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(Direktor: Prof. Dr. med. Alexander Marx)

Activin drives liver progenitor cells to take over coagulation function through upregulating HNF4α in acute-on-chronic liver failure

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Tao Lin

aus

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Dekan: Prof. Dr. med. Sergij Goerdt

Referent: Prof. Dr. med. Alexander Marx

CONTENTS

LIST OF ABBREVIATIONS

°C, grad Celsius

µ, Micro (10-6)

AC, activin A

ACLF, acute-on-chronic liver failure

AD, acute decompensation

ALF, acute liver failure

ALK4, activin receptor like kinase 4 (also known as Activin receptor type-1B)

ALK5, aurora-like kinase

ALK7, activin A receptor type 1C

ALT, alanine transaminase

APAP, acetaminophen

APS, ammoniumpersulfate

ARE, activin responsive element

AST, aspartate transaminase

ATP, adenosine triphosphate

BMP, bone morphogenetic protein

BSA, bovine serum albumin

CBP, cAMP response element binding protein- binding protein

CDE, choline-deficient ethionine-supplemented

CDH1, cadherin-1

CDKI, cyclin-dependent kinase inhibitor

ChIP, chromatin immunoprecipitation

CLIF, chronic Liver Failure

Co-IP, co-immunoprecipitation

CON, control

CYP2D6, cytochrome P450 2D6

DAB, 3,3'-diaminobenzidine

DC, decompensated cirrhosis

DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine

DMEM, dulbecco´s modified eagle medium

DMSO, dimethylsulfoxide

DNA, deoxyribonucleic acid

Dok-1, docking protein 1

DP, ductular plate

DR, ductular reaction

EDTA, ethylene diaminetetraacetic acid

EGF, epidermal growth factor

EpiSCs, epiblast stem cells

F2, coagulation factor II

F5, coagulation factor V

F7, coagulation factor VII

F8, coagulation factor VIII

F9, coagulation factor IX

F10, coagulation factor X

F11, coagulation factor XI

F12, coagulation factor XII

FBS, fetal bovine serum

FOXH1, forkhead box protein H1

FOXO1, forkhead Box O1

FSH, follicle stimulating hormone

FST, follistatin

GAL4, galactose-responsive transcription factor GAL4

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GDF, growth differentiation factor

GSC, goosecoid

H3K4me3, trimethylation of Histone H3 at Lysine 4

H3K27me3, trimethylation of Histone H3 at Lysine 27

H3K27ac, acetylation of Histone H3 at Lysine 27

HBV, hepatitis-B-virus

HDACs, histone deacetylase

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hESCs, human embryonic stem cells

HGF, hepatocyte growth factor

hiPSCs, human pluripotent stem cells

HNF1α, hepatocyte nuclear factor 1 homeobox A

HNF4α, hepatocyte nuclear factor 4α

HRP, horseradish peroxidase

IHC, immunohistochemistry

INR, international normalized ratio

KEGG, Kyoto Encyclopedia of Genes and Genomes

kg, kilo gram

LB-Medium, lysogeny broth medium

LDTF, lineage transcription factor

LPC, liver progenitor cell

LTx, liver transplantation

m, Milli (10^{-3})

M, molar (mol/l)

MELD, model for end-stage liver disease

MH1, mad homology 1

MH2, mad homology 2

MHN, massive hepatic necrosis

Mixl, mix paired-like homeobox

NAFLD, non-alcoholic fatty liver disease

NODAL, nodal growth differentiation factor

NP-40, Nonidet P-40

NTR, nitroreductase

PAGE, polyacrylamide-gel-electrophoresis

PBC, primary biliary cirrhosis

PBS, phosphate buffered saline

PPIA, peptidylprolyl isomerase A

PSC, primary sclerosing cholangitis

PVDF, polyvinylidene fluoride

PXR, pregnane X receptor

rpm, revolutions per minute

RNA, ribonucleic acid

RNAi, RNA interference

SDS, sodium dodecyl sulfate

siRNA, small interfering RNA

shRNA, small hairpin RNA

Skil, SKI-like oncogene

SMAD1, sma and Mad homolog 1

SMAD2, sma and Mad homolog 2

SMAD3, sma and Mad homolog 3

SMAD4, sma and Mad homolog 4

SAMD5, sma and Mad homolog 5

SMAD7, sma and Mad homolog 7

SMAD8, sma and Mad homolog 8

SMHN, submassive hepatic necrosis

TAA, thioacetamide

TBIL, total bilirubin

TBS, tris buffered saline

TBS-T, tris buffered saline with Tween20

TEMED, N,N,N´,N´-Tetramethylethylenediamine

TGF-β, transforming growth factor beta

TGIF, transforming growth factor beta induced factor

Tris, tris(hydroxymethyl)-aminoethane

UGT1A, UDP glucuronosyltransferase family 1 member A complex locus

YB-1, Y-Box Binding Protein 1

ZEB1, zinc finger E-box binding homeobox 1

1 INTRODUCTION

1.1 ACLF and MHN

Liver disease is one of the major health challenges worldwide due to its high prevalence, severe clinical outcome and huge economic burden. Liver insults, such as alcohol, hepatitis virus, drug toxicity, and fat accumulation, damage the liver, leading to different consequences, including inflammation, fibrosis, cirrhosis, steatohepatitis, hepatocellular carcinoma, acute liver failure (ALF) and acute-on-chronic liver failure (ACLF). Liver transplantation is the only treatment option for end-stage liver disease. Given that liver donation are very limited, most patients with end-stage liver disease have a few chances to receive liver transplantation.

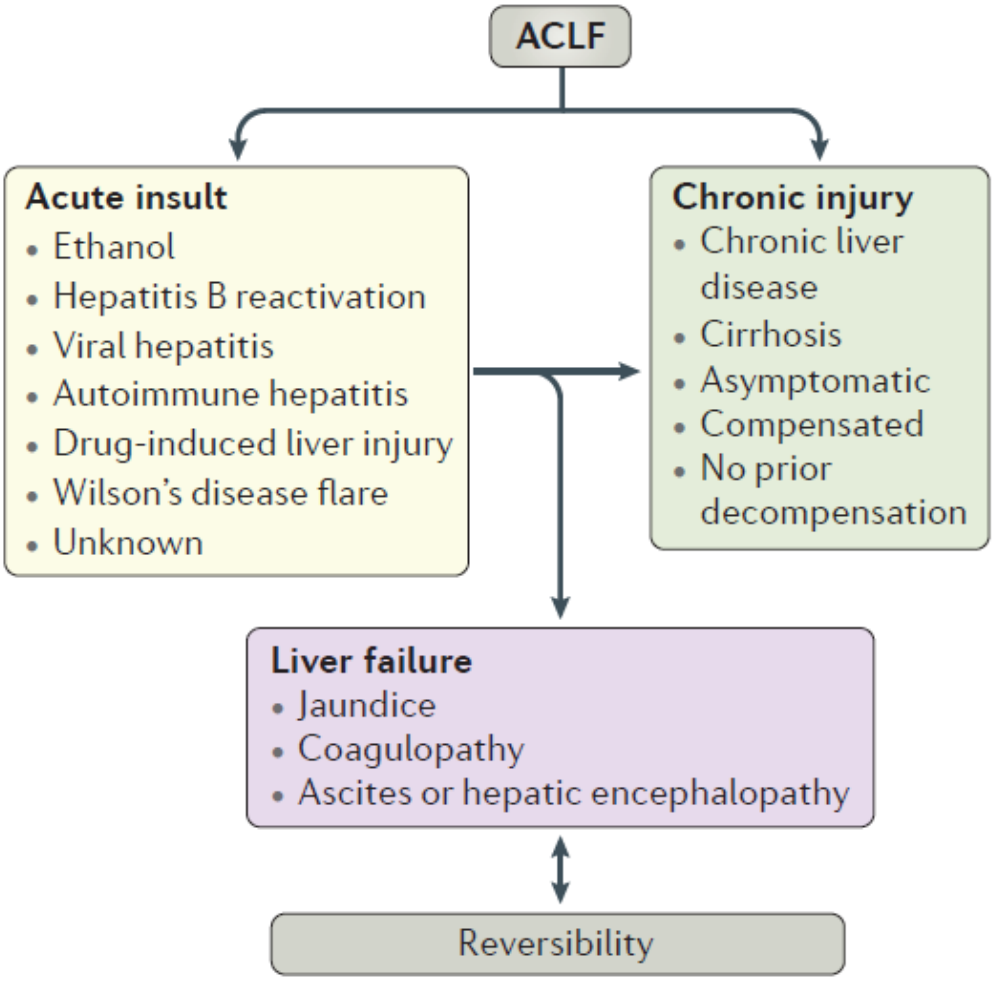


Figure 1. Definition and concept of ACLF (Sarin and Choudhury, 2016)

ACLF is a new redefined clinical syndrome characterized by acute and severe liver insufficiency (**Figure 1**), which results in near 50% three-month mortality (Weng et

al., 2015). In the United States, costs for one patient with ACLF receiving intensive care range between $116,000 and $180,000 (Olson et al., 2011). However, mortality in these patients has remained unchanged over the last 20 years. In Germany, precise epidemiological data on ALF and ACLF are not available to date. It is estimated that 200 to 500 patients develop life-threatening acute liver failure in the country each year, including ALF and ACLF (Canbay et al., 2011). The economic burden is huge even if the incidence of the disease remains stable.

ACLF mainly occurs in cirrhotic patients. Patients with chronic liver disease and chronic injury may also cause ACLF (Sarin and Choudhury, 2016). When these patients suffer from acute decompensation, the patient's condition quickly deteriorate and develop multi-organ failure within 1-2 weeks after the onset of the injury, and eventually lead to high short-term mortality (Sarin and Choudhury, 2016; Sarin et al., 2019). Therefore, the first week is a "golden window" period determining whether these patients can reduce acute injury, prevent the development of sepsis, and perform liver regeneration (Sarin and Choudhury, 2016) (**Figure 2**).

Massive hepatic necrosis (MHN) is defined as extensive diffuse necrosis in a healthy or diseased liver, which spans multiple acini or multiple lobes (Weng et al., 2015). MHN has many synonyms, includes massive liver loss, fulminant massive necrosis and acute yellow atrophy. Clinically, MHN or submassive hepatic necrosis (SMHN) is the defining histological feature of ALF and ACLF (Weng et al., 2015).

1.2 Liver progenitor cell-mediated liver regeneration

1.2.1 Liver progenitor cell

Liver progenitor cell (LPC) is a type of small hepatobiliary reactive cell with a diameter of about 10 μm, which has a large nucleus to cytoplasm ratio. LPC reside in the Canal of Hering and the smallest biliary tree (**Figure 3**). The nuclei of LPC present an oval shape, hence it is also known as oval cell (Dolle et al., 2010). These cells are activated by a signaling network mediated by a large number of growth factors and cytokines in response to severe liver damage (Conigliaro et al., 2010). LPC possess capability to differentiate into either bile duct epithelial cells or hepatocytes. LPC express specific makers of both bile ductular epithelial cells and

hepatocytes (Fausto, 2004; Lee et al., 2006).

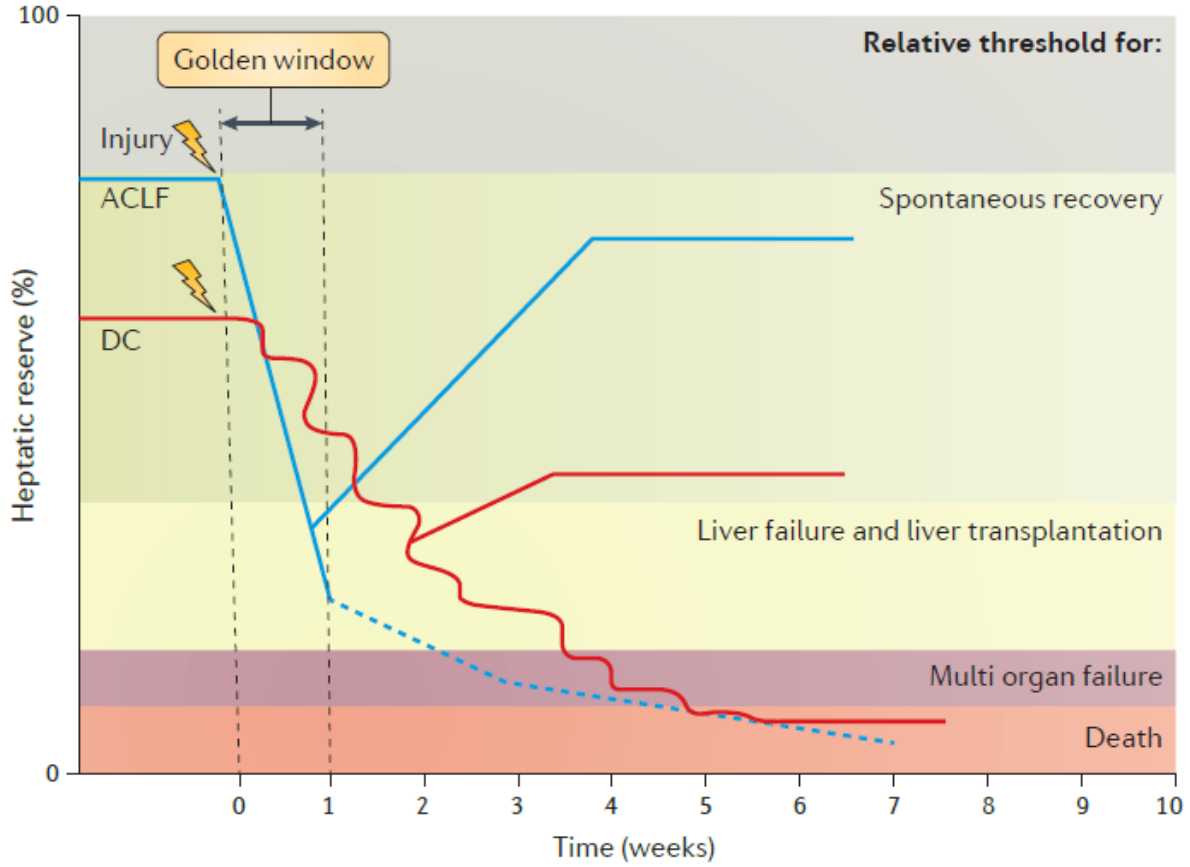


Figure 2. Golden window in ACLF (Sarin and Choudhury, 2016).

LPC differentiate into hepatocytes to restore lost liver mass in severe liver diseases characterized as hepatocyte extinction, such as severe viral hepatitis and acute-on-chronic liver failure (Falkowski et al., 2003; Fausto, 2004; Santoni-Rugiu et al., 2005). On the contrary, LPC mediate cholangiocyte regeneration in cases where the bile duct epithelial cells are most damaged, including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (Rodrigo-Torres et al., 2014). Studies on acute and chronic human liver disease have shown a correlation between the degrees of LPC activation and the severity of liver disease (Lowes et al., 1999).

1.2.2 Liver progenitor cell in liver regeneration

Liver regeneration is one of the most important repair mechanisms responding to liver damage. Once the liver is insulted, hepatocytes, the main functional cells of the liver, are the first cells responding to regenerative stimuli triggered by mitogenic growth factors, e.g. hepatocyte growth factor (HGF) and epidermal growth factor

(EGF) (Michalopoulos, 2013). In most conditions, hepatocytes proliferate rapidly, restoring liver mass and simultaneously providing all functions necessary to maintain body homeostasis (Michalopoulos, 2013). However, in severe liver damage that leads to massive loss of hepatic mass, the remaining hepatocytes lose the capacity for proliferation, or the proliferating hepatocytes cannot restore hepatic mass and maintain liver function. In such kind of emergency situations, LPC rapidly proliferate and transdifferentiate into hepatocytes in order to restore the parenchymal compartment (Michalopoulos, 2013; Weng et al., 2015). Katoonizadeh reported that 50% loss of hepatocytes is a threshold for extensive activation of LPC (Katoonizadeh et al., 2006). Actually, activation of LPC occurs not only in emergent liver diseases such as liver failure, but also in severe chronic liver disease, i.e., cirrhosis. Stueck and Wanless showed that up to 70% of cells in hepatocyte buds of non-biliary human cirrhotic livers are derived from LPC even without extensive loss of hepatic mass (Stueck and Wanless, 2015). In contrast to restoring the hepatocyte compartment from biliary epithelium, hepatocytes can also transdifferentiate to restore the biliary compartment in severe biliary damage (Katoonizadeh et al., 2006; Michalopoulos, 2013). Thus, cholangiocytes and hepatocytes seem to function as facultative stem cells for each other (Michalopoulos and Khan, 2015). Liver regeneration based on LPC proliferation and differentiation is a key event that determines the clinical outcome of patients suffering from ALF and ACLF. In the condition of MHN/SMHN, LPC are major cell sources that are capable of restoring the lost hepatic mass and recovering liver function. To achieve a successful LPC-dependent liver regeneration, the following three successive steps are required: (1) Rapid LPC activation and

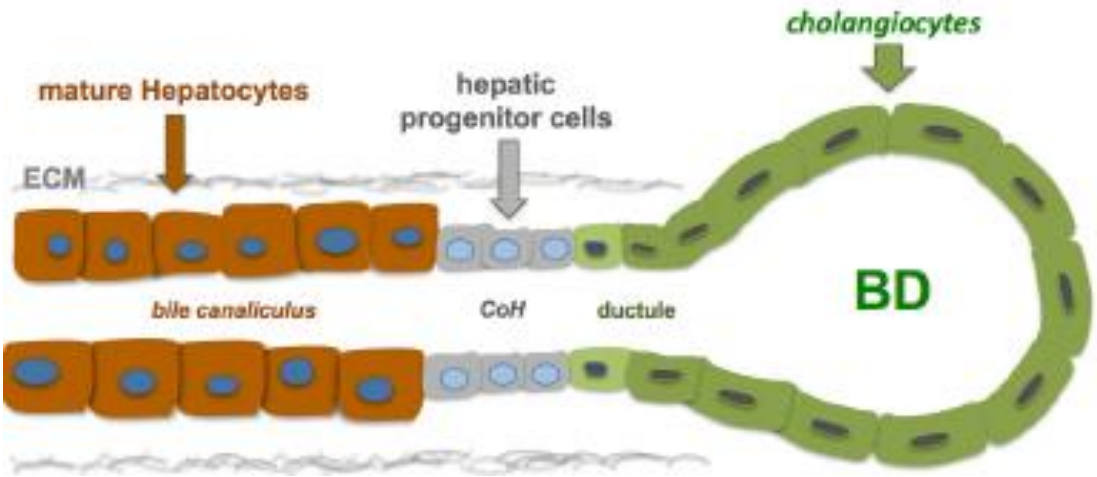


Figure 3. Depiction of liver progenitor cells (LPC) residing in the Canals of Herring (CoH) (Best et al., 2015)

proliferation, which is morphologically present as ductular reaction (DR); (2) LPC differentiation into hepatocytes; and (3) LPC-derived hepatocytes forming bile canaliculi (polarity) and connecting to the existing biliary tree (**Figure 4**) (Weng et al., 2015). If activated LPC can rapidly differentiate into functional hepatocytes, ALF/ACLF patients have a chance to recover from MHN/SMHN.

So far, few studies have investigated the molecular mechanisms underlying LPC-derived liver regeneration in ALF or ACLF. Knowledge regarding the detailed molecular mechanisms of LPC-mediated regeneration has mainly been derived from animal models of chronic liver damage, e.g. rodents fed with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or choline-deficient ethionine-supplemented (CDE) diet (Weng et al., 2015). In these animal models, chronic liver damage elicits a large number of oval cells (LPC in rodents) and DR. However, whether the regenerated hepatocytes are derived from these oval cells or remaining hepatocytes in these models has been controversial for decades (Michalopoulos and Khan, 2015). Jörs and colleagues had convincingly shown that the newly formed hepatocytes in these models were derived from neighbouring hepatocytes, but not from oval cells/DR (Jors et al., 2015). Only in mice fed with CDE for 4 months, a small fraction of regenerated hepatocytes were biliary-derived (Jors et al., 2015). Recently, two elegant studies further shed light on this issue. One study from Forbes' group reported that approximately 25% of hepatocytes were derived from cholangiocytes when hepatocyte proliferation was inhibited by β1-integrin knockdown or p21 overexpression in liver damaged mice (Raven et al., 2017). The second study from Deng and colleagues further observed mice fed with long-term thioacetamide (TAA) or DDC, respectively. Lineage tracing approach showed that cholangiocyte-derived hepatocytes accounted for 55.7% ± 3.9% in mice fed with 52-week TAA and 23.3% ± 3.8% in those fed with 24-week DDC (Deng et al., 2018). In contrast to rodent animals, zebrafish demonstrate robust cholangiocyte-derived liver regeneration when most hepatocytes were destroyed. Two elegant studies showed that administration of metronidazole destroyed near all hepatocytes of larval and adult zebrafish (Choi et al., 2014; He et al., 2014). Once the toxics were washed out, the liver mass in these zebrafish was rapidly restored through cholangiocyte proliferation and

transdifferentiation into hepatocyte-like cells. The phenomena are very similar to those observed in humans (Weng et al., 2015).

