial-to-mesenchymal transition of cholangiocytes in liver fibrosis in mice. *Gastroenterology* 2010;139(3):987-98. doi: 10.1053/j.gastro.2010.05.005 [published Online First: 2010/06/16]

59. Taura K, Miura K, Iwaisako K, et al. Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. *Hepatology* 2010;51(3):1027-36. doi: 10.1002/hep.23368 [published Online First: 2010/01/07]

60. Iredale JP, Thompson A, Henderson NC. Extracellular matrix degradation in liver fibrosis: Biochemistry and regulation. *Biochim Biophys Acta* 2013;1832(7):876-83. doi: 10.1016/j.bbadis.2012.11.002 [published Online First: 2012/11/15]

61. Lotersztajn S, Julien B, Teixeira-Clerc F, et al. Hepatic fibrosis: molecular mechanisms and drug targets. *Annu Rev Pharmacol Toxicol* 2005;45:605-28. doi: 10.1146/annurev.pharmtox.45.120403.095906 [published Online First: 2004/10/09]

62. Novo E, Cannito S, Paternostro C, et al. Cellular and molecular mechanisms in liver fibrogenesis. *Arch Biochem Biophys* 2014;548:20-37. doi: 10.1016/j.abb.2014.02.015 [published Online First: 2014/03/19]

63. Herrera B, Addante A, Sanchez A. BMP Signalling at the Crossroad of Liver Fibrosis and Regeneration. *Int J Mol Sci* 2017;19(1) doi: 10.3390/ijms19010039 [published Online First: 2018/01/04]

64. Walton KL, Johnson KE, Harrison CA. Targeting TGF-beta Mediated SMAD Signaling for the Prevention of Fibrosis. *Front Pharmacol* 2017;8:461. doi: 10.3389/fphar.2017.00461 [published Online First: 2017/08/05]

65. Weiskirchen R, Meurer SK. BMP-7 counteracting TGF-beta1 activities in organ fibrosis. *Front Biosci (Landmark Ed)* 2013;18:1407-34. [published Online First: 2013/06/12]

66. Horbelt D, Denkis A, Knaus P. A portrait of Transforming Growth Factor beta superfamily signalling: Background matters. *Int J Biochem Cell Biol* 2012;44(3):469-74. doi: 10.1016/j.biocel.2011.12.013 [published Online First: 2012/01/10]

67. Wrana JL, Attisano L, Carcamo J, et al. TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 1992;71(6):1003-14. [published Online First: 1992/12/11]

68. Yamashita H, ten Dijke P, Franzen P, et al. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. *J Biol Chem* 1994;269(31):20172-8. [published Online First: 1994/08/05]

69. Lee SJ, Reed LA, Davies MV, et al. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proc Natl Acad Sci U S A* 2005;102(50):18117-22. doi: 10.1073/pnas.0505996102 [published Online First: 2005/12/07]

70. de Caestecker M. The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev* 2004;15(1):1-11. [published Online First: 2004/01/30]

71. Greenwald J, Groppe J, Gray P, et al. The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. *Mol Cell* 2003;11(3):605-17. [published Online First: 2003/04/02]

72. Nohe A, Keating E, Knaus P, et al. Signal transduction of bone morphogenetic protein receptors. *Cell Signal* 2004;16(3):291-9. [published Online First: 2003/12/23]

73. Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. *J Biochem* 2010;147(1):35-51. doi: 10.1093/jb/mvp148 [published Online First: 2009/09/19]

74. Yan X, Lin Z, Chen F, et al. Human BAMBI cooperates with Smad7 to inhibit transforming growth factor-beta signaling. *J Biol Chem* 2009;284(44):30097-104. doi: 10.1074/jbc.M109.049304 [published Online First: 2009/09/18]

75. Xu X, Browning VL, Odorico JS. Activin, BMP and FGF pathways cooperate to promote endoderm and pancreatic lineage cell differentiation from human embryonic stem cells. *Mech Dev* 2011;128(7-10):412-27. doi: 10.1016/j.mod.2011.08.001 [published Online First: 2011/08/23]

76. Guo S, Mao X, He F, et al. Activin A supplement in the hESCs culture enhances the endoderm differentiation efficiency. *Cell Biol Int* 2014;38(7):849-56. doi: 10.1002/cbin.10274 [published Online First: 2014/03/08]