It has been long recognized that duct reaction derived from LPC proliferation display a similar morphological phenotype as the embryonic hepatoblast-originated ductular plate (DP) (Desmet, 2011). Thus, it is of great interest to find out whether differentiation of LPCs into mature hepatocytes following MHN/SMHN is governed by signaling pathways and regulatory networks similar to those used by hepatoblasts in the embryonic liver. It is well established that signaling from different TGF-β family members is indispensable for embryonic liver development (Wandzioch and Zaret, 2009). During the process, BMPs, Activin and TGF-βs provide the relevant signals controlling the formation of the foregut endoderm, hepatic specification and segregation of hepatobiliary lineage (Lemaigre, 2009). TGF-βs promote differentiation of hepatoblasts to biliary cells and repress hepatocyte differentiation (Lemaigre, 2009). Recently, Schaub and colleagues showed that in a mouse model mimicking human Alagille syndrome, TGF-β signaling is the driver that mediates hepatocyte transdifferentiation and the formation of de novo biliary system (Schaub et al., 2018). Therefore, it is of substantial interest to know whether and how signaling derived from different TGF-β family members play a crucial role in LPC-mediated regeneration after MHN/SMHN.

1.2.3 LPC take over hepatocyte function

In ACLF patients with massive hepatocyte loss, LPC have two potential options to take over hepatocyte function: (1) differentiating into hepatocytes and (2) running hepatocyte function with LPC identity. Previous observations showed that LPC-to-hepatocyte differentiation in ACLF requires at least three to four weeks (Weng et al., 2015). However, the clinical consequence of ACLF can be predicted at day-7 after the initiation of acute decompensation (Gustot et al., 2015; Hernaez et al., 2017). Therefore, survival of ACLF patients following massive hepatic necrosis should not be dependent on LPC-to-hepatocyte differentiation. The temporary performance of key hepatocyte functions by LPC might be a crucial survival mechanism in ACLF patients. Given that the liver is a unique organ undertaking multiple key physiological functions required for systemic homeostasis, it is interesting to clarify which crucial

hepatocyte functions are taken over by LPCs when massive hepatocyte loss occurs in ACLF.

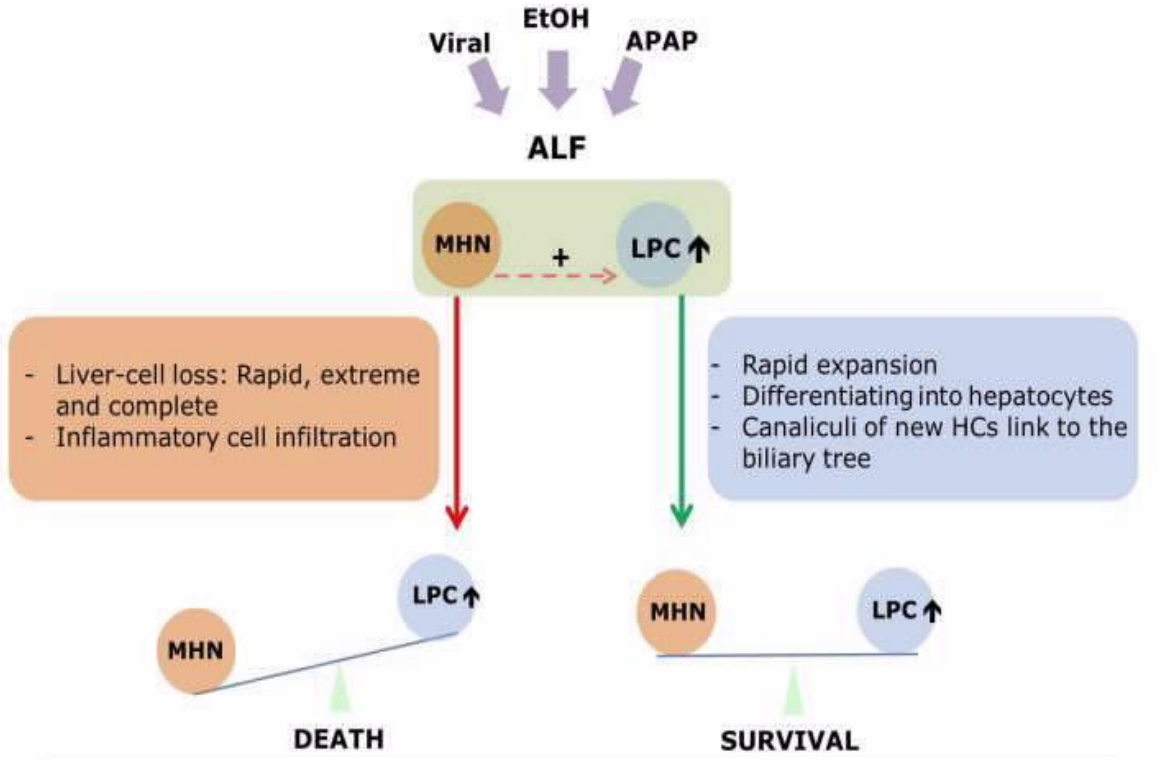
To date, liver transplantation (LTx) is the only approach to rescue patients with the end-stage liver diseases, including ACLF. In clinical practice, whether patients with ACLF are enrolled on the waiting list for LTx is determined by the Model for End-stage Liver Disease (MEDL) scores (European Association for the Study of the Liver. Electronic address, 2016). MELD score (MELD = 3.78×ln[serum bilirubin (mg/dL)] + 11.2×ln[INR] + 9.57×ln[serum creatinine (mg/dL)] + 6.43) consists of three variables, which reflects two key hepatocyte functions (coagulation and bilirubin metabolism). Among the three parameters, INR, which reflects coagulation function, accounts for the largest weight. With the exception of von Willebrand factor, all coagulation proteins are synthesized in hepatocytes (Alastair D. Burt, 2012a). Furthermore, the half-life of coagulation factors is only several hours. In the circumstance of massive hepatocyte loss, whether LPCs are rapidly capable of taking over synthesis of coagulation factors is crucial for ACLF patient survival. In hepatocytes, hepatocyte nuclear factor 4α (HNF4α), a hepatocyte specific lineage transcription factor (LDTF), is an essential transcription factor controlling multiple coagulation factors through directly binding to the gene promoters (Odom et al., 2004; Safdar et al., 2012). To date, it is unknown whether LPC can rapidly take over coagulation function through expressing HNF4α in ACLF.

1.3 HNF4α

HNF4α, also known as NR2A1, is among the most abundant transcription factors in the liver (Chandra et al., 2013). HNF4α is expressed in the nucleus at high levels in the liver, mainly hepatocytes, but less in other adjacent organs such as bile duct, intestine, colon, kidney and pancreas (Dean et al., 2010; Drewes et al., 1996). As a homodimer Zn-finger and DNA binding protein, HNF4α has conserved ligand binding domains and DNA binding domains (Mangelsdorf and Evans, 1995; Sladek et al., 1990). HNF4α can bind to its DNA recognition site and recruit transcriptional co-activators to regulate the expression of liver-specific genes (Wang et al., 2018). In the liver, more than 40% of actively transcribed genes have HNF4α response elements,

including a large number of hepatic function genes (Lau et al., 2018).

HNF4α is pivotal in liver development and maintaining a differentiated hepatocyte phenotype (Babeu et al., 2009). HNF4α knockout mice show a significantly decreased hepatic gene expression and fail to develop a fully functional liver (Li et al., 2000). Lack of HNF4α causes significant metabolic disorders and increases mortality. These mice exhibit high hepatic lipids (steatosis), elevated serum levels of bile acids and ammonia, which are relevant to the disrupted fatty acid metabolism, including ornithine transcarbamylase, and bile acid metabolizing enzymes (Hayhurst et al., 2001; Inoue et al., 2002; Inoue et al., 2006a; Inoue et al., 2004; Inoue et al., 2006b).



Questions:

- What is nature of hepatocyte death during MHN: apoptosis, necrosis , or necroptosis?
- Why does the destruction specifically affect hepatocytes, whereas the framework and sinusoids remain unaltered?
- How does MHN induce LPC-mediated regeneration?
- How do LPCs undergo activation, expansion, differentiation into hepatocytes and linking to the biliary tree?
- Which factors and signaling pathways contribute and modulate LPC-mediated regeneration after MHN?

Figure 4. A scheme depicting MHN and LPC-mediated regeneration in acute liver failure (Weng et al., 2015).

Further studies based on *in vitro* and rodent model systems revealed that HNF4α can bind to the promoter of the *CYP2D6* (Cairns et al., 1996) and *UGT1A* (Barbier et al., 2005) genes. Knockdown of HNF4α by RNAi in primary human hepatocytes affects glucuronosyltransferases, sulfotransferases, ATP-binding cassette transporters and Cytochrome P450 (Kamiyama et al., 2007). Lack of HNF4α leads to embryonic lethality in mice, indicating an essential role of the transcription factor in liver development (Duncan, 2003). Moreover, HNF4α activates expression of other transcription factors, thereby controls more liver-specific target genes, including hepatocyte nuclear factor 1 homeobox A (HNF1α) (Kuo et al., 1992) and pregnane X receptor (PXR) (Kamiya et al., 2003).

1.3.1 HNF4α mediates coagulation factor expression

HNF4α is a high-level steroid/thyroid hormone nuclear receptor expressed in the liver (Harish et al., 2001), and is essential for the synthesis of large amounts of coagulation factors. There are functional HNF4α binding site in the promoter or enhancer of human coagulation factor genes (Arbini et al., 1997; Ceelie et al., 2003; Erdmann and Heim, 1995; Farsetti et al., 1998; Hung and High, 1996; Miao et al., 1992; Naka and Brownlee, 1996). Ceelie revealed the contribution of HNF4α to the transcriptional activity of the coagulation factor II enhancer. Electrophoretic mobility shift assays revealed the binding site of hepatocyte nuclear factor HNF4α. Mutagenesis studies further showed that loss of HNF4α binding capacity could lead to reduced coagulation factor II activity (Ceelie et al., 2003). Erdmann et al. and Arbini et al. found that HNF4α plays a major positive regulatory role in the expression of factor VII through binding to factor VII promoter at a position of -71 to -49 bp (Arbini et al., 1997; Erdmann and Heim, 1995). The mutation of this binding site leads to reduced activity of factor VII (Arbini et al., 1997; Erdmann and Heim, 1995). *In vitro* DNase I footprint and gel shift analysis indicate that HNF4α regulates the transcription of the human factor IX promoter (Naka and Brownlee, 1996) and binds to ACTTTG element in the factor X promoter at the -73 to -44 bp (Hung and High, 1996; Miao et al., 1992). Miao and colleagues investigated the direct role of HNF4α

in regulating estrogen-dependent transcription of the factor XII gene promoter (Miao et al., 1992). In HNF4α knockdown hepatocyte and HNF4α-null mice, expression of F5, F9, F11, and F12 was decreased, whereas there was no alteration of F2, F7, F8, and F10 expression (Inoue et al., 2006a; Safdar et al., 2012; Safdar et al., 2010). In human liver samples, HNF4α expression positively correlates with coagulation factor levels (Salloum-Asfar et al., 2016).

1.4 Activin-R-SMADs-FoxH1 axis

1.4.1 TGF-β family members

The transforming growth factor beta (TGF-β) superfamily encompasses three subfamilies, TGF-β/Activin, bone morphogenetic protein (BMP) and growth differentiation factor (GDF), and consists of more than 45 members of signaling molecules (Morikawa et al., 2016). These subfamilies play fundamental roles in many cellular processes, including cell growth, proliferation, differentiation, and apoptosis (Zhang et al., 2017). In addition, the TGF-β signaling pathway is active in immunity, inflammation, fibrosis, and cancer (Prud'homme, 2007). TGF-β signals via serine/threonine kinase receptors (Hata and Chen, 2016). The TGF-β subfamily has two types of receptor (Hata and Chen, 2016). TGF-β binding to type II receptors elicits phosphorylation of type I receptors. The latter subsequently phosphorylates “receptor SMADs” (R-SMADs), which belong to the transcription factor Smad family (Hata and Chen, 2016). The TGF-β type I receptor family has 7 members. ALK5 (TGF-β1/2/3 type I receptor) and ALK4 and ALK7 (Activin A/B/AB type I receptor) phosphorylate SMAD 2 and SMAD3, whereas ALK1, ALK2, ALK3 and ALK6 (BMP and GDF type I receptors) phosphorylate SMAD1, SMAD5, and SMAD8 (Massague et al., 2005). Once activated, the R-SMADs bind to SMAD4, a common binding partner to all R-SMADs, and form complexes that translocate into the nuclear compartment (**Figure 5**). In order to achieve high affinity and selectivity to specific target genes, the SMAD complexes require additional DNA-binding cofactors (Massague, 2012). Each SMADs-cofactor complex targets a particular set of genes (Massague, 2012). TGF-β can activate or repress several hundreds of target genes at the same time by virtue of combinatorial interactions (Massague, 2012). Interestingly, except for the negative feedback regulators Smad7 and SKIL (SKI-like

oncogene), few TGF-β target genes are common to all cell types, suggesting the highly context-dependent nature of TGF-β action (Massague, 2012).

1.4.2 The characteristics of Activin

Activin is a homodimer or heterodimer of various β subunit isotypes, which are linked by disulfide bond (Wang et al., 2016). The Activins subfamily comprises Activin A (beta A - beta A), Activin B (beta B - beta B) and Activin AB (beta A - beta B) (Ying, 1987). Activin A represents the most widely studied Activin. Activin A was identified as a gonadal protein in the 1980s. It induces follicle-stimulating hormone (FSH) biosynthesis and secretion, which plays a pivotal role in the regulation of the menstrual cycle (Vale et al., 1986). In the following decades, extensive research has revealed that Activin A is expressed and functioned in many different cell types (Morianos et al., 2019). In embryonic development, Activin signaling is required for germ layer specification in mouse (Zhou et al., 1993), Xenopus (Jones et al., 1995) and zebrafish (Feldman et al., 1998). Lack of Activin signal leads to expression of ectopic neuroectodermal markers and loss of pluripotency markers in epiblast cells of mice (Mesnard et al., 2006). Epiblast stem cells (EpiSCs) from mouse embryos express Activin A (Brons et al., 2007). Activin was also confirmed in human embryonic stem cells (hESCs), which maintains the pluripotent status of the epiblast (Vallier et al., 2004). Furthermore, Activin can induce differentiation of hESCs and human pluripotent stem cells (hiPSCs) into the three germ layers. These findings suggest that Activin signaling alters cell fates of ESCs (Vallier et al., 2009a; Vallier et al., 2009b).

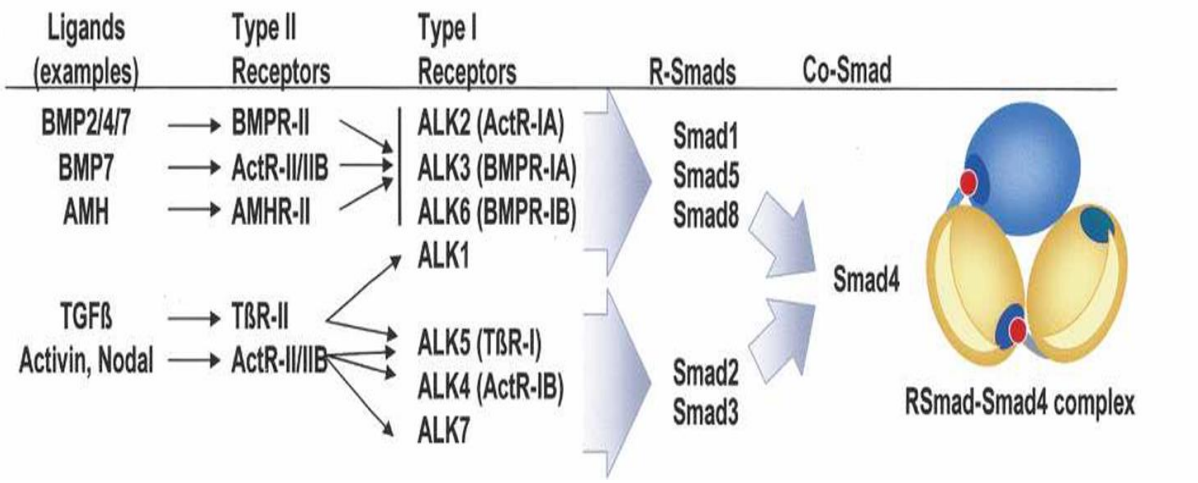


Figure 5. TGF-β family members (Massague et al., 2005)

1.4.3 Activin in the liver

Activin A inhibits hepatocyte proliferation and induces apoptosis *in vivo* and *in vitro* (Hully et al., 1994; Schwall et al., 1993; Yasuda et al., 1993). Knocking out Activin A in mice leads to death shortly after birth (Matzuk et al., 1995). Joanne et al. reported a pivotal role of Activin in cell cycle arrest in hepatocyte (Ho et al., 2004). Activin A treatment leads to increased gene expression of the cyclin-dependent kinase inhibitor (CDKI) p15INK4B and Sp1, which lead to induce hepatocyte cell growth arrest (Ho et al., 2004). Activin A also upregulates p21WAF1/Cip1/Sdi1, a cyclin-dependent kinases inhibitor, to inhibit the proliferation and induce apoptosis of hepatocytes (Oh et al., 2007). When hepatocyte replication occurs in a regenerating liver, Activin A gene expression is decreased firstly, but is increased when liver regeneration terminates (Gold et al., 2005). However, other studies report that expression of Activin A is increased at an earlier phase following partial hepatectomy (Date et al., 2000; Zhang et al., 1997b). Increased levels of circulating Activin A have been found in patients with various liver diseases, including chronic viral hepatitis (Patella et al., 2001), hepatocellular carcinoma (Elsammak et al., 2006; Pirisi et al., 2000; Yuen et al., 2002), alcoholic cirrhosis (Yuen et al., 2002), ALF (Hughes and Evans, 2003; Lin et al., 2006) and non-alcoholic fatty liver (NAFLD) (Yndestad et al., 2009).

LPC have a higher resistance to the anti-proliferative effect of TGF-β compared with hepatocytes (Nguyen et al., 2007; Sanchez and Fabregat, 2010). However, the role of Activin A in LPCs has not been elucidated (del Castillo et al., 2008; Michalopoulos, 2010; Preisegger et al., 1999). Notably, the gradient of Activin signaling has been shown to control the differentiation of hepatoblasts into hepatocytes and biliary tract cells in mice (Clotman et al., 2005).

1.4.4 SMADs in transcription

SMADs are transcriptional activators (Liu et al., 1996). Fusion of the SMAD MH2 domain and GAL4 can induce expression of a reporter construct containing a Gal4 binding site (Liu et al., 1996). SMADs interact with CBP / p300 to co-activate

transcription induced by TGFβ signals through chromosomal structural modifications (Ross and Hill, 2008). Moreover, numerous co-activators, including Axin, Dok-1 and Zeb1 have been revealed to interact with activated SMADs through their MH1 or/ and MH2 domains (Furuhashi et al., 2001; Postigo, 2003; Yamakawa et al., 2002). The transcriptional activation of target genes is increased under the influence of these interaction complexes by the recruiting of the RNA Polymerase II complex to promoters or promoting SMADs/CBP/p300 interaction (Feng and Derynck, 2005; Ross and Hill, 2008). SMADs also can silence the transcription by interacting with co-repressors. Homeodomain protein TGIF could be recruited by activated Smad2 and histone deacetylase (HDACs) to repress transcription (Wotton et al., 1999). YB-1 represses transcription through interaction with the MH1 domain of SMAD3 to interrupt the Smad3-p300 interaction (Higashi et al., 2003).

1.4.5 SMAD co-factor: FOXH1

FOXH1 (forkhead box protein H1, also known as FAST1) is a pioneer factor that contains three domains: SMADs interacting motif, forkhead motif, and FOXH1 motif (Attisano et al., 2001). The Nodal/Activin signaling pathway has many similarities with other TGF-β signaling pathways because it utilizes the core Smad-dependent signaling component (Barnes and Black, 2016) (**Figure 6**). The proline-rich SMADs interacting motif at the carboxyl terminal region is able to bind to the MH2 domain of SMAD2 and SMAD3. The sequence of the FOXH1 forkhead motif is highly conserved and features TGT(G/T)(T/G)ATT as the consensus sequence (Attisano et al., 2001; Liu et al., 1997; Whitman, 2001; Zhou et al., 1998). The binding of FOXH1 to SMADs is essential for regulating TGF-β signaling-dependent transcription. FOXH1 binding to DNA recruits active SMAD complexes to target gene enhancer or promoter, which in turn bind to FOXH1, allowing transcriptional activation (Chen et al., 1997; Labbe et al., 1998; Liu et al., 1999; Zhou et al., 1998). In embryonic stem cells, TGF-β signaling drives pSmad2-Smad4 to bind to promoters of target genes that are pre-loaded with Smad3 and FoxH1 for transcription activation (Aragon et al., 2019).