77. Dutta DJ, Zameer A, Mariani JN, et al. Combinatorial actions of Tgfbeta and Activin ligands promote oligodendrocyte development and CNS myelination. *Development* 2014;141(12):2414-28. doi: 10.1242/dev.106492 [published Online First: 2014/06/12]

78. Xu F, Liu C, Zhou D, et al. TGF-beta/SMAD Pathway and Its Regulation in Hepatic Fibrosis. *J Histochem Cytochem* 2016;64(3):157-67. doi: 10.1369/0022155415627681 [published Online First: 2016/01/10]

79. Sakai K, Jawaid S, Sasaki T, et al. Transforming growth factor-beta-independent role of connective tissue growth factor in the development of liver fibrosis. *Am J Pathol* 2014;184(10):2611-7. doi: 10.1016/j.ajpath.2014.06.009 [published Online First: 2014/08/12]

80. Fabregat I, Moreno-Caceres J, Sanchez A, et al. TGF-beta signalling and liver disease. *FEBS J* 2016;283(12):2219-32. doi: 10.1111/febs.13665 [published Online First: 2016/01/26]

81. Occleston NL, Laverty HG, O'Kane S, et al. Prevention and reduction of scarring in the skin by Transforming Growth Factor beta 3 (TGFbeta3): from laboratory discovery to clinical pharmaceutical. *J Biomater Sci Polym Ed* 2008;19(8):1047-63. doi: 10.1163/156856208784909345 [published Online First: 2008/07/23]

82. Ferguson MW, Duncan J, Bond J, et al. Prophylactic administration of avotermin for improvement of skin scarring: three double-blind, placebo-controlled, phase I/II studies. *Lancet* 2009;373(9671):1264-74. doi: 10.1016/S0140-6736(09)60322-6 [published Online First: 2009/04/14]

83. Zeisberg M, Hanai J, Sugimoto H, et al. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003;9(7):964-8. doi: 10.1038/nm888 [published Online First: 2003/06/17]

84. Namwanje M, Brown CW. Activins and Inhibins: Roles in Development, Physiology, and Disease. *Cold Spring Harb Perspect Biol* 2016;8(7) doi: 10.1101/cshperspect.a021881 [published Online First: 2016/06/23]

85. Risbridger GP, Schmitt JF, Robertson DM. Activins and inhibins in endocrine and other tumors. *Endocr Rev* 2001;22(6):836-58. doi: 10.1210/edrv.22.6.0450 [published Online First: 2001/12/12]

86. Matzuk MM, Kumar TR, Vassalli A, et al. Functional analysis of activins during mammalian development. *Nature* 1995;374(6520):354-6. doi: 10.1038/374354a0 [published Online First: 1995/03/23]

87. Schrewe H, Gendron-Maguire M, Harbison ML, et al. Mice homozygous for a null mutation of activin beta B are viable and fertile. *Mech Dev* 1994;47(1):43-51. [published Online First: 1994/07/01]

88. Vassalli A, Matzuk MM, Gardner HA, et al. Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev* 1994;8(4):414-27. [published Online First: 1994/02/15]

89. Lau AL, Kumar TR, Nishimori K, et al. Activin betaC and betaE genes are not essential for mouse liver growth, differentiation, and regeneration. *Mol Cell Biol* 2000;20(16):6127-37. [published Online First: 2000/07/27]

90. Deli A, Kreidl E, Santifaller S, et al. Activins and activin antagonists in hepatocellular carcinoma. *World J Gastroenterol* 2008;14(11):1699-709. [published Online First: 2008/03/20]

91. Mellor SL, Ball EM, O'Connor AE, et al. Activin betaC-subunit heterodimers provide a new mechanism of regulating activin levels in the prostate. *Endocrinology* 2003;144(10):4410-9. doi: 10.1210/en.2003-0225 [published Online First: 2003/09/10]

92. Wijayarathna R, de Kretser DM. Activins in reproductive biology and beyond. *Hum Reprod Update* 2016;22(3):342-57. doi: 10.1093/humupd/dmv058 [published Online First: 2016/02/18]

93. Tsuchida K, Nakatani M, Yamakawa N, et al. Activin isoforms signal through type I receptor serine/threonine kinase ALK7. *Mol Cell Endocrinol* 2004;220(1-2):59-65. doi: 10.1016/j.mce.2004.03.009 [published Online First: 2004/06/16]