FOXH1, the first SMAD binding partner, exploits an Activin responsive element (ARE) to regulate the activation of Mix.2 expression in the Xenopus development. Activin treatment induces the formation of a SMADs and FOXH1 complex, which binds to the ARE of target genes (Huang et al., 1995). Subsequent studies elucidated a

comprehensive genome-wide interaction between FOXH1 and SMAD2/3 in mediating Activin signaling-dependent mesendoderm development. Inhibition of Activin activity or knockdown of FOXH1 not only significantly reduces mesodermal gene expression, but also greatly affects embryonic patterning. Afterwards, Labbe found that FOXH1 is required to induce the mouse goosecoid-like (GSC) gene promoter. FOXH1, SMAD2 and SMAD4 form a complex that binds to the GSC gene promoter to initiate transcription. Interestingly, SMAD3 competes with SMAD2 to bind to Smad4 thereby altering the conformation of the DNA-binding complex to prevent GSC gene transcription (Zhou et al., 1998). Mix 1 homeobox-like 1, an Activin signaling dependent homeodomain protein, is also regulated by the FOXH1-SMADs DNA binding complex (Chen et al., 1996). In addition, Silvestri found that FOXH1 activates the transcriptional regulatory network in the anterior neuroectoderm development (Silvestri et al., 2008). RNA-seq and ChIP-seq analyses further show that the FOXH1-SMAD2/3 complex regulates a large number of Activin signaling targeted mesodermal genes in Xenopus tropicalis embryos (Chiu et al., 2014).

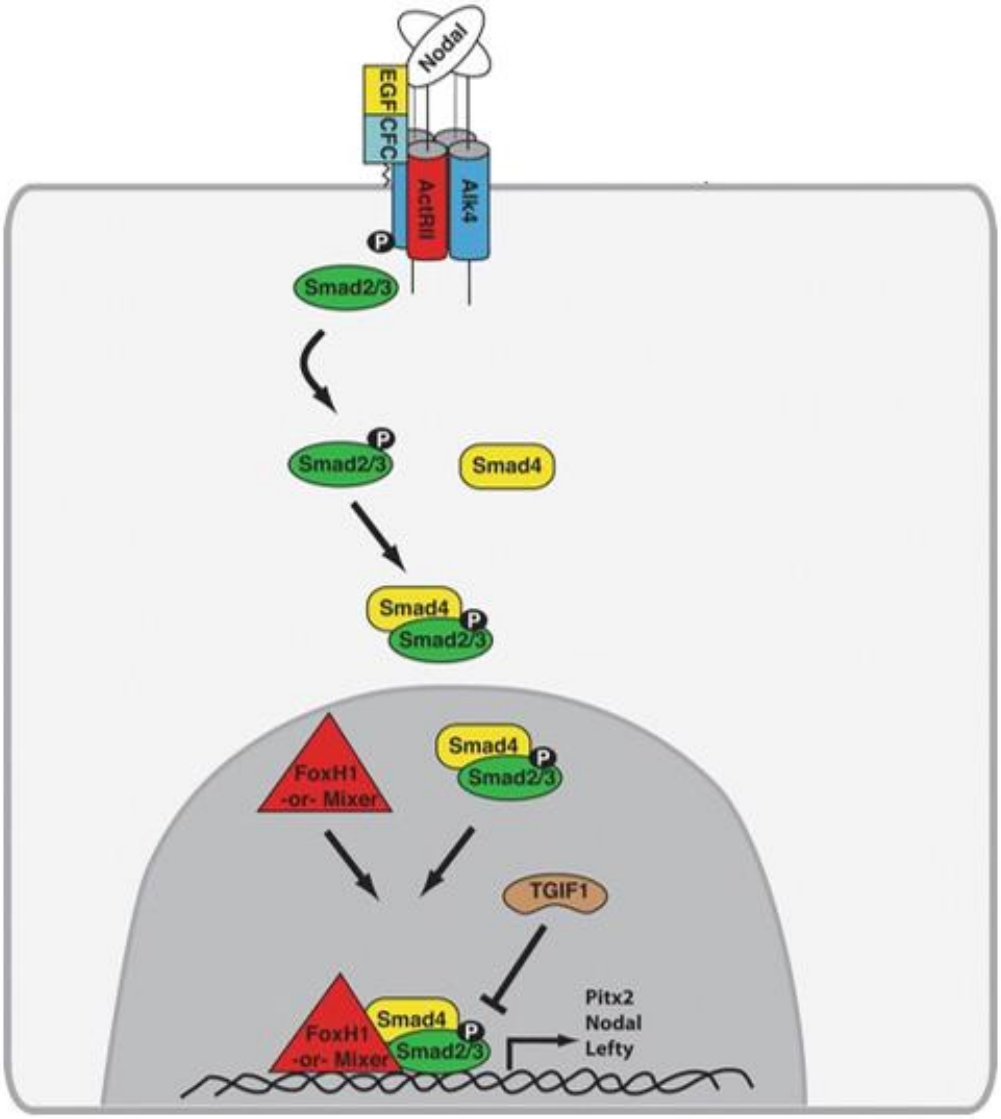


Figure 6. The Nodal/Activin-Foxh1 signaling pathway (Barnes and Black, 2016)

1.5 Follistatin

Follistatin, also known as Activin-binding protein, is a plasma protein, the function of which is the biological neutralization of members of the TGF-β superfamily, with a particular focus on Activin (Amthor et al., 2004; Harrington et al., 2006; Hedger and de Kretser, 2013; Iemura et al., 1998; Nakamura et al., 1990). Follistatin has three isoforms, FS-288, FS-300 and FS-315. FS-288 and FS-315 are produced by alternative splicing of the original mRNA transcript. F-300 is the product of post-translational modification (Hinck et al., 2016). Thompson reported that two follistatin molecules surround Activin, burying its residues and receptor binding sites to neutralize Activin (Refaat, 2014; Thompson et al., 2005)(**Figure 7**).

The Activin/follistatin system plays a key role in the homeostasis of liver (Ooe et al., 2012). Hepatocytes are the main contributors of circulating follistatin in the body (Hansen et al., 2016). The functions of Activin and follistatin in the liver have been investigated. Dysregulation of Activin and follistatin expression is associated with liver diseases, including fibrosis (Yuen et al., 2002), hepatocellular carcinoma (Elsammak et al., 2006; Pirisi et al., 2000; Yuen et al., 2002), and NAFLD (Hughes and Evans, 2003; Polyzos et al., 2016; Yndestad et al., 2009). Hughes and Lin reported an increased Activin A/follistatin mRNA ratio in patients with ALF (Hughes and Evans, 2003; Lin et al., 2006). In addition, elevated levels of circulating Activin A and follistatin are demonstrated after partial hepatectomy (Date et al., 2000; Endo et al., 2006; Kogure et al., 1995; Kogure et al., 1996; Kogure et al., 1998; Zhang et al., 1997b).

1.6 ACLF/sepsis-like circumstance: insulin and glucagon

ACLF is a sepsis-like clinical syndrome. In patients with sepsis, Michel found the concentration of Activin and follistatin increased simultaneously in the serum (Michel et al., 2003). Given that both Activin and follistatin are produced by hepatocytes, it is not surprise that massive hepatocyte death release a large amount of the two factors. The mechanism of hepatocytes-secreted follistatin has not yet been elucidated completely. *In vitro*, glucagon and insulin regulate follistatin expression in hepatocytes (Hansen et al., 2016; Zhang et al., 1997a) (**Figure 8**). The secretion of follistatin in the hepatocyte is controlled by the ratio of glucagon to insulin. Glucagon stimulates the expression and secretion of follistatin in cultured human hepatocytes (Hansen et al., 2016). The transcription factor FOXO1 is the key regulatory factor in controlling follistatin secretion in the hepatocyte. Glucagon and insulin have opposite effects on FOXO1 levels (Smati et al., 2020; Tao et al., 2018). In addition, the increase of circulating follistatin is closely related to the degree of insulin resistance. Elevated follistatin in plasma is observed in the case of fasting hyperinsulinemia. Physiological concentrations of insulin regulate the plasma levels of Activin A and follistatin (Sylow et al., 2020).

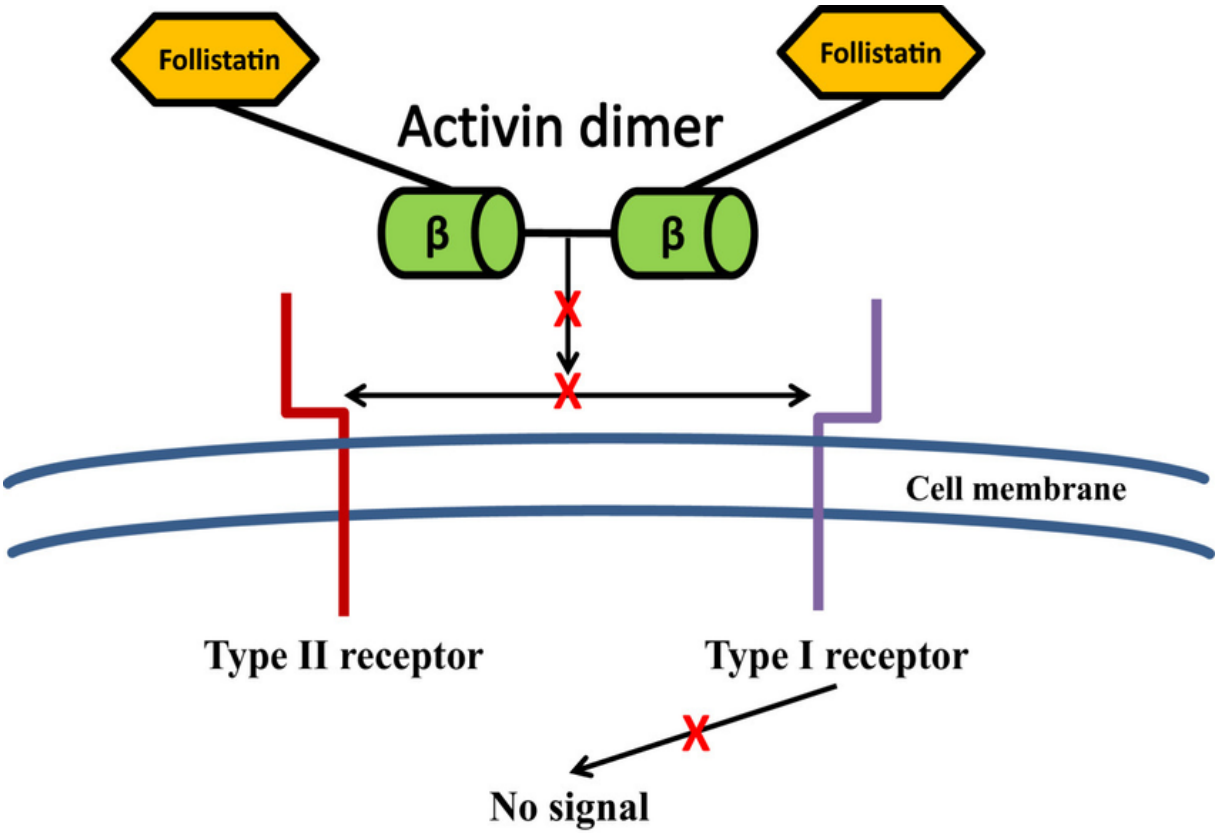


Figure 7. The signalling mechanism of follistatin (Refaat, 2014)

1.7 Aims of this study

On the basis of the described state of the art, the following hypotheses are proposed:
(1) HNF4α expression in LPC is a critical transcription factor for taking over hepatocyte functions, e.g. producing coagulation factors, in ACLF patients following MHN.
(2) The expression of HNF4α in LPCs requires the formation of extracellular Activin signal-driven transcription factor complex FOXH1-SMAD2/3/4.
(3) In ACLF patients, Activin signaling is negatively regulated by follistatin, a hepatocyte-derived hormone determined by the balance of insulin and glucagon.

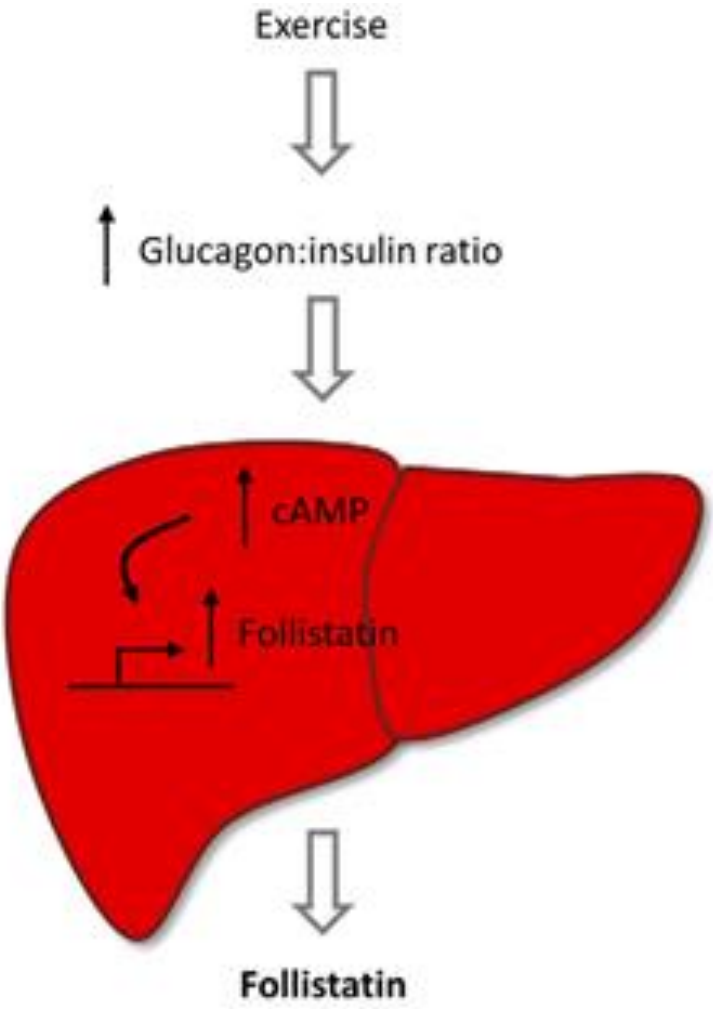


Figure 8. Schematic presentation of the regulation of follistatin by the ratio of glucagon and insulin (Hansen et al., 2016)

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagent kits (Table.1)

Acetic acid	LC-7167.2	Labochem International
Agarose	50004	Lonza
Agar-Agar	5210.1	Carl Roth
AgeI restriction enzyme	ER1461	Thermo Fisher Scientific
APS	A3678	Sigma-Aldrich
BSA	A8806	Sigma-Aldrich
Chemiluminescent Substrate	34580	Thermo Fisher Scientific
DAB	D-5905	Sigma-Aldrich
DMEM	BE12-709F	Lonza
DMSO	41639	Sigma-Aldrich
DNA ladder marker	BIO-33029-BL	Bioline
DNA Gel Loading Dye	R0611	Thermo Fisher Scientific
dNTP	R0191	Thermo Fisher Scientific
EcoRI	FD0274	Thermo Fisher Scientific
EDTA	324503	Calbiochem
Ethanol 100%	K928.4	Carl Roth
Fetal Bovine Serum (FBS)	10270-098	Invitrogen
Formaldehyde	F1635	Sigma-Aldrich
Glycine	3790.2	Carl Roth
Glucagon	G2044	Sigma-Aldrich
HEPES-KOH	9105.4	ROTH
Herring sperm DNA	15634017	Thermo Fisher Scientific
Hydrogen chloride (HCl)	1.09057	Merck
H_2O_2	H1009	Sigma-Aldrich
Insulin	I0516	Sigma-Aldrich
InviTrap Spin Universal RNA Mini Kit	1060100300	Stratec

MinElute PCR Purification Kit	28004	Qiagen
Laemmli-buffer	161-0737	BioRad
LB-Medium	X968.1	Carl Roth
L-glutamine	BE17-605E	Lonza
Lithium chloride (LiCl)	L4408	Sigma-Aldrich
Lipofectamine 2000	11668027	Thermo Fisher Scientific
Lipofectamine RNAiMAX	13778-075	Thermo Fisher Scientific
LDS smaple buffer (4x)	2020067	Invitrogen
2-β-Mercaptoethanol	516732	Sigma-Aldrich
Mounting medium	S3023	Dako
Methanol	8388	Carl Roth
Milk powder	T145	Carl Roth
NP-40	11754599001	Sigma-Aldrich
NaCl NaHCO3	S7653 S5761	Sigma-Aldrich Sigma-Aldrich
Penicillin/streptomycin	A2210	Biochrom KG
Phos-Inhibitor Cocktail 2	P5726	Sigma-Aldrich
Protease Inhibitor Cocktail	S8820	Sigma-Aldrich
Puromycin	A1113802	Gibco®
Peroxidase Blocking Reagent	S2003	Dako
PBS	D8537	Sigma Aldrich
Protein A/G PLUS-Agarose	SC-2003	Santa Cruz (USA)
Protein Assay Reagent A	5000113	Bio Rad
Protein Assay Reagent B	5000114	Bio Rad
Protein Assay Reagent S	5000115	Bio Rad

POWRUP SYBR MASTER MIX	A25780	Thermo Fisher Scientific
Proteinase K	EO0491	Sigma-Aldrich
Protein ladder marker	26619	Thermo Fisher Scientific
PureLink Quick Plasmid Miniprep Kit	K210010	Invitrogen
PureLink® HiPure Plasmid Maxiprep Kit	K210007	Invitrogen
QIAquick Gel Extraction Kit	28704	Qiagen
RNase A	EN0531	Sigma-Aldrich
RiboLock RNase Inhibitor	EO0382	Thermo Fisher Scientific
Recombinant TGF-β1	100-21	Peprotech
Recombinant Activin A	338-AC-050	R&D Systems
Recombinant Follistatin	769-FS-025	R&D Systems
RevertAid H Minus Reverse Transcriptase	EP0451	Thermo Fisher Scientific
Random hexamer primer	SO142	Thermo Fisher Scientific
Sample reducing agent (10x)	2148880	Invitrogen
Supersignal Ultra	34095	Thermo Fisher Scientific
Sodium Deoxycholate	D6750	Sigma-Aldrich
TEMED	T9821	Sigma-Aldrich
TRIS	4855	Carl Roth
Triton® X-100	T-9284	Sigma-Aldrich
Trypsin/EDTA 10x	T4174	Sigma-Aldrich
Tween® 20	9127.2	Carl Roth
TGF-β1/ Activin A inhibitor	SB431532	Selleckchem
Williams' Medium E	W1878	Sigma-Aldrich

2.1.2 Antibodies (Table.2)

Antibodies	Product no.	Company
Phospho-SMAD2	3108S	Cell Signaling
SMAD2/3	8685S	Cell Signaling
SMAD2	5339S	Cell Signaling
SMAD3	9523S	Cell Signaling
SMAD4	46535	Cell Signaling
FOXH1	ab49133	Abcam
FOXH1	AF4248	R&Dsystems
HNF4α	ab181604	Abcam
HNF4α	sc-374229	Santa Cruz
Follistatin	PA5-79284	Thermo Fisher
Coagulation factor 5	20963-1-AP	Proteintech
Coagulation factor 2	ab92621	Abcam
Rabbit IgG	2729S	Cell Signaling
α-TUBULIN	ab4074	Abcam
Albumin	MAB1455	R&Dsystems
Cdh1	sc-8426	Santa Cruz
Goat anti rabbit IgG HRP	P0217	DAKO
Goat anti mouse IgG HRP	P0447	DAKO
Anti-Sheep HRP	31480	Thermo Fisher

2.1.3 Oligonucleotides (Table.3)

	Forward	Reverse
mRNA		
mALK4	TGCTTGAGCTTTCTGTGCAT	GAGAAGCAGCAGCACTCAGAG
mHNF4α	AGAGGTTCTGTCCCAGCAGATC	CGTCTGTGATGTTGGCAATC
mFactor 2	GGACGCTGAGAAGGGTATCG	CCCCACACAGCAGCTCTTG
mFactor 5	AATGGATCGTCAGTGAGGA	TGCCTTTCTTGCAGATAAGC