94. Gressner OA. Intracrine signaling mechanisms of activin A and TGF-beta. *Vitam Horm* 2011;85:59-77. doi: 10.1016/B978-0-12-385961-7.00004-4 [published Online First: 2011/03/01]

95. Hedger MP, Winnall WR, Phillips DJ, et al. The regulation and functions of activin and follistatin in inflammation and immunity. *Vitam Horm* 2011;85:255-97. doi: 10.1016/B978-0-12-385961-7.00013-5 [published Online First: 2011/03/01]

96. Bao YL, Tsuchida K, Liu B, et al. Synergistic activity of activin A and basic fibroblast growth factor on tyrosine hydroxylase expression through Smad3 and ERK1/ERK2 MAPK signaling pathways. *J Endocrinol* 2005;184(3):493-504. doi: 10.1677/joe.1.05978 [published Online First: 2005/03/08]

97. de Guise C, Lacerte A, Rafiei S, et al. Activin inhibits the human Pit-1 gene promoter through the p38 kinase pathway in a Smad-independent manner. *Endocrinology* 2006;147(9):4351-62. doi: 10.1210/en.2006-0444 [published Online First: 2006/06/03]

98. Vallee A, Lecarpentier Y, Guillevin R, et al. Interactions between TGF-beta1, canonical WNT/beta-catenin pathway and PPAR gamma in radiation-induced fibrosis. *Oncotarget* 2017;8(52):90579-604. doi: 10.18632/oncotarget.21234 [published Online First: 2017/11/23]

99. De Bleser PJ, Niki T, Xu G, et al. Localization and cellular sources of activins in normal and fibrotic rat liver. *Hepatology* 1997;26(4):905-12. doi: 10.1002/hep.510260416 [published Online First: 1997/10/27]

100. Schwall RH, Robbins K, Jardieu P, et al. Activin induces cell death in hepatocytes in vivo and in vitro. *Hepatology* 1993;18(2):347-56. [published Online First: 1993/08/01]

101. Yasuda H, Mine T, Shibata H, et al. Activin A: an autocrine inhibitor of initiation of DNA synthesis in rat hepatocytes. *J Clin Invest* 1993;92(3):1491-6. doi: 10.1172/JCI116727 [published Online First: 1993/09/01]

102. Date M, Matsuzaki K, Matsushita M, et al. Differential regulation of activin A for hepatocyte growth and fibronectin synthesis in rat liver injury. *J Hepatol* 2000;32(2):251-60. [published Online First: 2000/03/09]

103. Werner S, Alzheimer C. Roles of activin in tissue repair, fibrosis, and inflammatory disease. *Cytokine Growth Factor Rev* 2006;17(3):157-71. doi: 10.1016/j.cytogfr.2006.01.001 [published Online First: 2006/02/17]

104. Zhang HJ, Tai GX, Zhou J, et al. Regulation of activin receptor-interacting protein 2 expression in mouse hepatoma Hepa1-6 cells and its relationship with collagen type IV. *World J Gastroenterol* 2007;13(41):5501-5. [published Online First: 2007/10/02]

105. Hully JR, Chang L, Schwall RH, et al. Induction of apoptosis in the murine liver with recombinant human activin A. *Hepatology* 1994;20(4 Pt 1):854-62. [published Online First: 1994/10/01]

106. Wada W, Kuwano H, Hasegawa Y, et al. The dependence of transforming growth factor-beta-induced collagen production on autocrine factor activin A in hepatic stellate cells. *Endocrinology* 2004;145(6):2753-9. doi: 10.1210/en.2003-1663 [published Online First: 2004/03/06]

107. Kiagiadaki F, Kampa M, Voumvouraki A, et al. Activin-A causes Hepatic stellate cell activation via the induction of TNFalpha and TGFbeta in Kupffer cells. *Biochim Biophys Acta Mol Basis Dis* 2018;1864(3):891-99. doi: 10.1016/j.bbadis.2017.12.031 [published Online First: 2017/12/31]

108. Wang DH, Wang YN, Ge JY, et al. Role of activin A in carbon tetrachloride-induced acute liver injury. *World J Gastroenterol* 2013;19(24):3802-9. doi: 10.3748/wjg.v19.i24.3802 [published Online First: 2013/07/11]