	CA	A
mAlbumin	TGTCCGTCAGAGAATGAAG TGC	AAGACATCCTTGGCCTCAGC A
mCdh1	GGTTTTCTACAGCATCACC G	GCTTCCCCATTTGATGACAC
mFollistatin	TGCTGCTACTCTGCCAGTT C	GTGCTGCAACACTCTTCCTT G
mPPIA	GAGCTGTTTGCAGACAAAG TT	CCCTGGCACATGAATCCTGG
hALK4	ATCATCAGCGTGTCTATCAC AAC	ACAAAGAGGGGTAACCCTGA G
hHNF4α	CAGGCTCAAGAAATGCTTCC	GGCTGCTGTCCTCATAGCTT
hFactor 2	CACGGCTACGGATGTGTTCT G	ACCCTCAGCACAGTTACCTT C
hFactor 5	GAACCATCATAAGGTCTCAG CC	CCTCTGCTCACGAGTTATTTT CT
hFollistatin	ACGTGTGAGAACGTGGACTG	CACATTCATTGCGGTAGGTTT TC
hPPIA	AGCATGTGGTGTTTGGCAAA	TCGAGTTGTCCACAGTCAGC
ChIP		
mFactor 2	GGAGCCTCCAGAAGGACTT ATT	GCGGACGTGCGACATAGTG
mFactor 5	GGGGGAAATGCTGCTTTGTG	CTCAGTGCTCTGTCCCTGTT
mHNF4α	AGTTCCATGTAGAGAGGG	TGGGATCACCGTGCTAGC
hFactor 2	GGGGGTGACAGTGACCTTTT	GCCATAGTGTGTCAGCTCCT
hFactor 5	ACTGCACTGCACAGAAGGT	ACCAGAGGTATTAGGGAGAG CA
hHNF4α	GGTTGGACTCTCACCTCTCC	TCCACCAGGAAGGCGGT
shRNA		
mFoxh1-shRNA	CCGGCCCTGGGAAAGAATC CACATGCTCGAGCATGTGGA TTCTTTCCCAGGGTTTTTG	AATTCAAAAACCCTGGGAA AGAATCCACATGCTCGAGCAT GTGGATTCTTTCCCAGGG
Control-shRNA	CCGGCCTAAGGTTAAGTCGC CCTCGCTCGAGCGAGGGCG ACTTAACCTTAGGTTTTTG	AATTCAAAAACCTAAGGTTAA GTCGCCCTCGCTCGAGCGA GGGCGACTTAACCTTAGG

2.1.4 Buffers (Table.4)

	Ingredient
10% APS	1g APS in 10ml H_2O
RIPA buffer	50 mM Tris-HCl pH 7.4 150 mM NaCl 1% Nonidet P-40

	0.1 % SDS 0.5 % Sodium-Desoxycholate 2 mM EDTA
Running buffer 10X	30 g of Tris base 144 g of glycine 10g of SDS 1000 ml of H2O
Transfer buffer	3 g of Tris base 14.4 g of glycine 1g of SDS 200ml methanol 1000 ml of H2O
Co-IP lysis buffer	20 mM Tris HCl pH 8 137 mM NaCl 1% NP-40 2 mM EDTA
PBS-T	1ml Tween20 in 1L PBS
Co-IP Wash buffers	10mM Tris pH 7.4 1mM EDTA 1mM EGTA pH 8.0 150mM NaCl 1% Triton X-100 1X Protease inhibitor cocktail
ChIP Lysis Buffer	50 mM HEPES-KOH pH7.5 140 mM NaCl 1 mM EDTA pH8 1% Triton X-100 0.1% Sodium Deoxycholate 0.1% SDS 1X Protease Inhibitors
ChIP dilution buffer	1% Triton X-100 150 mM NaCl 2 mM EDTA

	20 mM Tris-HCl pH 8.0
Low salt wash buffer	150 mM NaCl 1% Triton X-100 0.1% SDS, 2 mM EDTA 20 mM Tris-HCl pH 8.0
High salt wash buffer	500 mM NaCl 1% Triton X-100 0.1% SDS 2 mM EDTA 20 mM Tris-HCl pH 8.0
LiCl wash buffer	0.25 M LiCl 1% NP-40 1% Sodium Deoxycholate 1 mM EDTA 10 mM Tris-HCl pH 8.0
TE Buffer	1 mM EDTA 10 mM Tris-HCl pH 8.0
Elution buffer	1% SDS 100 mM NaHCO3
TAE buffer 10X	48.4 g of Tris base 11.4 mL of acetic acid 3.7 g of EDTA 1L H2O
Annealing buffer 10X	1M NaCl, 100 mM Tris-HCl, pH=7.4

2.1.5 Cell culture materials (Table.5)

Material	Product no.	Company
25 cm^2 flask	690175	Greiner Bio one
75 cm^2 flask	658175	Greiner Bio-one
175 cm^2 flask	660175	Greiner Bio-one
96 well plate	655180	Greiner Bio-one
24 well plate	662160	Greiner Bio-one

12 well plate	665180	Greiner Bio-one
6 well plate	657160	Greiner Bio-one
10 cm^2 plate	664160	Greiner Bio-one
PCR tube	0030 124.359	Eppendorf
96-well qPCR plate	4346907	Life Technology
0.5ml tube	0030 121.023	Eppendorf
1.5ml tube	0030 120.086	Eppendorf
2ml tube	0030 123.344	Eppendorf
15ml tube	188271	Greiner Bio-one
50ml tube	227261	Greiner Bio one
10µl tip	S1111-3000	StarLab
200µl tip	S1111-0006	StarLab
1ml tip	S1111-6001	StarLab
5ml Pipette	606180	StarLab
10ml Pipette	607180	StarLab
20ml Pipette	760180	StarLab
50ml Pipette	768180	StarLab
Cell scraper	83.1830	SARSTEDT

2.1.6 Instruments and Softwares (Table.6)

Apparatus or softwares	Company
Cell culture Incubator	Heraeus GmbH (Germany)
Centrifugation	Eppendorf (Germany)
Chemismart 5100	PEQLAB (Germany)
GraphPad Prism 8.0	GraphPad Software (USA)
Immunofluoerscence optical Microscopy	Olympus (Germany)
Infinite M200	Tecan

Inverted microscopy	Zeiss (Germany)
Light microscope	Leica (Germany)
Microwave oven	Sharp (USA)
pH-Meter 538 Multical	WTW (Germany)
Real-time PCR	Biosystems
TCS SP2 Confocal Microscope	Leica (Germany)

2.2 Methods

2.2.1 Patients

A total 19 ACLF patients were enrolled in this study. Ten patients were from the Department of Gastroenterology and Hepatology, Beijing You'an Hospital, Affiliated with Capital Medical University and another 10 from the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School. Liver tissue specimens were obtained by surgery (e.g. liver transplantation) or transjugular liver biopsy (for recovered ACLF patients). Given the very limited liver tissue obtained from transjugular liver biopsies, 10 liver biopsies obtained from Beijing (5 recovered and 5 receiving LTx) were perform IHC staining for HNF4α and p-SMAD2. Nine liver tissues collected from Hannover (4 recovered and 5 receiving LTx) were used for measuring p-SMAD2, FOXH1 and HNF4α.

All enrolled patients were hospitalized for at least 1 day due to ACLF. ACLF was defined as a syndrome that develops in patients with an acute decompensation of chronic liver disease and is characterized by development of organ failure and high short-term mortality. The diagnostic criteria in brief were: (i) TBIL> 10 ULN or increased TBIL 1mg/d with or without grade 2 to 3 ascites within <2 weeks; (ii) overt hepatic encephalopathy; (iii) gastrointestinal hemorrhage; (iv) INR>≥1.5, (v) bacterial infections (spontaneous bacterial peritonitis, spontaneous bacteremia, urinary tract infection, pneumonia, cellulitis) (Moreau et al., 2013). The study protocol was approved by three local Ethics Committees. Written informed consent was obtained from patients or their representatives. In Beijing, allocation and timing of LTx was governed by China Liver Transplant Registry (CLTR) (Wan et al., 2016), an official organization for scientific registry authorized by the Chinese Health Ministry, according to the Model for End-Stage Liver Disease (MELD) score of the patient. In Hannover, liver transplantations were organized by Eurotransplant.

2.2.2 Immunohistochemical staining

1) Deparaffinization

Performed the following washing on the slides

(1) 3 x 5 minutes in Xylene

(2) 3 minutes in Xylene 1:1 with 100% ethanol

(3) 5 minutes in 100% ethanol

(4) 5 minutes in 95% ethanol

(5) 5 minutes in 70% ethanol

(6) 5 minutes in 50% ethanol

(7) 2 x 5 minutes in PBS

2) Antigen Unmasking

Antigen retrieval was performed using a microwave with Tris/EDTA pH 9.0 buffer

(1) 10 to15 seconds boiling
(2) 45 to 50 seconds waiting } 10 cycles

3) Blocked

(1) 3 x 5 minutes in PBS

(2) 30 minutes in DAKO Blocking Peroxide

(3) 5 minutes in PBS

(4) 15 minutes in 0.3 %H2O2

4) Stained primary antibody

(1) 3 x 10 minutes in PBS

(2) Incubated with primary antibodies overnight at 4°C

5) Stained secondary antibody

(1) 3 x 10 minutes in PBS

(2) Incubated with secondary antibodies 45 minutes at room temperature

(3) 3 x 10 minutes in PBS

6) Stained horse radish peroxidase (HRP)

(1) Incubated with DAB solution (10mg DAB in 15ml 50mM pH 7.6 Tris (hydroxymethyl)-aminomethean, 12µl H_2O_2) 10 minutes.

(2) Stained in hematoxylin 20 seconds.

(3) Washed in tap water 10 minutes.

7) Dehydrate sections

(1) 2 x 10 seconds in 95% ethanol

(2) 2 x 10 seconds in 100% ethanol

(3) 2 x 10 seconds in 100% xylene

(4) Mounted slides with malinol mounting medium.

(5) Analyzed staining results with microscope.

2.2.3 Cell culture and treatment

HEK293T cells were used to construct lentivirus and generate stable cell lines expressing target genes. The cells were grown in DMEM medium with 10% FBS, 1% L-glutamine and 100U/ml penicillin G/streptomycin sulfate. BMOL cells were kindly provided by Dr. George Yeoh (University of Western Australia). BMOL cells were maintained in Williams E medium supplemented with 10% (v/v) heat inactivated FBS, 1% L-glutamine and 100U/ml penicillin G/streptomycin sulfate. HepaRG cells were purchased from Biopredic International (Saint Gregoire, France). The cells were cultured in Williams E medium supplemented with 10% (v/v) heat inactivated FBS, 5 µg/ml insulin, 50 µM hydrocortisone hemisuccinate, 1% L-glutamine and 100U/ml penicillin G/streptomycin sulfate. Primary hepatocyte isolation was performed as previous described (Godoy et al., 2009). Hepatocytes were cultured in Williams E medium with 10% FBS, 4 mM L-glutamine, 100 U/ml Pen/Strep, 5.5 µg/ml transferrin, 5 ng/ml selenium and 40 ng/ml Dexamethasone in a collagen I coated plate.

2.2.4 Plasmids

1) Foxh1 and Control shRNA oligonucleotides were cloned into the pLKO.1 vector.
2) Reconstituted oligos to 0.1 nmol/µl with ddH_2O.
3) Mixed 11.25 µl of each oligo and 2.5 µl 10x annealing buffer.
4) Incubated oligo mixtures in a >95°C water bath and naturally cool down to room temperature.
5) Mixed 1 µl of the oligo mixture with 0.5X annealing buffer in 1:400.
6) Digested pLKO.1 with AgeI and EcoRI and gel-purify the digested vector.
7) Ligation reaction at 16°C overnight:

   Oligo mixture or 0.5X buffer 1 µl
   Purified digested pLKO.1 vector 1 µl (10 ng/µl)
   10X ligase buffer 1 µl
   T4 DNA ligase 1 µl
   ddH_2O 6 µl

   } 10 reaction volume

8) Transformed the ligation mixture into competent DH5α cells.

(1) Added 10 µl of ligation mixture to DH5α cells, incubate for 20-30 min.
(2) Heat shock for 20 second at 42°C.
(3) Incubated on ice for 5 min.

(4) Added 1ml of LB growth media.
(5) Shaked for 1h at 37°C.
(6) Plated DH5α cells onto 50μg/mL Ampicillin LB plates.
9) Picked individual colonies and culture in in Ampicillin LB medium. Isolate DNA for sequencing.

2.2.5 Production of Lentivirus and generation of stable cell lines

For lentivirus production, the process was as follows:

1) HEK293T cells were co-transfected with pLKO.1 shFoxh1/shControl vectors, psPAX2 and a VSV-G–encoding plasmid at a 1:1:0.5 ratio. Plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen, USA). Transfection medium was changed 8 h since co-transfection.
2) 72 hours later, medium containing Lentivirus were filtered through a 0.22 μm sterile, centrifuged at 30000g for 4h, harvested and stored at −80 °C until use.
3) BMOL cells were incubated with Foxh1 shRNA or Control shRNA lentivirus for 2d, followed by selection with 10 μg/ml puromycin.

2.2.6 RNA interference

Human ALK4 siRNA (162106, Ambion, DE), Mouse ALK4 siRNA (M-043507-01, Dharmacon, USA), Human/Mouse SMAD2 siRNA (156216, Ambion, DE), human SMAD3 siRNA (SI05062645, QIAGEN, DE), Mouse SMAD3 siRNA (156947, Ambion, DE), Human/Mouse SMAD4 siRNA (M-040687-00, Dharmacon, USA), Human FOXH1 siRNA(M-008636-00, Dharmacon, USA), human HNF4α (M-003406-02, Dharmacon, USA), mouse HNF4α siRNA (M-065463-00, Dharmacon, USA) and control siRNA (1027281, Qiagen) were transfected into cells with Lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's instruction:

1) Mixed 30-pmol siRNA pool in 500 μl Opti-MEM Medium in a 6-well cell culture plate.
2) Added 5 μl Lipofectamine ™ RNAiMAX to each well, mixed gently and incubated for 10 minutes.
3) Added 2-4 x 10^5 cells to each well containing the diluted siRNA-RNAiMAX complex and rocked the plate back and forth to mix well. Incubated the cells 24-72 hours in the incubator.

2.2.7 Western blot

1) RIPA buffer with protease and phosphatase inhibitors extracted cell protein lysates.
2) Added 2 μl of protein sample to 25 μl of the mixture of Reagent A and Reagent S (Reagent A to Reagent B: 1:50), then added 200 μl of Reagent B, and incubated at room temperature for 10-60 minutes. Measured the absorbance of the sample at the absorbance of 595nm, and compared the standard concentration of BSA to get the concentration of the sample.
3) 20μg samples were separated by 8%-12% SDS-PAGE and then transferred to PVDF membranes.
4) Incubated with 5% BSA for 1h at room temperature.
5) Incubated with primary antibodies overnight at 4°C.
6) Washed with PBS-T for 10 minutes three times.
7) Probed with secondary antibodies 1h at room temperature.
8) Washed with PBS-T for 10 minutes three times.
9) Developed with a chemiluminescent substrate (Amersham, Freiburg, DE).

2.2.8 RNA extraction and quantitative real time-PCR (qRT-PCR)

Total RNA was extracted from cells with InviTrap Spin Universal RNA Mini Kit (Stratec, DE) according to the manufacturer's instructions.

1) Lysed the samples with 350μl or 700 μl 1% 2-Mercaptoethanol-containing lysis solution TR and mixed thoroughly at room temperature.
2) Transfered the lysate onto a DNA-binding spin filter with a 2.0 ml receiver tube and centrifuged for 1 min at 13000 rpm at room temperature.
3) Keep the solution, added 1 volume of 70% ethanol and transfer to a RNA-RTA spin filter. Centrifuged for 2 min at 13000 rpm at room temperature.
4) Added 600μl wash buffer R1 onto the RNA-RTA spin filter and centrifuged for 1 min at 13000 rpm at room temperature.
5) Added 700μl wash buffer R2 onto the RNA-RTA spin filter and centrifuged for 1 min at 13000 rpm at room temperature. Repeated this step one time.
6) Centrifuged for 4 min at 13000 rpm at room temperature to eliminate the remnant of ethanol.

7) Added 30 µl elution buffer R onto the RNA-RTA spin filter and centrifuge for 1 min at 13000 rpm at room temperature.
8) Measured the RNA concentration at 260nm with Tecan infinite M200.

Reverse transcription was performed to synthesis cDNA using RevertAid H Minus Reverse Transcriptase (Thermo Fischer Scientific, USA).

1) Mixed 1µg RNA sample, 1µl random hexamer and appropriate volume of sterile, nuclease-free water to 13µl total volume.
2) Incubated at 65 °C for 5 min and chill on ice.
3) Added 4 µl 5X Reaction Buffer, 0.5 µl Thermo Scientific™ RiboLock™ RNase Inhibitor, 2µL 10 mM dNTP mix, and 0.5 µl RevertAid H Minus Reverse Transcriptase.
4) Performed the following process
(1) 10 min at 25 °C
(2) 1h at 42 °C
(3) 10 min at 70 °C
5) Used the cDNA directly in qPCR or store at -20 °C.

The qRT-PCR assays were performed using POWRUP SYBR MASTER MIX (Life Technologies, USA) by a StepOnePlus Real-time PCR instrument (Applied Biosystems, USA).

1) Number of reactions

	Volume (20 µl/well)
PowerUp™ SYBR™ Green Master Mix (2X)	10 µl
Forward and reverse primers	0.5 µl+0.5 µl (10µM)
Diluted cDNA template	2-4 µl
Nuclease-Free water	To 20 µl

2) Standard cycling mode

Step	Temperature	Time	Cycles
UDG activation	50°C	2 minutes	Hold
Dual-Lock™ DNA polymerase	95°C	2 minutes	Hold
Denature	95°C	15 seconds	40
Anneal/extend	60°C	1 minute	

3) Dissociation curve conditions (melt curve stage)

Step	Temperature	Time
1	95°C	15 seconds
2	60°C	1 minute
3	95°C	15 seconds

2.2.9 Histone extract

1) Cells were lysed in Triton Extraction Buffer (TEB: PBS containing 0.02% (w/v) NaN3, 0.5% Triton X 100 (v/v), 2mM phenylmethylsulfonyl fluoride) with proteinase inhibitor (Roche) on ice for 10 minutes with gentle stirring and centrifuged at 2000rpm for 10 minutes at 4°C.
2) After discarding the supernatant and washing the pellets with cold TEB buffer with proteinase inhibitor twice, suspended and incubated the pellet at 4°C overnight in 0.2N HCl.
3) After adding 100% trichloroacetic acid, incubating on ice for 2h, centrifugation at 13000rpm for 10 min at 4°C and washing by cold acetone, the histone pellets were dissolved with 150Mm NaCl.

2.2.10 Protein complex immunoprecipitation (Co-IP)

1) Ten cm plates cultured cells were lysed with 1ml ice-cold IP lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 1% Nonidet P-40 (NP-40), 2 mM EDTA) with protease and phosphatase inhibitors.

2) After centrifugation, the supernatants were transferred to a new tube with 1 μg appropriate control IgG and 20 μl Protein A/G PLUS-Agarose beads for pre-clearing.
3) Following incubation at 4° C for 1h, the supernatants were centrifuged at 1000xg for 10 min at 4° C.
4) Subsequently, 1mg of the cell lysis supernatants were incubated with 3 μg primary antibody or IgG for 3-4 hour at 4° C.
5) Add 40 μl Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, USA) to the tubes and incubated the samples at 4° C on a rotating device overnight.
6) Centrifuged the tubes, removed the supernatant and washed the beads with lysis buffer three times.
7) After last wash, discard supernatant and suspended beads in 40 μl 1x SDS sample buffer.
8) Samples were analyzed using Western blotting as previously described.

2.2.11 ChIP

1) Cells were cross-linked by 1% formaldehyde-PBS solution for 10 minutes at room temperature and 125 mM glycine for 5 minutes, respectively.
2) Pellets were washed by cold PBS twice, centrifuged at 4 °C, resuspended in Lysis Buffer (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA pH8, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS and 1X fresh protease Inhibitors) and incubated for 10 min on ice.
3) Cell pellets were sonicated to obtain an average fragment size of 500 bp DNA by the BioRuptor water bath sonicator (Diagenode) with 30 seconds on/30 seconds off cycles for 20-30 times.
4) Then, supernatants were collected by centrifugation. Use 50 μL of each sonicated sample as inputs.
5) Immunoprecipitation aliquots were diluted into 1:10 with ChIP dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, and 20 mM Tris-HCl pH 8.0) and then incubated on rotation at 4 °C with 5μg of the primary antibody or IgG overnight.
6) Next day, 50μL Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, USA) and 4μg single-stranded herring sperm DNA were added to each sample and incubated at 4° C on a rotating device for 3-4 hours.

7) After centrifugation, removed supernatant and washed beads for 5 minutes at 4 °C with rotation with the following buffers sequentially: low salt wash buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl pH 8.0), high salt wash buffer (500 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, and 20 mM Tris-HCl pH 8.0), LiCl wash buffer(0.25 M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0), and TE Buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0).
8) Subsequently, the beads were suspended in 120 uL fresh elution buffer (1% SDS, 100 mM NaHCO3) and vortex slowly for 15 min at 30°C.
9) The supernatants and inputs samples were incubated with 4.8 µL of 5 M NaCl and 2 µL RNase A (10 mg/mL) (Sigma Aldrich, USA) at 65 °C overnight to reverse the crosslinks.
10) Following RNase digestion, samples were incubated by 2 µL proteinase K (20 mg/mL) (Sigma Aldrich, USA) at 60 °C for 1 hour.
11) All samples were purified using a MinElute PCR Purification Kit (Qiagen, DE). The PCR productions were showed by 2% Agarose gel electrophoresis.

2.2.12 ELISA

Insulin, glucagon, follistatin and Activin in patient serums were measured by ELISA according to the manufacturer's protocols.

1) Dilute the capture antibodies with PBS to the working concentration and coat them into 96-well microplates with 100 µL per well. Incubate overnight at room temperature.
2) Discard the diluted capture antibodies and wash three times with 400 µL wash buffer. After the last wash, remove any remaining wash buffer completely.
3) Block plates by 300 µL of reagent diluent and incubate 2 hours at room temperature.
4) Discard the diluted reagent diluent and wash three times with 400 µL wash buffer. After the last wash, remove any remaining wash buffer completely.
5) Add 100 µL of serum samples or kit-provided standard samples per well. Add 100 µL/well of prepared 1M Urea in PBS to samples and standards, gently mix and incubate 3 hours at room temperature.
6) Discard the samples and wash three times with 400 µL wash buffer. After the last

wash, remove any remaining wash buffer completely.

7) Add 100 µL of the reagent diluent diluted detection antibodies to each well and incubate 3 hours at room temperature.
8) Discard the detection antibodies and wash three times with 400 µL wash buffer. After the last wash, remove any remaining wash buffer completely.
9) Add 100 µL of the working dilution of Streptavidin-HRP C to each well and incubate for 20 minutes at room temperature. Avoid light.
10) Discard the Streptavidin-HRP C and wash three times with 400 µL wash buffer. After the last wash, remove any remaining wash buffer completely.
11) Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid light.
12) Add 50 µL of Stop Solution to each well. Gently mix.
13) Plates were read at 450 nm by a microplate reader.
14) Prepare a standard curve based on the serial dilutions data with concentration on the x axis (log scale) versus absorbance on the Y axis (linear). Calculate the concentration of the samples from this standard curve.

2.2.13 Statistics analysis

Results were expressed as mean ± standard deviation (SD). Two-tailed unpaired Student t-test was used to calculate P values between groups. P values less than 0.05 were considered significant and represented graphically as * $p<0.05$; ** $p<0.01$; *** $p<0.001$, unless otherwise indicated. All data analyses were conducted using GraphPad Prism version 8.0.

3 RESULTS

3.1 Recovered ACLF patients robustly express HNF4α in liver progenitor cells and hepatocytes

To clarify why a proportion of patients survive ACLF, we first compared clinical parameters and liver histological alterations between 5 recovered and 5 irreversible ACLF patients. All patients had known etiology and clinical duration, i.e. the interval between the first symptoms of acute decompensation and the time of tissue sampling. Both cohorts were of similar age and clinical duration (*P*>0.05, **Figure 9**). Among the 10 patients, 5 were HBV-induced ACLF whereas 5 developed liver failure due to herbal toxicity. Clinical duration in the recovered and irreversible patients was 20 – 180 days and 10 – 270 days, respectively (*P*>0.05, **Figure 9**). Meanwhile, key biochemical and clinical parameters were examined 24 hours before liver histological examination (transjugular liver biopsy in the recovered patients *versus* liver explant in the irreversible patients). There were no statistical differences in serum ALT, AST and albumin levels between the two cohorts (*P*>0.05, **Figure 9**).

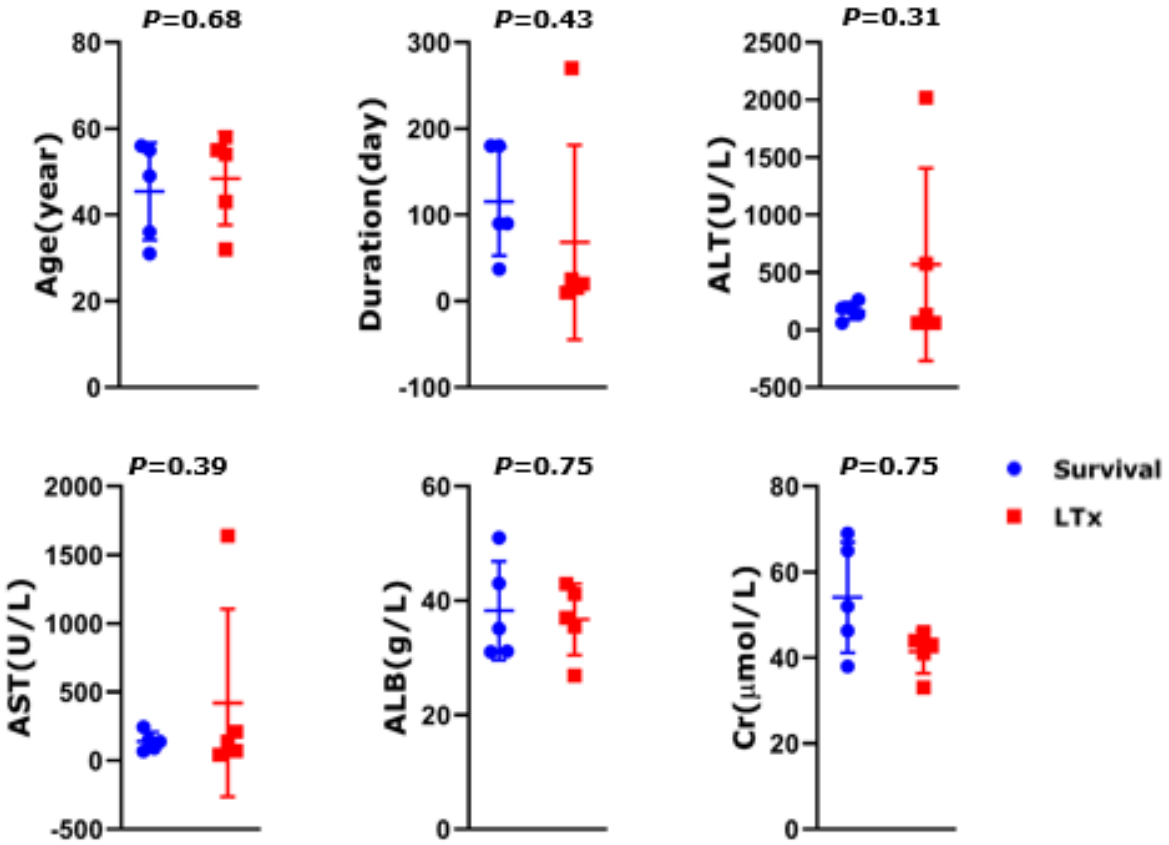


Figure 9. Age, clinical duration, serum ALT, AST, albumin and creatinine (Cr) are listed for 5 survival

and 5 irreversible ACLF patients receiving liver transplantation (LTx).

However, three parameters were strikingly different: total bilirubin (TBIL, 41.8 ± 18.2 *vs* 422.5 ± 187.7, *P*=0.02), INR (1.15 ± 0.13 *vs* 3.52 ± 1.06, *P*=0.001) and MELD score (6.43 ± 2.57 *vs* 28.64 ± 3.25, *P*<0.001, **Figure 10**). These results demonstrate that compared to the recovered ACLF patients, bilirubin metabolism and coagulation function were insufficient in the irreversible cohort.

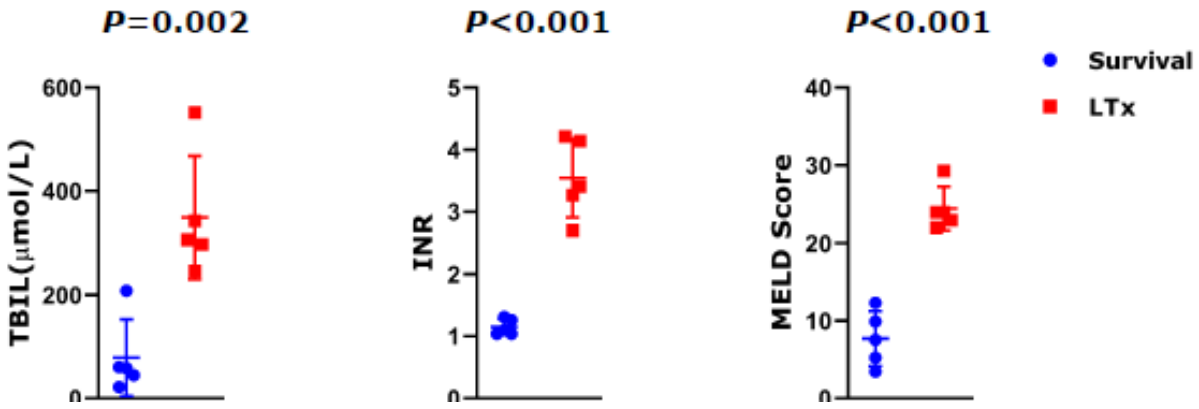


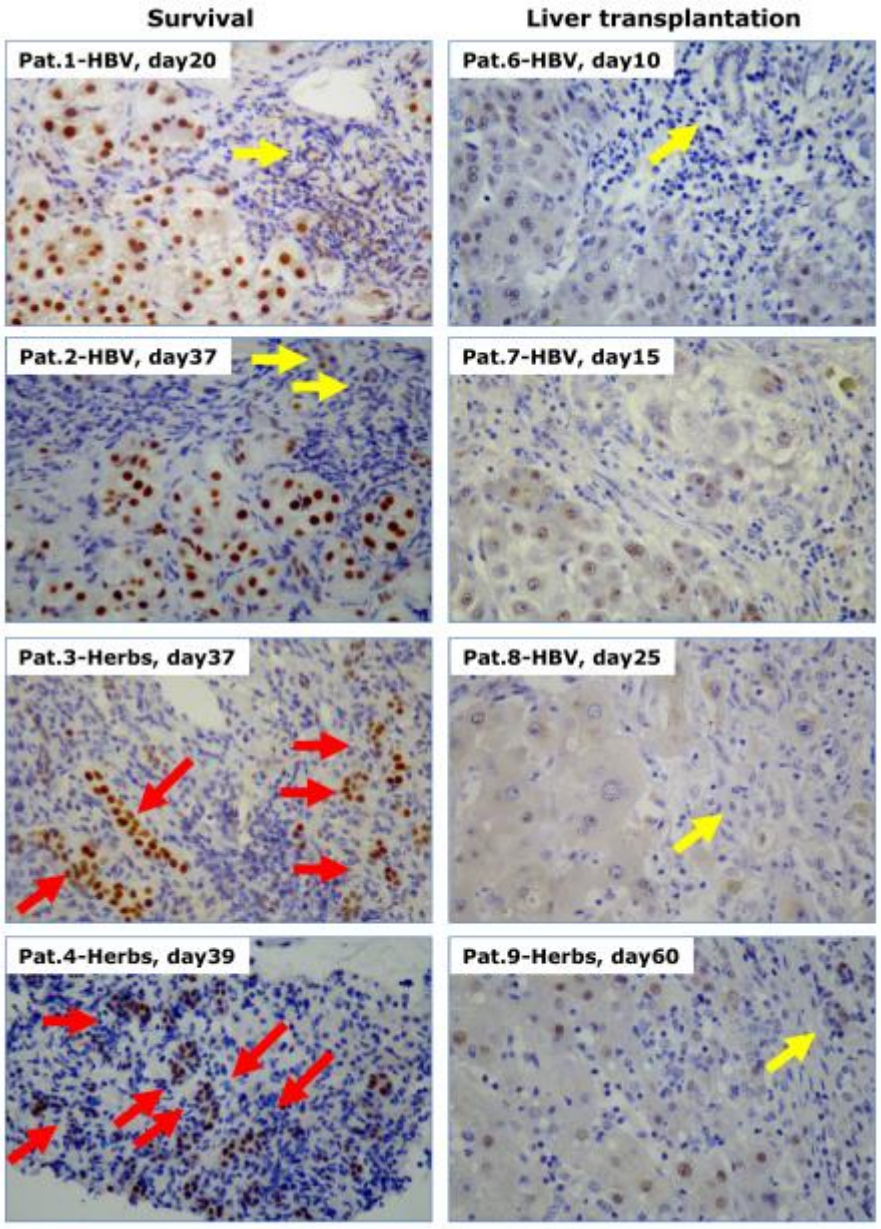
Figure 10. Serum total bilirubin (TBIL), international normalized ratio (INR) and the Model for End-stage Liver Disease (MELD) scores are compared between 5 survival and 5 irreversible ACLF patients receiving liver transplantation (LTx).

Given the crucial role of HNF4α in the regulation of coagulation (Odom et al., 2004), we subsequently performed immunohistochemical staining (IHC) for HNF4α in the collected liver tissues. As shown in **Figure 11A**, the recovered patients displayed intensive HNF4α immune positivity in the nuclei of both hepatocytes and LPC. Notably, in areas with remaining hepatocytes, immune reactivity of HNF4α in LPC was very weak (yellow arrows in Pat.1 and 2, **Figure 11A**). However, in those areas lacking hepatocytes, robust HNF4α immune reactivity was only observed in the nuclei of LPC (red arrows in Pat.3 and 4, **Figure 11A**). In contrast to the recovered patients, the irreversible patients demonstrated rather weak immune reactivity of HNF4α in both hepatocytes and LPC (yellow arrows depicting LPC, **Figure 11A**). We further quantified positive HNF4α immune reactions in LPC and hepatocytes. In biopsied specimens collected from 5 recovered patients, 3 demonstrated remaining hepatocytes and proliferative LPC while 2 only displayed active LPC, but no hepatocytes. In the former, all remaining hepatocytes, but no LPC, showed strong HNF4α immune reactivity, whereas in the latter, LPC displayed robust HNF4α

immune positivity (**Figure 11B**). In contrast to the recovered patients, HNF4α positivity was displayed only in a portion of hepatocytes in the 5 irreversible patients (**Figure 11B**). In these patients, only a few LPC demonstrated detectable HNF4α immune reaction (**Figure 11B**). Noteworthy, IHC showed that LPC in ACLF patients express F5 (**Figure 11C**).

These results imply a potential link between HNF4α and expression of coagulation factors in LPC.

A



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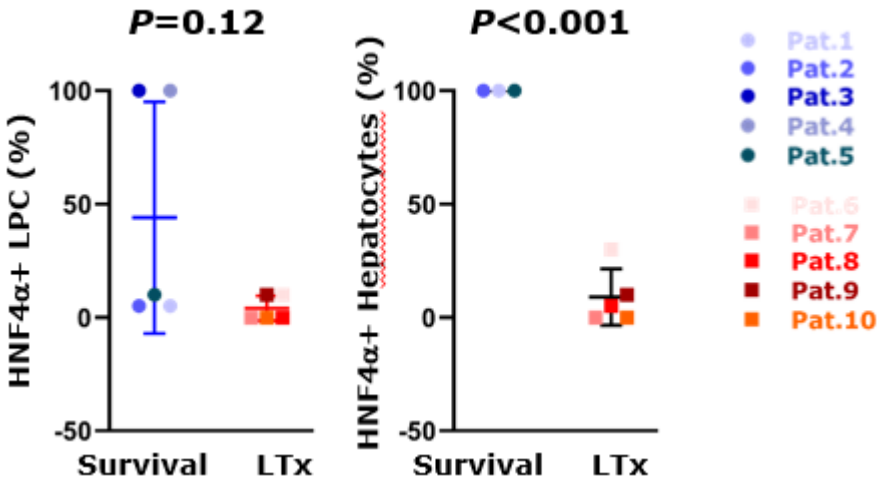


C

F5

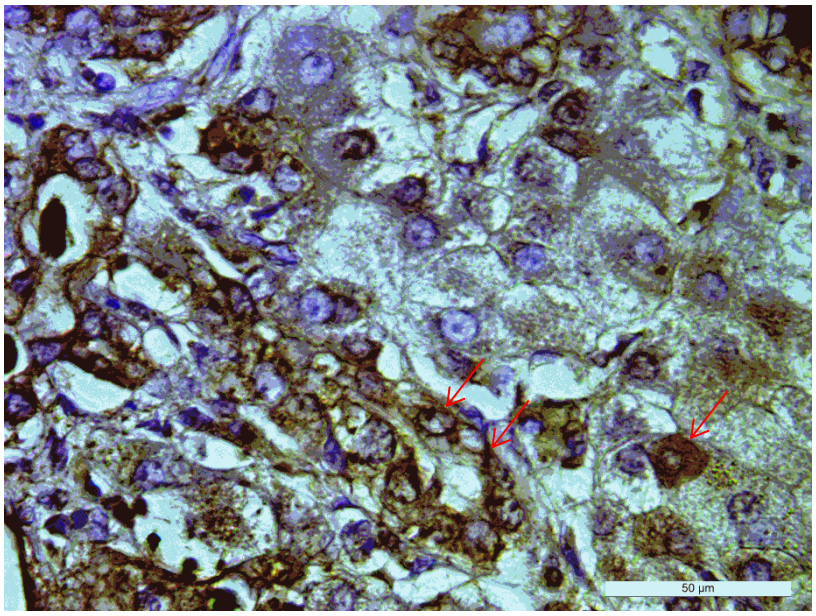


Figure 11. Recovered ACLF patients robustly express HNF4α in either LPC or hepatocytes. (**A**) Immunohistochemical staining for HNF4α was performed in these patients. Yellow and red arrows depict negative or positive HNF4α expression in liver progenitor cells (LPC). (**B**) Positive HNF4α hepatocytes and LPC were counted in the collected patients as described in Materials and Methods. (**C**) Immunohistochemical staining for coagulation factor V (F5) in liver progenitor cells (LPC) is shown in a representative patient (arrows).

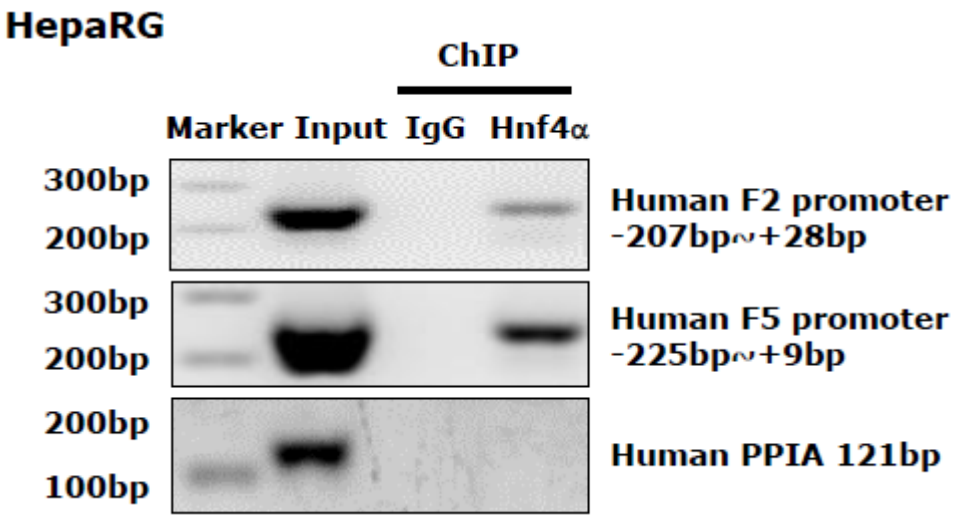
3.2 HNF4α regulates expression of multiple coagulation factors by binding to promoters in LPC

Next, we examined the effects of HNF4α on expression of coagulation factors in human HepaRG LPC cells (Gripon et al., 2002) and murine oval BMOL cells (Tirnitz-

Parker et al., 2007). Chromatin immunoprecipitation (ChIP) assays revealed that HNF4α bound to the promoters of the coagulation factor *F2* and *F5* genes in both human and mouse LPC (**Figure 12A-B**). Knockdown of HNF4α in both cell lines (>80% efficacy) significantly reduced mRNA and protein expression of coagulation factor genes *F2* and *F5* (**Figure 12C-D**).

These results suggest that HNF4α controls coagulation factor *F2* and *F5* gene expression through directly binding to their promoters in LPC.