109. Kobayashi T, Niimi S, Hashimoto O, et al. Expression of inhibin betaA, betaB and follistatin mRNAs in the carbon tetrachloride induced rat liver regeneration model. *Biol Pharm Bull* 2000;23(6):755-7. [published Online First: 2000/06/23]

110. 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(3):285-95. doi: 10.1016/j.cytogfr.2013.03.003 [published Online First: 2013/04/02]

111. 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(2):702-11. doi: 10.1210/endo.135.2.8033818 [published Online First: 1994/08/01]

112. Canali S, Core AB, Zumbrennen-Bullough KB, 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(3):1146-62. doi: 10.1210/en.2015-1747 [published Online First: 2016/01/07]

113. Pavlov CS, Casazza G, Nikolova D, et al. Transient elastography for diagnosis of stages of hepatic fibrosis and cirrhosis in people with alcoholic liver disease. *Cochrane Database Syst Rev* 2015;1:CD010542. doi: 10.1002/14651858.CD010542.pub2 [published Online First: 2015/01/23]

114. Lee H, Jeong H, Park S, et al. Fusion protein of retinol-binding protein and albumin domain III reduces liver fibrosis. *EMBO Mol Med* 2015;7(6):819-30. doi: 10.15252/emmm.201404527 [published Online First: 2015/04/13]

115. Knockaert L, Berson A, Ribault C, et al. Carbon tetrachloride-mediated lipid peroxidation induces early mitochondrial alterations in mouse liver. *Lab Invest* 2012;92(3):396-410. doi: 10.1038/labinvest.2011.193 [published Online First: 2011/12/14]

116. Scholten D, Trebicka J, Liedtke C, et al. The carbon tetrachloride model in mice. *Lab Anim* 2015;49(1 Suppl):4-11. doi: 10.1177/0023677215571192 [published Online First: 2015/04/04]

117. Yanguas SC, Cogliati B, Willebrords J, et al. Experimental models of liver fibrosis. *Arch Toxicol* 2016;90(5):1025-48. doi: 10.1007/s00204-015-1543-4 [published Online First: 2015/06/07]

118. Tag CG, Sauer-Lehnen S, Weiskirchen S, et al. Bile duct ligation in mice: induction of inflammatory liver injury and fibrosis by obstructive cholestasis. *J Vis Exp* 2015(96) doi: 10.3791/52438 [published Online First: 2015/03/06]

119. Lamas-Paz A, Hao F, Nelson LJ, et al. Alcoholic liver disease: Utility of animal models. *World J Gastroenterol* 2018;24(45):5063-75. doi: 10.3748/wjg.v24.i45.5063 [published Online First: 2018/12/21]

120. Nagy LE, Ding WX, Cresci G, et al. Linking Pathogenic Mechanisms of Alcoholic Liver Disease With Clinical Phenotypes. *Gastroenterology* 2016;150(8):1756-68. doi: 10.1053/j.gastro.2016.02.035 [published Online First: 2016/02/28]

121. Crabb DW, Matsumoto M, Chang D, et al. Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. *Proc Nutr Soc* 2004;63(1):49-63. [published Online First: 2004/04/22]

122. Zelickson BR, Benavides GA, Johnson MS, et al. Nitric oxide and hypoxia exacerbate alcohol-induced mitochondrial dysfunction in hepatocytes. *Biochim Biophys Acta* 2011;1807(12):1573-82. doi: 10.1016/j.bbabio.2011.09.011 [published Online First: 2011/10/06]

123. You M, Liang X, Ajmo JM, et al. Involvement of mammalian sirtuin 1 in the action of ethanol in the liver. *Am J Physiol Gastrointest Liver Physiol* 2008;294(4):G892-8. doi: 10.1152/ajpgi.00575.2007 [published Online First: 2008/02/02]

124. Wang HJ, Gao B, Zakhari S, et al. Inflammation in alcoholic liver disease. *Annu Rev Nutr* 2012;32:343-68. doi: 10.1146/annurev-nutr-072610-145138 [published Online First: 2012/04/25]

125. Akie TE, Cooper MP. Determination of Fatty Acid Oxidation and Lipogenesis in Mouse Primary Hepatocytes. *J Vis Exp* 2015(102):e52982. doi: 10.3791/52982 [published Online First: 2015/09/19]

126. Higashi T, Friedman SL, Hoshida Y. Hepatic stellate cells as key target in liver fibrosis. *Adv Drug Deliv Rev* 2017;121:27-42. doi: 10.1016/j.addr.2017.05.007 [published Online First: 2017/05/17]

127. Bitto N, Liguori E, La Mura V. Coagulation, Microenvironment and Liver Fibrosis. *Cells* 2018;7(8) doi: 10.3390/cells7080085 [published Online First: 2018/07/26]

128. Sanyal AJ. Novel therapeutic targets for steatohepatitis. *Clin Res Hepatol Gastroenterol* 2015;39 Suppl 1:S46-50. doi: 10.1016/j.clinre.2015.05.012 [published Online First: 2015/07/15]

129. Friedman SL. Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol* 2010;7(8):425-36. doi: 10.1038/nrgastro.2010.97 [published Online First: 2010/06/30]

130. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008;214(2):199-210. doi: 10.1002/path.2277 [published Online First: 2007/12/29]

131. Yaden BC, Wang YX, Wilson JM, et al. Inhibition of activin A ameliorates skeletal muscle injury and rescues contractile properties by inducing efficient remodeling in female mice. *Am J Pathol* 2014;184(4):1152-66. doi: 10.1016/j.ajpath.2013.12.029 [published Online First: 2014/03/25]

132. Noble PW, Albera C, Bradford WZ, et al. Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials. *Lancet* 2011;377(9779):1760-9. doi: 10.1016/S0140-6736(11)60405-4 [published Online First: 2011/05/17]

133. Chang B, Xu MJ, Zhou Z, et al. Short- or long-term high-fat diet feeding plus acute ethanol binge synergistically induce acute liver injury in mice: an important role for CXCL1. *Hepatology* 2015;62(4):1070-85. doi: 10.1002/hep.27921 [published Online First: 2015/06/03]

134. Vale W, Rivier J, Vaughan J, et al. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* 1986;321(6072):776-9. doi: 10.1038/321776a0 [published Online First: 1986/06/19]

135. Nakamura T, Asashima M, Eto Y, et al. Isolation and characterization of native activin B. *J Biol Chem* 1992;267(23):16385-9. [published Online First: 1992/08/15]

136. Tomaszewski J, Joseph A, Archambeault D, et al. Essential roles of inhibin beta A in mouse epididymal coiling. *Proc Natl Acad Sci U S A* 2007;104(27):11322-7. doi: 10.1073/pnas.0703445104 [published Online First: 2007/06/27]

137. Mithraprabhu S, Mendis S, Meachem SJ, et al. Activin bioactivity affects germ cell differentiation in the postnatal mouse testis in vivo. *Biol Reprod* 2010;82(5):980-90. doi: 10.1095/biolreprod.109.079855 [published Online First: 2010/02/05]

138. Sokol S, Christian JL, Moon RT, et al. Injected Wnt RNA induces a complete body axis in Xenopus embryos. *Cell* 1991;67(4):741-52. [published Online First: 1991/11/15]

139. Feijen A, Goumans MJ, van den Eijnden-van Raaij AJ. Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. *Development* 1994;120(12):3621-37. [published Online First: 1994/12/01]

140. Kanamori Y, Sugiyama M, Hashimoto O, et al. Regulation of hepcidin expression by inflammation-induced activin B. *Sci Rep* 2016;6:38702. doi: 10.1038/srep38702 [published Online First: 2016/12/07]

141. Besson-Fournier C, Gineste A, Latour C, et al. Hepcidin upregulation by inflammation is independent of Smad1/5/8 signaling by activin B. *Blood* 2017;129(4):533-36. doi: 10.1182/blood-2016-10-748541 [published Online First: 2016/12/03]

142. Carracedo S, Lu N, Popova SN, et al. The fibroblast integrin alpha11beta1 is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation. *J Biol Chem* 2010;285(14):10434-45. doi: 10.1074/jbc.M109.078766 [published Online First: 2010/02/05]

143. Ohga E, Matsuse T, Teramoto S, et al. Effects of activin A on proliferation and differentiation of human lung fibroblasts. *Biochem Biophys Res Commun* 1996;228(2):391-6. doi: 10.1006/bbrc.1996.1672 [published Online First: 1996/11/12]