A



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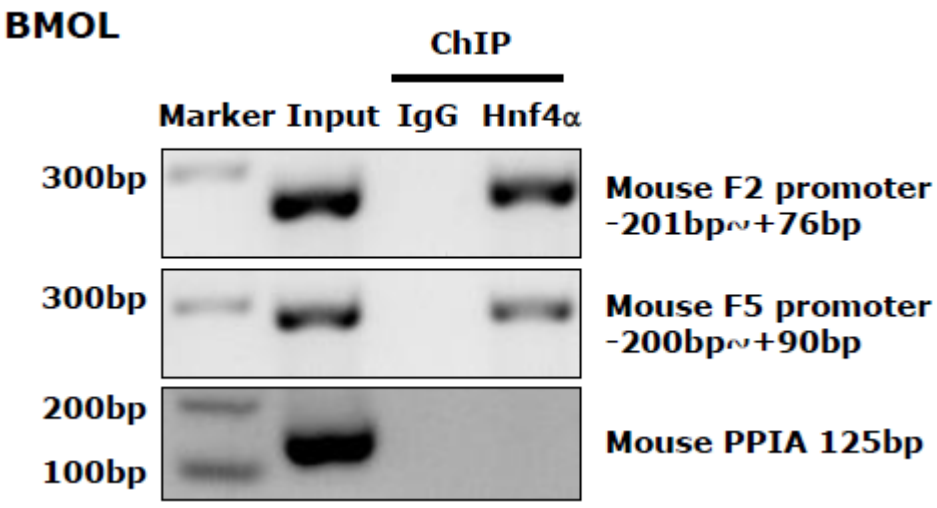

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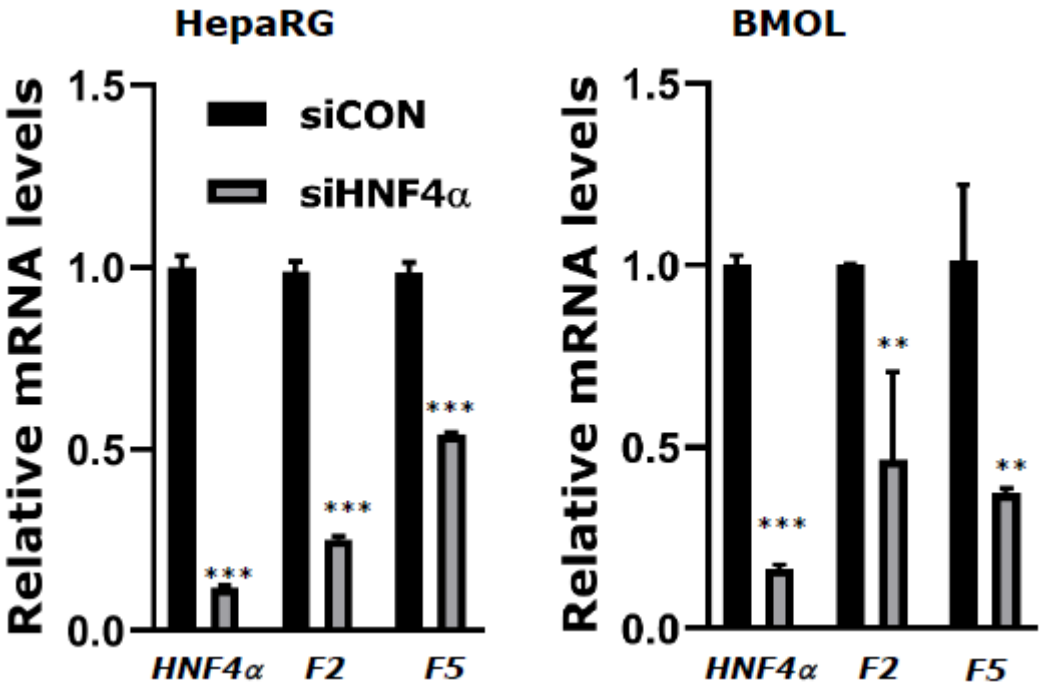


D

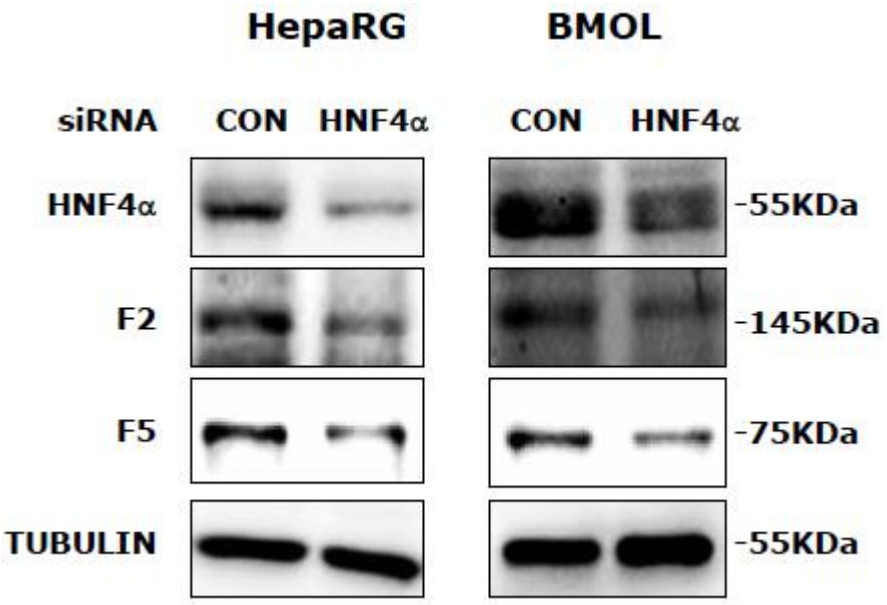


Figure 12. (**A-B**) ChIP assay was performed to measure HNF4α binding to the promoter of coagulation factor 2 (*F2*) and 5 (*F5*) genes in HepaRG and BMOL cells. (**C-D**) qPCR and Western blotting were used to measure mRNA expression of coagulation factors in HepaRG and BMOL cells with or without HNF4α RNAi. Tubulin was used as loading control in Western blotting. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

3.3 A SMADs-FOXH1 complex controls HNF4α expression in LPC

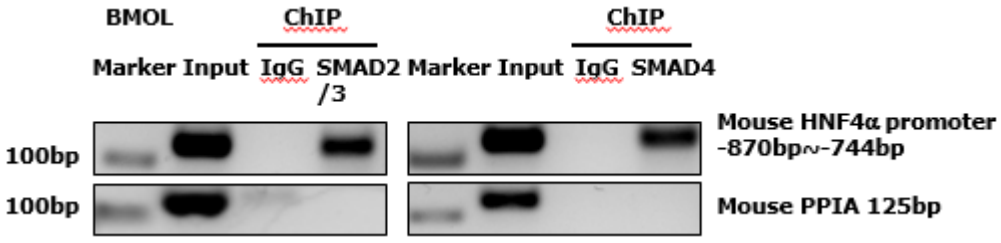
Next, we investigated potential transcription factors that control HNF4α expression in LPC. *In silico* analysis for putative transcription factors indicated that several SMAD transcription factors (SMAD2, SMAD3 and SMAD4) and FOXH1 possess binding sites in the promoter of the *HNF4A* gene (https://www.genecards.org/cgi-bin/carddisp.pl?gene=HNF4A).

ChIP assays validated the binding of SMAD2/3 or SMAD4 protein to the promoter of *HNF4A* in HepaRG and BMOL cells (**Figure 13A-B**). SMAD2/3 and SMAD4 were capable of binding to the *HNF4A* gene promoter in both cells. We also observed binding of FOXH1 to the promoter of the *HNF4A* gene in HepaRG cells (**Figure 13A**). Immunoprecipitation further showed that FOXH1 combined with SMAD2/3-SMAD4 in HepaRG cells (**Figure 13C**).

These results imply that a transcription factor complex formed by FOXH1 and SMAD proteins is required for the transcription of the *HNF4A* gene in LPC.

A

B



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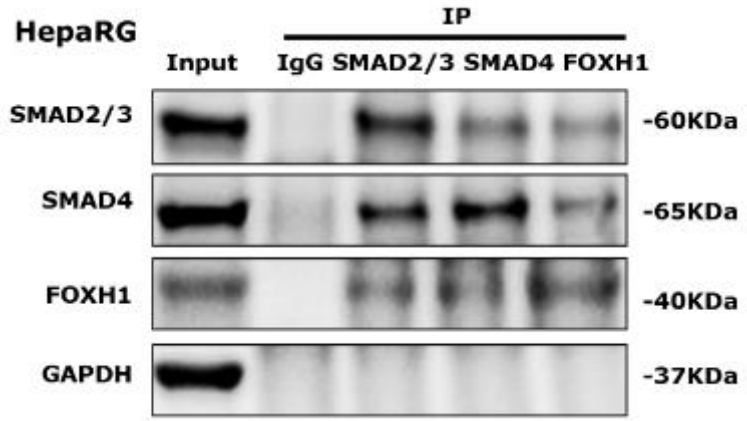
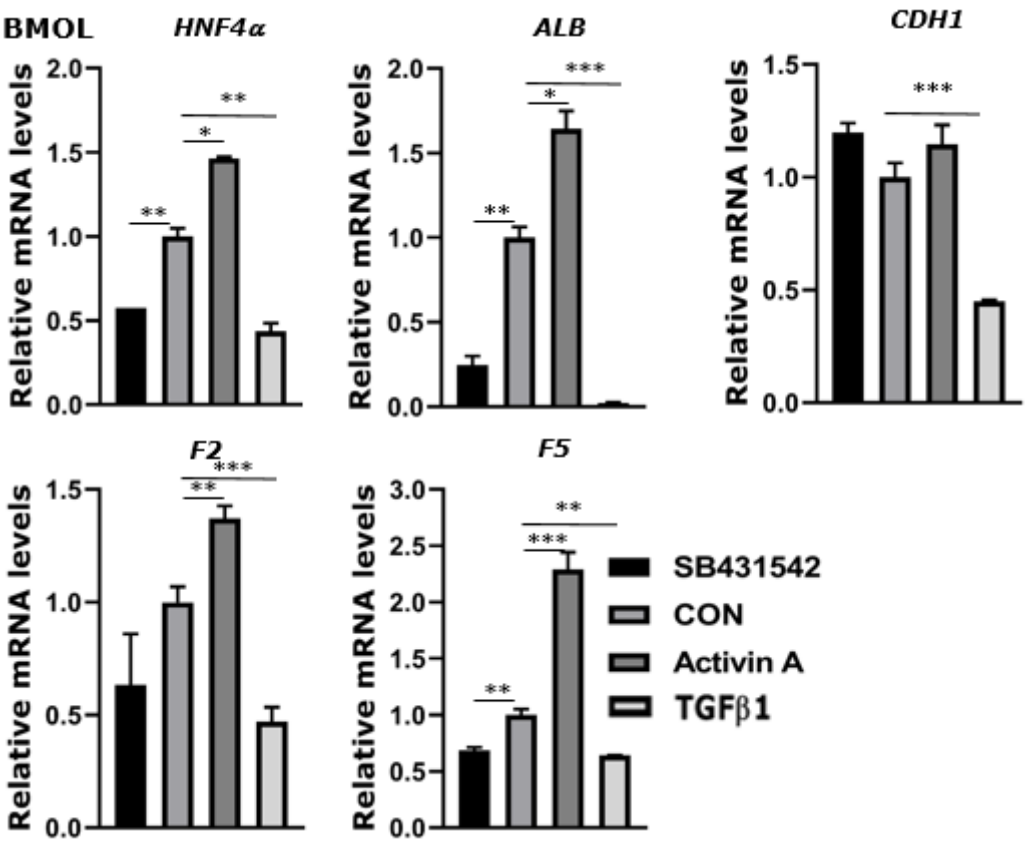


Figure 13. (**A-B**) ChIP assay was performed to measure SMAD2/3, SMAD4 and FOXH1 binding to the promoter of *hnf4α* genes in HepaRG and BMOL cells. (**C**) CoIP was performed to measure SMADs binding to FOXH1 in HepaRG cells. The expression of the cyclophilin A (PPIA, peptidylprolyl isomerase A) was used as loading control in ChIP assay.

3.4 Activin controls HNF4α and coagulation factors in LPC

Given that both TGF-β and Activin can activate SMAD2 and SMAD3 (Heldin and Moustakas, 2016; Shi and Massague, 2003), we assessed the effects of TGF-β1 and Activin A (termed TGF-β and Activin in the following) on the expression of HNF4α and coagulation factor genes in BMOL cells. Interestingly, BMOL cells displayed significantly different responses to stimulation with TGF-β or Activin. qPCR and Western blot analyses showed that 24 hours TGF-β incubation significantly inhibited expression of mRNA and protein expression of E-cadherin, HNF4α, albumin and F5 in LPC (**Figure 14A-B**). In contrast to TGF-β, Activin induced mRNA and protein expression of HNF4α, albumin and F5, but did not affect E-cadherin expression (**Figure 14A-B**). The intrinsic effects of both TGF-β and Activin were inhibited by SB431542, a cell permeable small molecule TGF-β/Activin type I receptor kinase inhibitor (**Figure 14A-B**).

A



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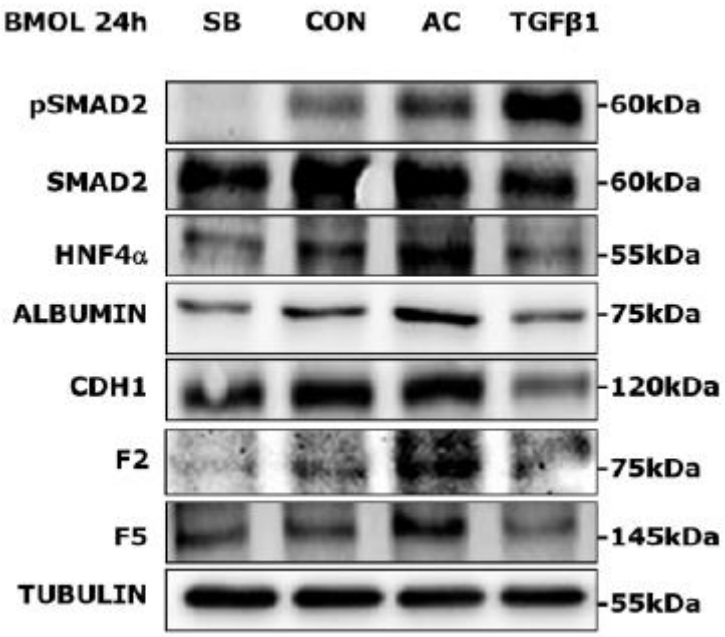
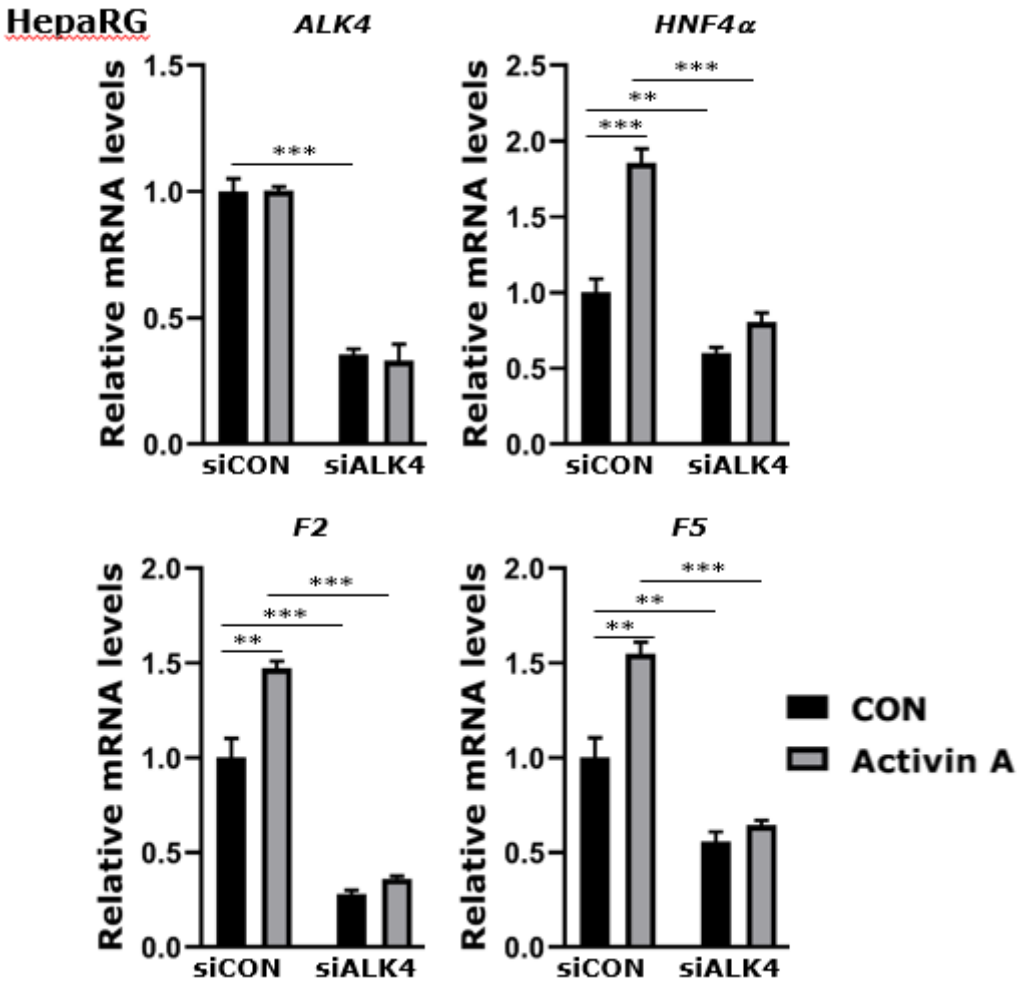


Figure 14. (**A**) qPCR was used to measure the effects of Activin, TGF-β and SB431542 administration for 24h on mRNA and protein expression of *cdh1*/E-cadherin, HNF4α, albumin and F5 in BMOL cells.

(**B**) Western blot was used to measure the effects of Activin, TGF-β and SB431542 administration for 24h on mRNA and protein expression of *cdh1*/E-cadherin, HNF4α, albumin and F5 in BMOL cells. Tubulin was used as loading control in Western blotting. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

To further investigate whether Activin plays a crucial role in HNF4α expression, we knocked down the Activin type I receptor ALK4 by siRNA in HepaRG and BMOL cells (**Figure 15A-B**). Concomitant with reduced ALK4 expression, p-SMAD2 expression was inhibited in both cell lines (**Figure 15C**). ALK4 RNAi significantly reduced Activin-dependent mRNA and protein expression of HNF4α, F2 and F5 (**Figure 15A-C**), indicating Activin specific effects towards HNF4α, albumin, F2 and F5, but not on E-cadherin. ChIP assays further showed that Activin stimulation for 24h increased binding of HNF4α protein in the gene promoters of coagulation factors *F2* and *F5* (**Figure 15D**). In contrast, the administration of SB421542 inhibited the binding (**Figure 15D**).