144. Yamashita S, Maeshima A, Kojima I, et al. Activin A is a potent activator of renal interstitial fibroblasts. *J Am Soc Nephrol* 2004;15(1):91-101. doi: 10.1097/01.asn.0000103225.68136.e6 [published Online First: 2003/12/25]

145. Ohnishi N, Miyata T, Ohnishi H, et al. Activin A is an autocrine activator of rat pancreatic stellate cells: potential therapeutic role of follistatin for pancreatic fibrosis. *Gut* 2003;52(10):1487-93. doi: 10.1136/gut.52.10.1487 [published Online First: 2003/09/13]

146. Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology* 2000;32(3):477-81. doi: 10.1053/jhep.2000.16602 [published Online First: 2000/08/29]

147. Le TA, Chen J, Changchien C, et al. Effect of colesevelam on liver fat quantified by magnetic resonance in nonalcoholic steatohepatitis: a randomized controlled trial. *Hepatology* 2012;56(3):922-32. doi: 10.1002/hep.25731 [published Online First: 2012/03/21]

148. Thiele M, Kjaergaard M, Thielsen P, et al. Contemporary use of elastography in liver fibrosis and portal hypertension. *Clin Physiol Funct Imaging* 2017;37(3):235-42. doi: 10.1111/cpf.12297 [published Online First: 2015/10/16]

149. Gressner OA, Weiskirchen R, Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. *Comp Hepatol* 2007;6:7. doi: 10.1186/1476-5926-6-7 [published Online First: 2007/08/01]

150. Gressner OA, Weiskirchen R, Gressner AM. Biomarkers of liver fibrosis: clinical translation of molecular pathogenesis or based on liver-dependent malfunction tests. *Clin Chim Acta* 2007;381(2):107-13. doi: 10.1016/j.cca.2007.02.038 [published Online First: 2007/04/03]

151. Adams LA, Angulo P. Recent concepts in non-alcoholic fatty liver disease. *Diabet Med* 2005;22(9):1129-33. doi: 10.1111/j.1464-5491.2005.01748.x [published Online First: 2005/08/20]

152. Adams LA, Sanderson S, Lindor KD, et al. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005;42(1):132-8. doi: 10.1016/j.jhep.2004.09.012 [published Online First: 2005/01/05]

153. Moreno C, Mueller S, Szabo G. Non-invasive diagnosis and biomarkers in alcohol-related liver disease. *J Hepatol* 2019;70(2):273-83. doi: 10.1016/j.jhep.2018.11.025 [published Online First: 2019/01/20]

154. Afdhal NH. Biopsy or biomarkers: is there a gold standard for diagnosis of liver fibrosis? *Clin Chem* 2004;50(8):1299-300. doi: 10.1373/clinchem.2004.035899 [published Online First: 2004/07/28]

155. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115(2):209-18. doi: 10.1172/JCI24282 [published Online First: 2005/02/04]

156. Madrigal-Matute J, Fernandez-Laso V, Sastre C, et al. TWEAK/Fn14 interaction promotes oxidative stress through NADPH oxidase activation in macrophages. *Cardiovasc Res* 2015;108(1):139-47. doi: 10.1093/cvr/cvv204 [published Online First: 2015/08/01]

157. Viebahn CS, Benseler V, Holz LE, et al. Invading macrophages play a major role in the liver progenitor cell response to chronic liver injury. *J Hepatol* 2010;53(3):500-7. doi: 10.1016/j.jhep.2010.04.010 [published Online First: 2010/06/22]

158. Dwyer BJ, Olynyk JK, Ramm GA, et al. TWEAK and LTbeta Signaling during Chronic Liver Disease. *Front Immunol* 2014;5:39. doi: 10.3389/fimmu.2014.00039 [published Online First: 2014/03/05]

159. Tirnitz-Parker JE, Olynyk JK, Ramm GA. Role of TWEAK in coregulating liver progenitor cell and fibrogenic responses. *Hepatology* 2014;59(3):1198-201. doi: 10.1002/hep.26701 [published Online First: 2013/09/17]