A



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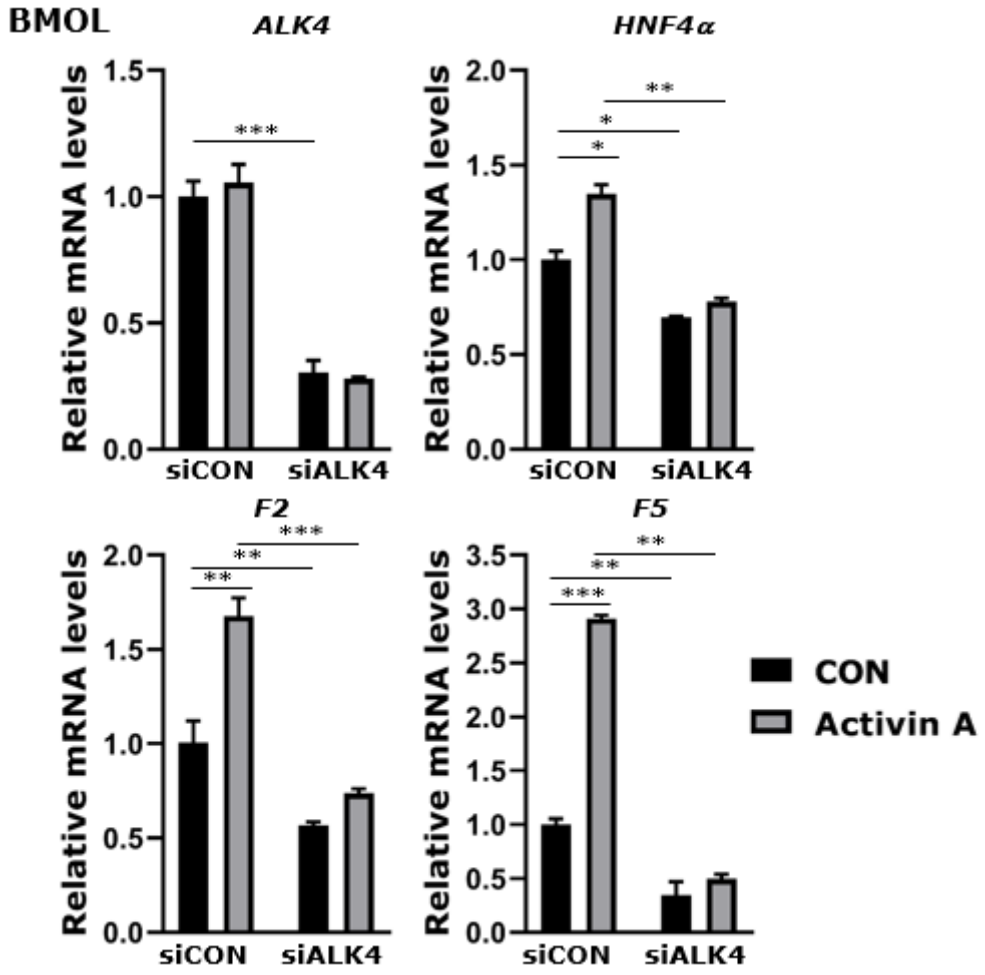
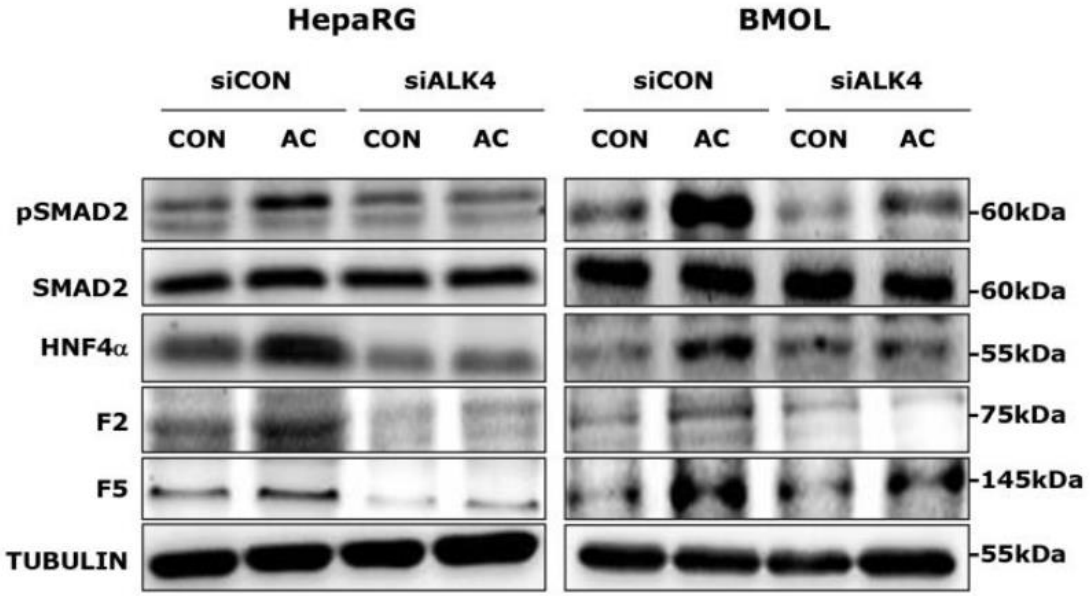

BMOL
ALK4
HNF4α
F2
F5
Relative mRNA levels
siCON
siALK4
CON
Activin A

C


HepaRG
BMOL
siCON
siALK4
CON
AC
pSMAD2
SMAD2
HNF4α
F2
F5
TUBULIN
60kDa
55kDa
75kDa
145kDa

D

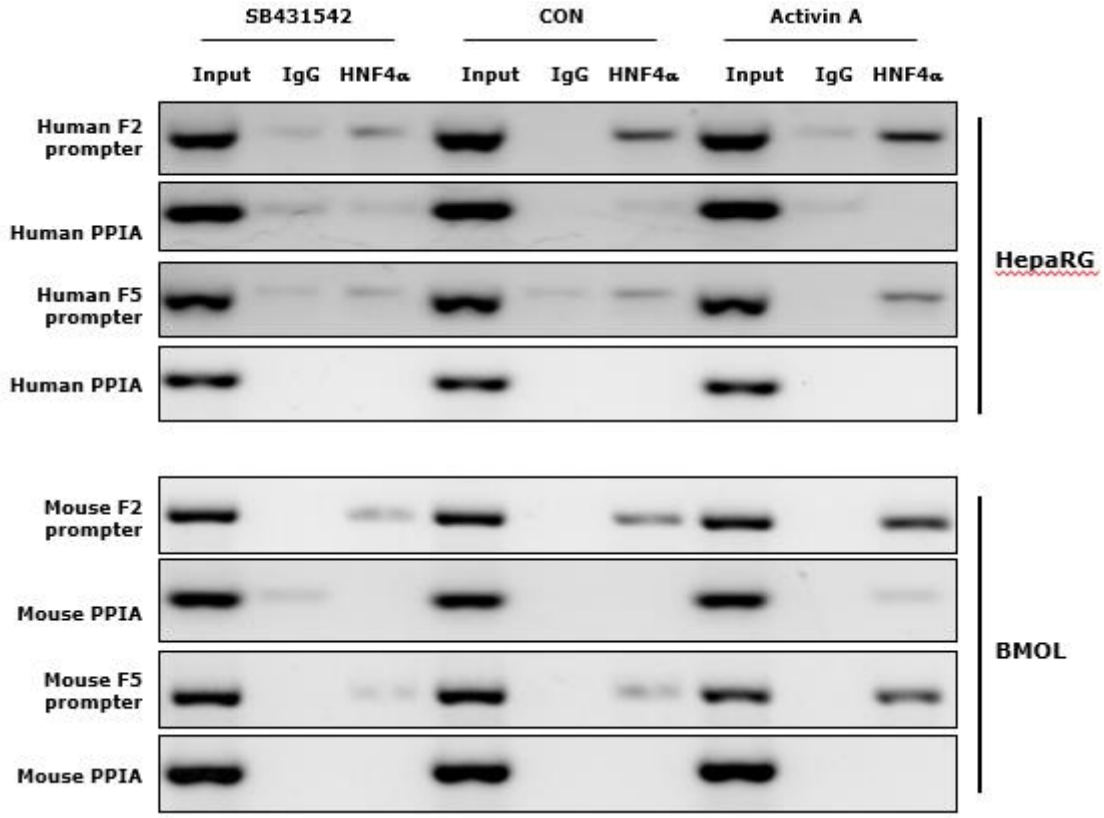
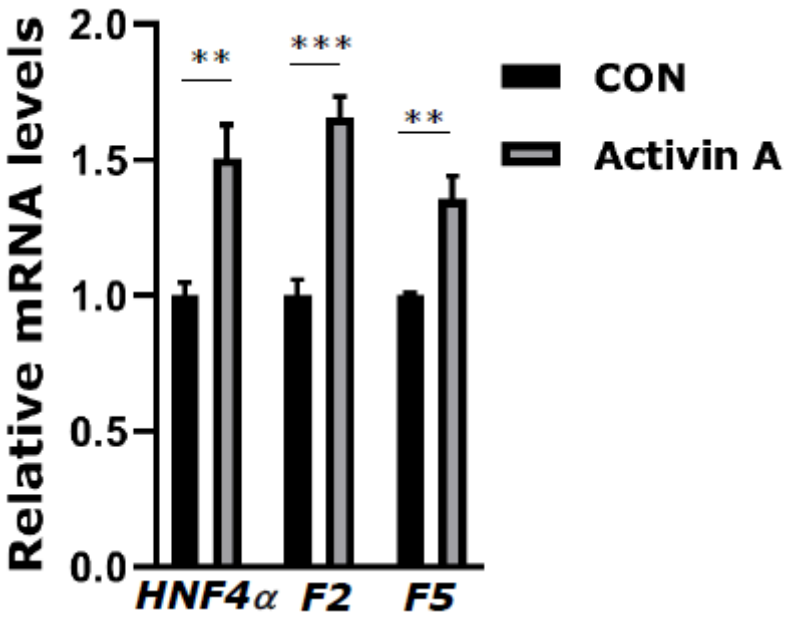


Figure 15. (**A-C**) qPCR and Western blot were performed to measure the impact of ALK4 knockdown on mRNA and protein expression of HNF4α, albumin and F5 in Activin-treated HepaRG and BMOL cells. (**D**) ChIP assay was performed to examine the impact of Activin and SB431542 on the binding of HNF4α to *F2* and *F5* gene promoters in HepaRG and BMOL cells. The expression of the cyclophilin A (PPIA, peptidylprolyl isomerase A) was used as loading control in ChIP assays. Tubulin was used as loading control in Western blotting. **P* < 0.05, ***P* < 0.01, and *****P* < 0.001.

Furthermore, we examined the effect of Activin in primary human LPC, which were isolated from healthy tissues surrounding tumor in a patient with hepatocellular carcinoma. As in LPC lines, Activin significantly induced mRNA expression of HNF4α, F2 and F5 in human primary LPC *in vitro* (**Figure 16A**). On knockdown of HNF4α in human LPC, Activin-induced F2 and F5 mRNA expression was reduced (**Figure 16B**).

These results suggest that expression of HNF4α and coagulation factors F2 and F5 in LPC is driven by Activin receptor signaling.

A



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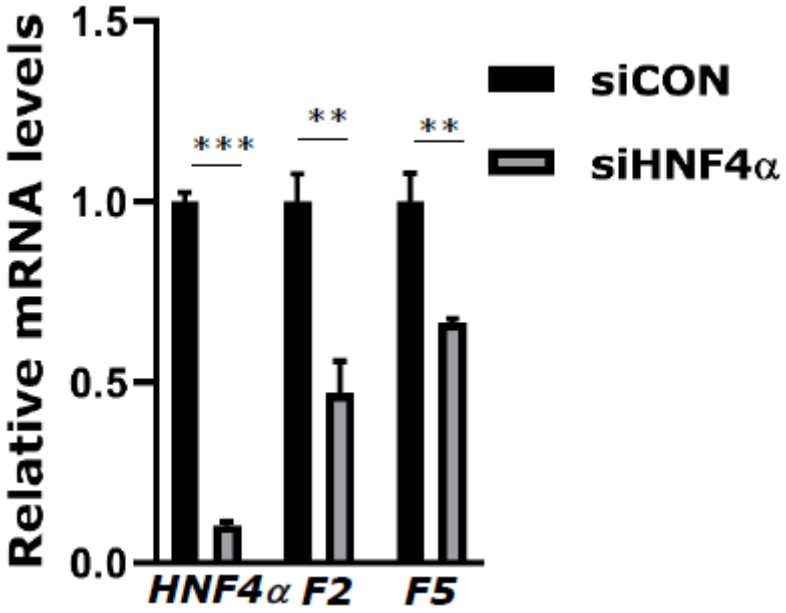


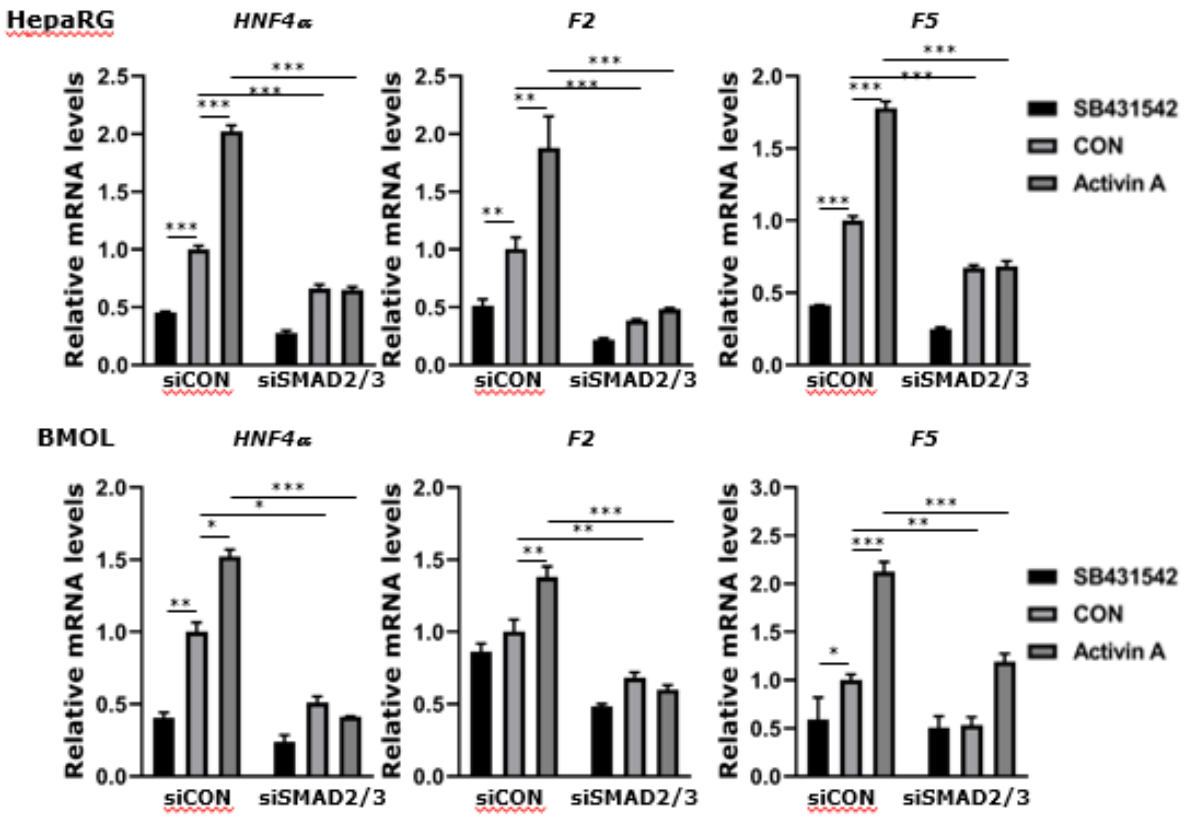
Figure 16. (**A**) qPCR measures the effects of Activin on mRNA expression of HNF4α, F2 and F5 in primary human LPC. (**B**) qPCR measures the effects of Activin on mRNA expression of F2 and F5 in primary human LPC with or without RNAi- mediated depletion of HNF4. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.5 The Activin-FOXH1-SMADs-HNF4α axis controls the expression of coagulation factors in LPC

Based on these observations, we speculated that the Activin-FOXH1-SMADs complex-HNF4α axis is controlling the expression of coagulation factors in LPC. Subsequently, we examined the effects of SMAD2, SMAD3, SMAD4, FOXH1 and HNF4α on the expression of coagulation factors F2 and F5 in LPC.

In both HepaRG and BMOL cells, knockdown of SMAD2/3 by RNAi significantly inhibited Activin-induced mRNA and protein expression of HNF4α, F2 and F5 (**Figure 17A-B**). ChIP assay further showed that Activin administration increased the binding activity of SMAD2/3 and SMAD4 in the *HNF4A* gene promoter (**Figure 17C**). In contrast, SMAD binding to the *HNF4A* gene promoter was inhibited by SB431542 (**Figure 17C**).

A



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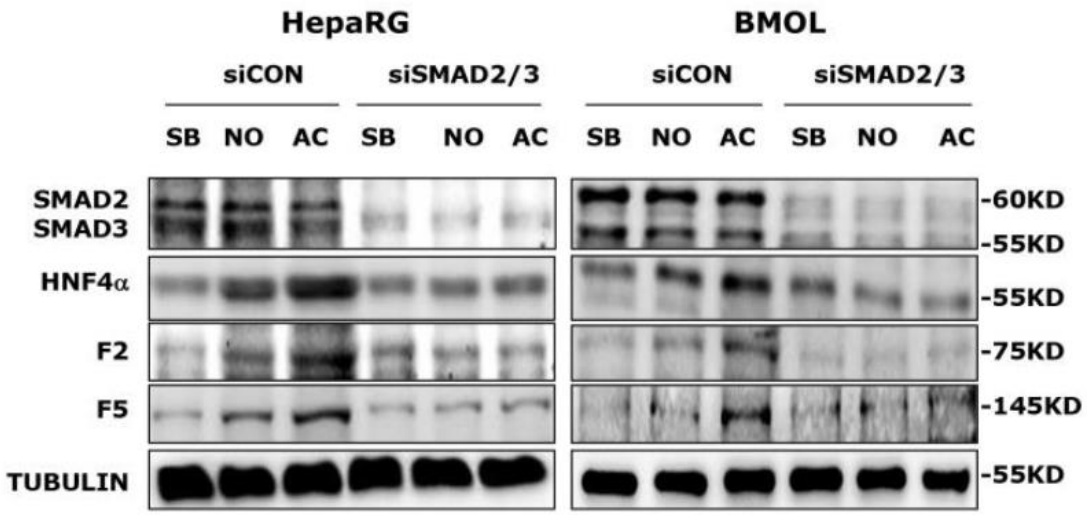

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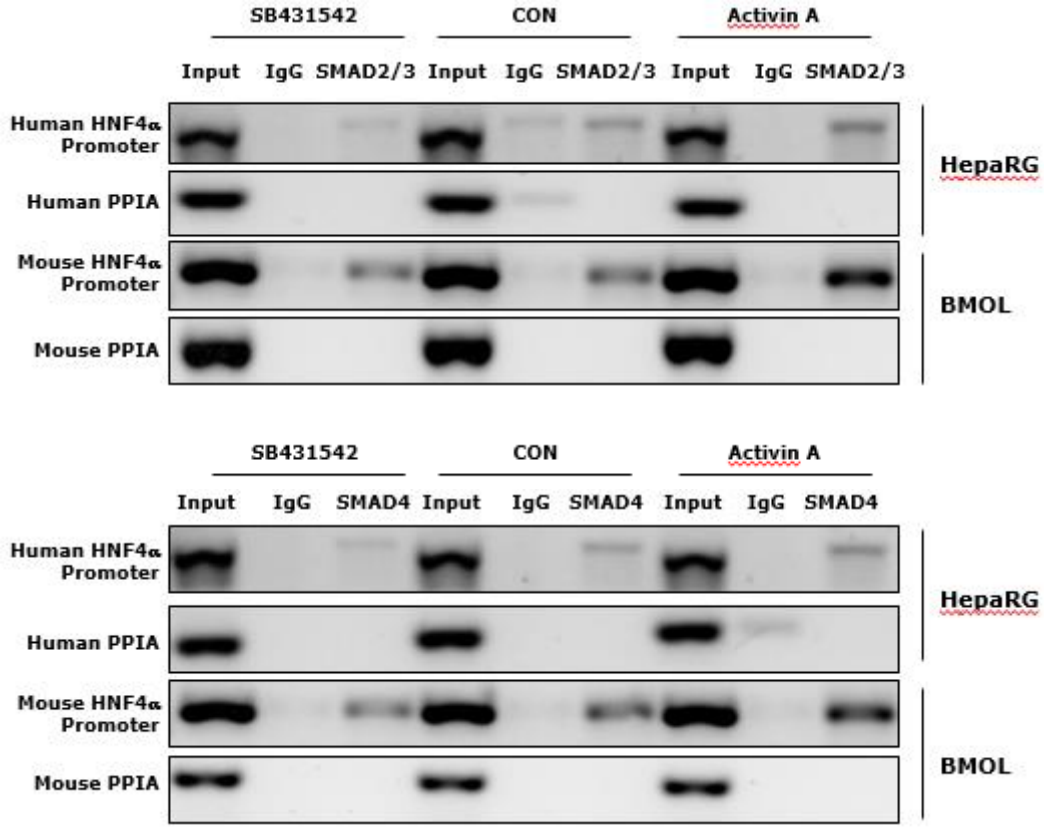
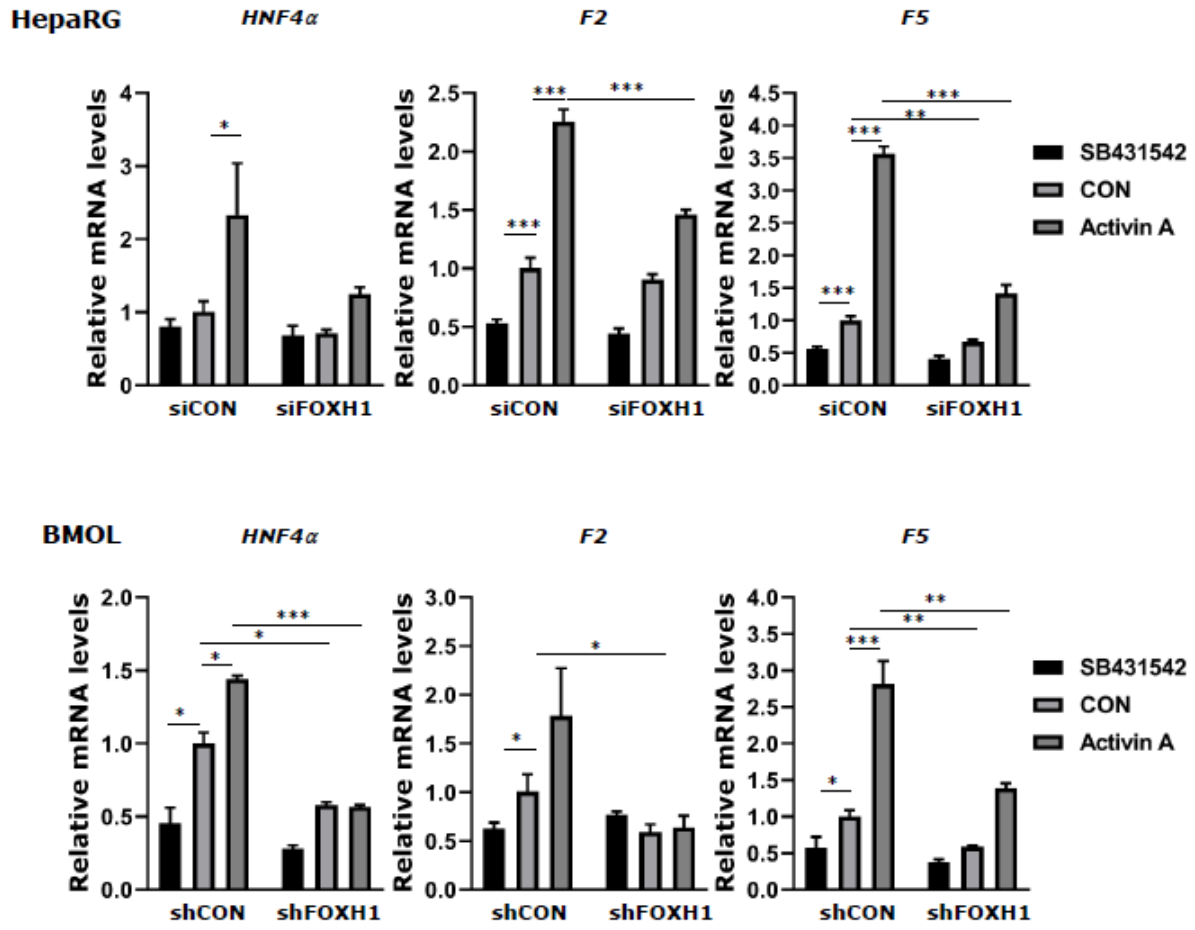