160. Iwakiri Y. Nitric oxide in liver fibrosis: The role of inducible nitric oxide synthase. *Clin Mol Hepatol* 2015;21(4):319-25. doi: 10.3350/cmh.2015.21.4.319 [published Online First: 2016/01/16]

161. Anavi S, Eisenberg-Bord M, Hahn-Obercyger M, et al. The role of iNOS in cholesterol-induced liver fibrosis. *Lab Invest* 2015;95(8):914-24. doi: 10.1038/labinvest.2015.67 [published Online First: 2015/06/23]

162. Aram G, Potter JJ, Liu X, et al. Lack of inducible nitric oxide synthase leads to increased hepatic apoptosis and decreased fibrosis in mice after chronic carbon tetrachloride administration. *Hepatology* 2008;47(6):2051-8. doi: 10.1002/hep.22278 [published Online First: 2008/05/29]

163. Attie KM, Borgstein NG, Yang Y, et al. A single ascending-dose study of muscle regulator ACE-031 in healthy volunteers. *Muscle Nerve* 2013;47(3):416-23. doi: 10.1002/mus.23539 [published Online First: 2012/11/22]

164. Garber K. No longer going to waste. *Nat Biotechnol* 2016;34(5):458-61. doi: 10.1038/nbt.3557 [published Online First: 2016/05/08]

165. Page A, Mann DA. Epigenetic regulation of liver fibrosis. *Clin Res Hepatol Gastroenterol* 2015;39 Suppl 1:S64-8. doi: 10.1016/j.clinre.2015.05.013 [published Online First: 2015/07/21]

166. Yang JJ, Tao H, Deng ZY, et al. Non-coding RNA-mediated epigenetic regulation of liver fibrosis. *Metabolism* 2015;64(11):1386-94. doi: 10.1016/j.metabol.2015.08.004 [published Online First: 2015/09/13]

167. El Taghdouini A, van Grunsven LA. Epigenetic regulation of hepatic stellate cell activation and liver fibrosis. *Expert Rev Gastroenterol Hepatol* 2016;10(12):1397-408. doi: 10.1080/17474124.2016.1251309 [published Online First: 2016/11/03]

168. Lilja-Maula L, Syrja P, Laurila HP, et al. Upregulation of alveolar levels of activin B, but not activin A, in lungs of west highland white terriers with idiopathic pulmonary fibrosis and diffuse alveolar damage. *J Comp Pathol* 2015;152(2-3):192-200. doi: 10.1016/j.jcpa.2014.11.006 [published Online First: 2015/01/04]

169. Myllarniemi M, Tikkanen J, Hulmi JJ, et al. Upregulation of activin-B and follistatin in pulmonary fibrosis - a translational study using human biopsies and a specific inhibitor in mouse fibrosis models. *BMC Pulm Med* 2014;14:170. doi: 10.1186/1471-2466-14-170 [published Online First: 2014/11/02]

170. Poncy A, Antoniou A, Cordi S, et al. Transcription factors SOX4 and SOX9 cooperatively control development of bile ducts. *Dev Biol* 2015;404(2):136-48. doi: 10.1016/j.ydbio.2015.05.012 [published Online First: 2015/06/03]

VITA

Education

Purdue University, Indianapolis	PhD Biology	May 2014–Aug 2019
Kansas State University, Manhattan	M.S. Molecular Biology	Aug 2000–Sept 2002
China Medical University, Shenyang	M.S. Internal Medicine	Aug 1995–Aug 1998
China Medical University, Shenyang	B.S. Clinical Medicine	Aug 1986–Aug 1991

Research experience

Purdue University, Indianapolis PhD Biology May 2014–Aug 2019

- Hypothesizing that activin B participates in modulating the initiation and progression of liver fibrogenesis.
- Investigating activin B mRNA and protein expression in the liver and blood of patients with liver fibrosis and in multiple liver fibrosis mouse models compared with the related ligand activin A.
- Delineating the detailed molecular mechanisms by which activin B directly stimulates different liver cells, including hepatocytes, macrophages, and HSCs, along with the well-known pro-fibrotic regulator TGFβ1 in liver cells.
- Determining the effect of the inhibition of activin B and A individually or in combination by using specific monoclonal antibodies on liver function and fibrosis in CCl_4- and BDL-induced liver fibrosis models.

Publications

1. Yaden BC, **Wang Y**, Wilson JM, Culver AE, Milner A, Datta-Mannan A, Shetler P, Croy JE, Dai G, Krishnan V. Inhibition of activin A ameliorates skeletal muscle injury and rescues contractile properties by inducing efficient remodeling in female mice. *Am J Pathol*. 2014; 184(4):1152-66

2. Yaden BC, Croy JE, **Wang Y**, Wilson JM, Datta-Mannan A, Shetler P, Milner A, Bryant HU, Andrews J, Dai G, Krishnan V. Follistatin: a novel therapeutic for the improvement of muscle regeneration. *J Pharmacol Exp Ther.* 2014; 349(2):355-71.

3. Istrate MA, Spicer TP, **Wang Y**, Bernard JA, Helvering LM, Bocchinfuso WP, Richardson TI, Zink R, Kumar N, Montrose-Rafizadeh C, Dodge J, Hodder P, Griffin PR. Development of an HTS-compatible assay for discovery of RORα modulators using AlphaScreen® technology. *J Biomol Screen.* 2011; 16(2):183-91.

4. **Wang Y**, Zhang M, Middleton FA, Horton JA, Pritchard M, Spadaro JA, Farnum CE, Damron TA. Connective tissue growth factor and insulin-like growth factor 2 show upregulation in early growth plate radio-recovery response following irradiation. *Cells Tissues Organs*. 2007; 186(3):192-203.

5. Zhang M, **Wang Y**, Middleton FA, Horton JA, Farnum CE, Damron TA. Growth plate zonal microarray analysis shows upregulation of extracellular matrix genes and downregulation of metalloproteinases and cathepsins following irradiation. *Calcif Tissue Int*. 2007 Jul; 81(1):26-38.

6. **Wang Y**, Lee Reichel, Middleton F, Horton J, Damron T. Microarray analysis of proliferative and hypertrophic zone of growth plate identifies differentiation markers and signal pathways. *Bone*. 2004 Dec; 35(6):1273-93.

7. Lu N, **Wang Y**, Blecha F, Fels RJ, Hoch HP, Kenney MJ. Central interleukin–1 beta antibody increases renal and splenic sympathetic nerve discharge. *Am J Physiol Heart Circ Physiol.* 2003, Jan 16.

8. Kenney MJ, Weiss ML, Mendes T, **Wang Y**, Fels RJ. Role of the paraventricular nucleus in regulation of sympathetic nerve frequency components. *Am J Physiol Heart Circ Physiol.* 2003, Jan 9.

9. Kenney MJ, Blecha F, **Wang Y**, McMurphy R, Fels RJ. Sympatho-excitation to intravenous interleukin-1β is dependent on forebrain neural circuits. *Am J Physiol Heart Circ Physiol.* 2000; 283(2):H501-H505.

10. Kenney MJ, Weiss ML, Patel KP, **Wang Y**, Fels RJ. Paraventricular nucleus bicuculline alters frequency components of sympathetic nerve discharge burst. *Am J Physiol Heart Circ Physiol.* 2001; 281:H1233-H1241.

11. **Wang Y**, Liu Y, Jia H. Hyperbaric oxygen comparing with normo-baric oxygen in the treatment of carbon monoxide poisoning rat. *J of China Medical University.* 2000; 29(suppl):41-2.

Honors received

- Outstanding Graduating Student Award, July 1991, China Medical University
- Outstanding Young Doctor Award, November 1994, China Medical University
- Jane Westfall Travel Award, April 2002, Kansas State University

Grant received

The role of connective tissue growth factor in osteosarcoma development, 2005 to 2006, Children miracle network

Oral and poster presentations

Experimental biology conference	(1 poster 2001)
Orthopedic Research Society annual meeting	(2 posters 2004)
American Society for Bone and Mineral Research annual meeting	(1 poster 2005)
American Society for Bone and Mineral Research annual meeting	(1 oral and 2 posters 2006)
Neuroscience meeting	(1 poster 2013)
Keystone Symposia	(1 poster 2015)
Keystone Symposia	(1 poster 2016)
Keystone Symposia	(1 poster 2017)

Leadership/Outreach

Project SEED	June 2014–Aug 2014
Project SEED	June 2015–Aug 2015
Project SEED	June 2016–Aug 2016
Project SEED	June 2018–Aug 2018