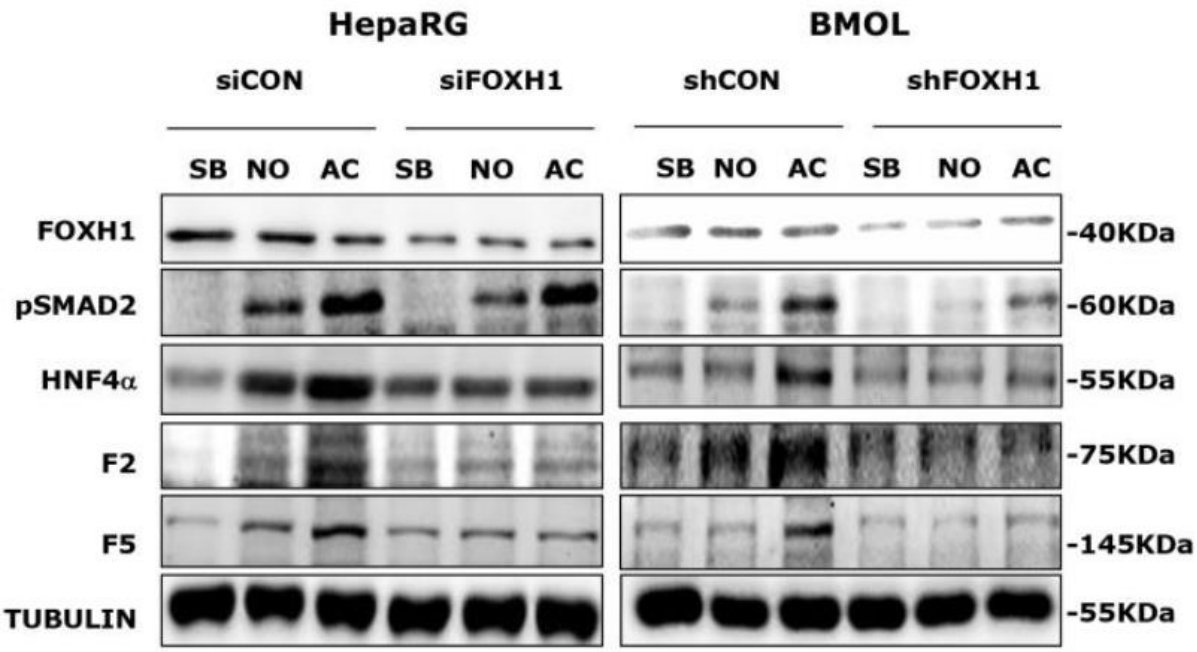
Figure 17. (**A-B**) qPCR and Western blot measure mRNA and protein expression of HNF4α, F2 and F5 in HepaRG and BMOL cells with or without SMAD2/3 knockdown by RNAi. (**C**) ChIP assay was performed to examine the role of Activin and SB431642 in the binding of SMAD2/3 and SMAD4 to the *HNF4A* gene promoters in indicated LPC. The expression of the cyclophilin A (PPIA, peptidylprolyl isomerase A) was used as loading control in ChIP assay. Tubulin was used as loading control in Western blotting. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Besides SMADs, we investigated the role of FOXH1 in the expression of HNF4α and coagulation factors in LPC. Knockdown of FOXH1 significantly inhibited Activin-induced mRNA and protein expression of HNF4α, F2 and F5 in both HepaRG and BMOL cells (**Figure 18A-B**). ChIP assay also revealed that Activin administration increased the binding activity of FOXH1 in the *HNF4A* gene promoter in HepaRG cells (**Figure 18C**). Co-IP analyses further showed that Activin increased FOXH1 complexing with SMAD2/3-SMAD4 in HepaRG and BMOL cells (**Figure 18D**). SB431542 inhibited the formation of the transcription factor complex (**Figure 18D**).

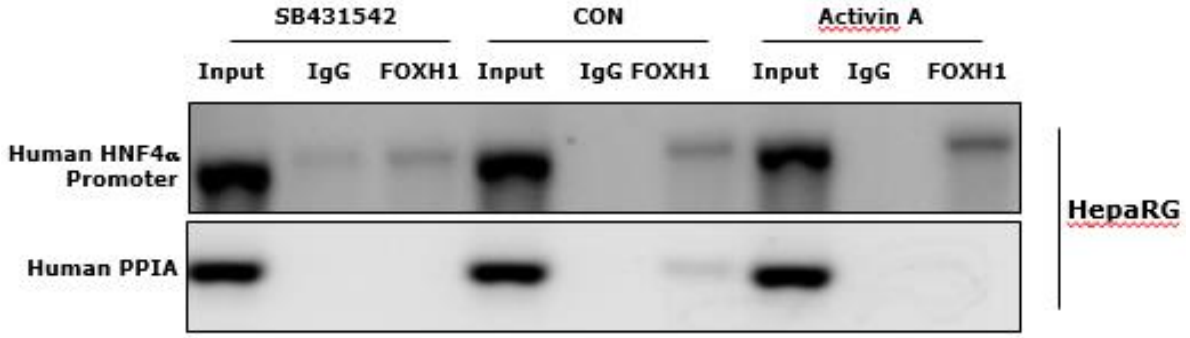
A



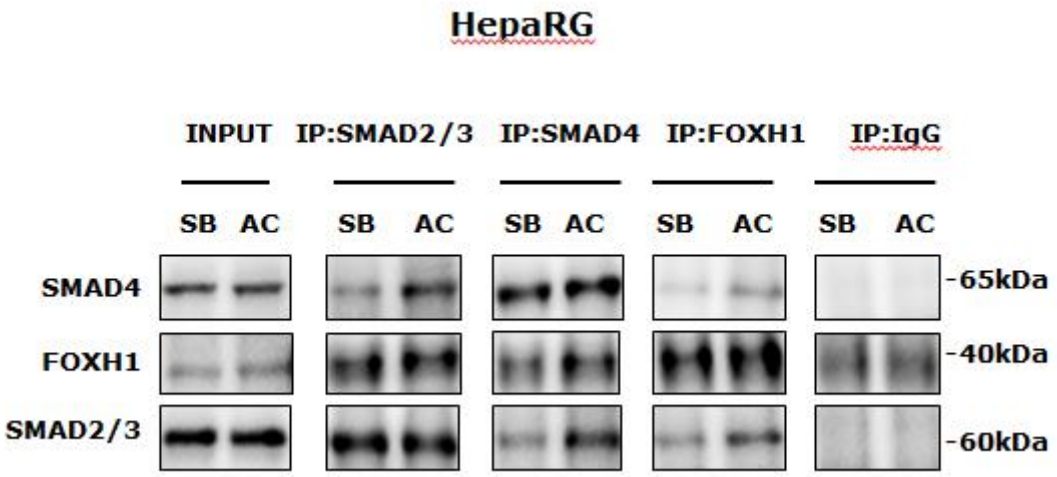
B


HepaRG
BMOL
siCON
siFOXH1
shCON
shFOXH1
SB NO AC SB NO AC
SB NO AC SB NO AC
FOXH1
pSMAD2
HNF4α
F2
F5
TUBULIN
-40KDa
-60KDa
-55KDa
-75KDa
-145KDa
-55KDa

C


SB431542
CON
Activin A
Input IgG FOXH1 Input IgG FOXH1 Input IgG FOXH1
Human HNF4α Promoter
Human PPIA
HepaRG

D


HepaRG
INPUT
IP:SMAD2/3
IP:SMAD4
IP:FOXH1
IP:IgG
SB AC SB AC SB AC SB AC SB AC
SMAD4
FOXH1
SMAD2/3
-65kDa
-40kDa
-60kDa

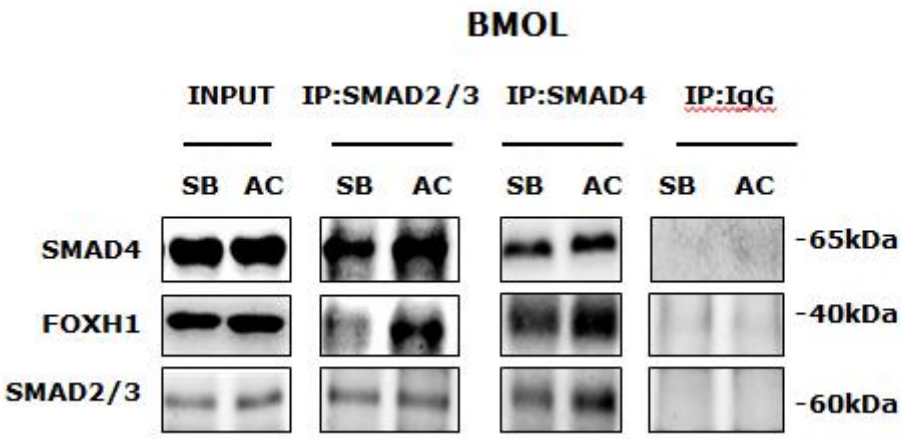
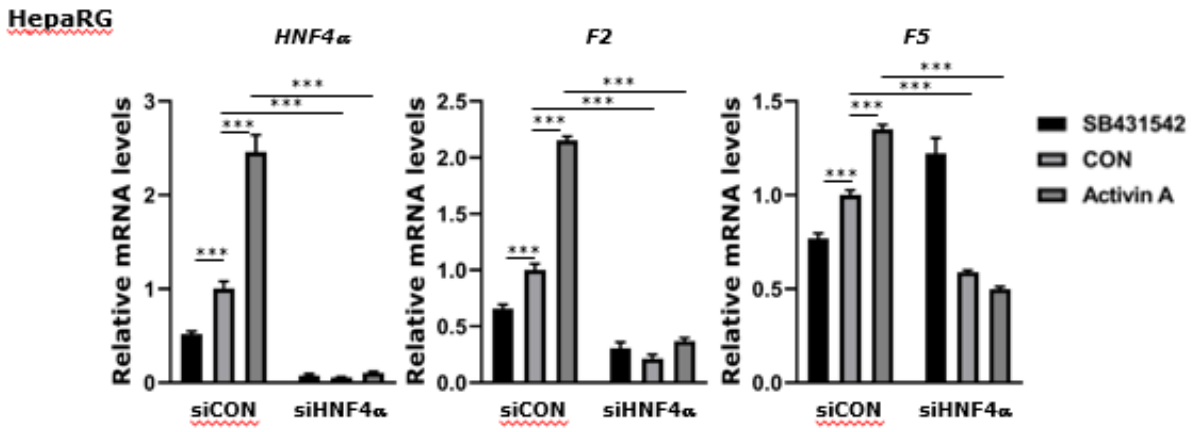


Figure 18. (**A-B**) qPCR and Western blot measure mRNA and protein expression of HNF4α, F2 and F5 in HepaRG and BMOL cells with or without FOXH1 knockdown by RNAi. (**C**) ChIP assay examined the role of Activin and SB431642 in the binding of FOXH1 to the *HNF4A* gene promoters in HepaRG cells. (**D**) Co-IP was performed to measure SMADs-FOXH1 complex formation in HepaRG and BMOL cells. The expression of the cyclophilin A (PPIA, peptidylprolyl isomerase A) was used as loading control in ChIP assay. Tubulin was used as loading control in Western blotting. **P* < 0.05, ***P* < 0.01, and *****P* < 0.001.

Upon HNF4α knockdown, Activin-induced mRNA and protein expression of F2 and F5 were significantly inhibited in both HepaRG and BMOL cells (**Figure 19A-B**). In addition, SB431542 inhibited Activin-dependent F2 and F5 expression (**Figure 19A-B**).

These results demonstrate the functionality of an Activin-SMADs-FOXH1 complex-HNF4α-coagulation factor axis in LPC.

A



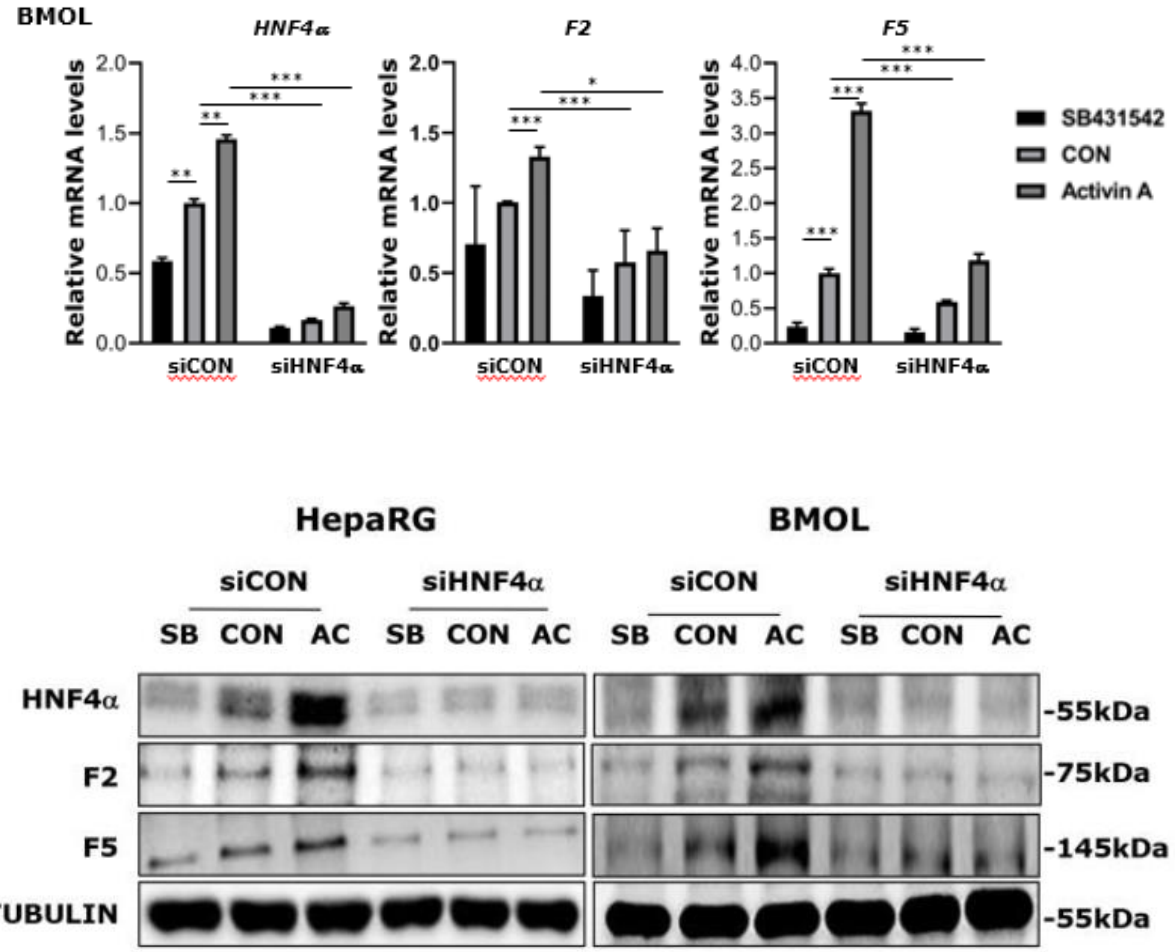
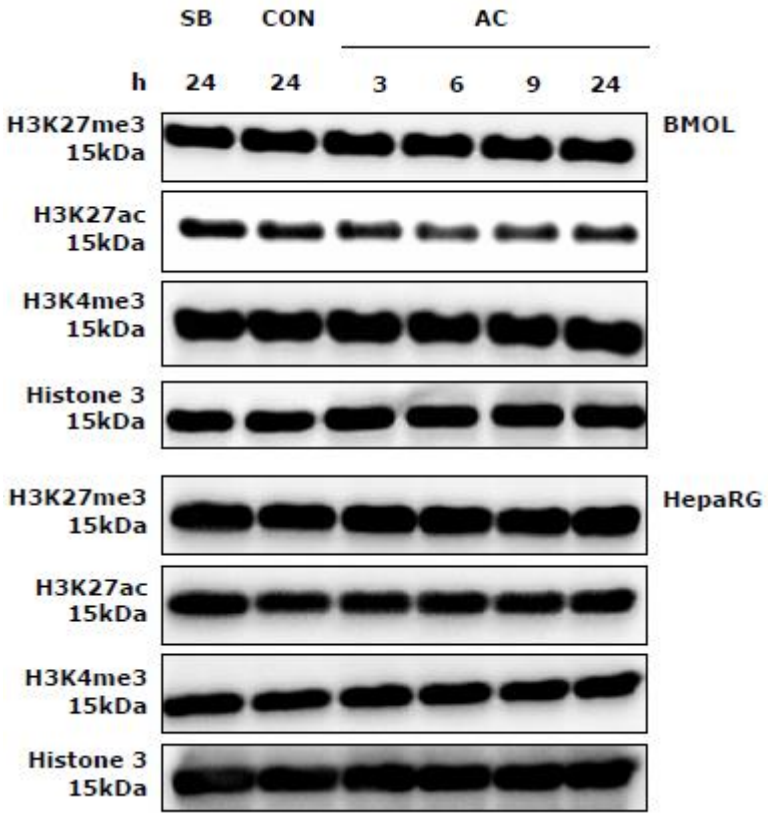


Figure 19. (**A-B**) qPCR and Western blot measure mRNA and protein expression of HNF4α, F2 and F5 in HepaRG and BMOL cells with or without HNF4α knockdown by RNAi. Tubulin was used as loading control in Western blotting. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

We also examined the role of the Activin-HNF4α axis in expression of epigenetic hallmarks, such as H3K4me3, H3K27me3 and H3K27ac, in HepaRG and BMOL cells. As shown in **Fig. 20A**, H3K4me3, H3K27me3 and H3K27ac patterns were not impacted by incubation with Activin or SB431542. In addition, disruption of components of the Activin-HNF4α axis, e.g. ALK4, SMAD2/3/4, FOXH1 and HNF4α, by RNAi also did not significantly influence these epigenetic hallmarks of BMOL cells (**Fig. 20B**).

These results suggest that activation of the Activin-HNF4α axis does not alter the epigenetic phenotypes of LPC.

A



B

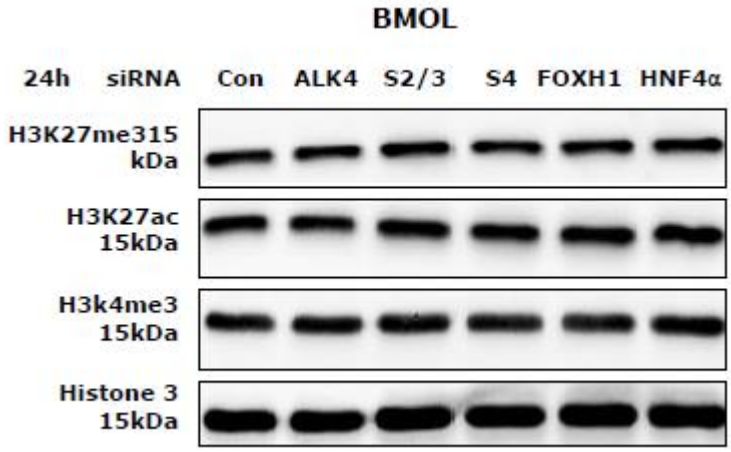


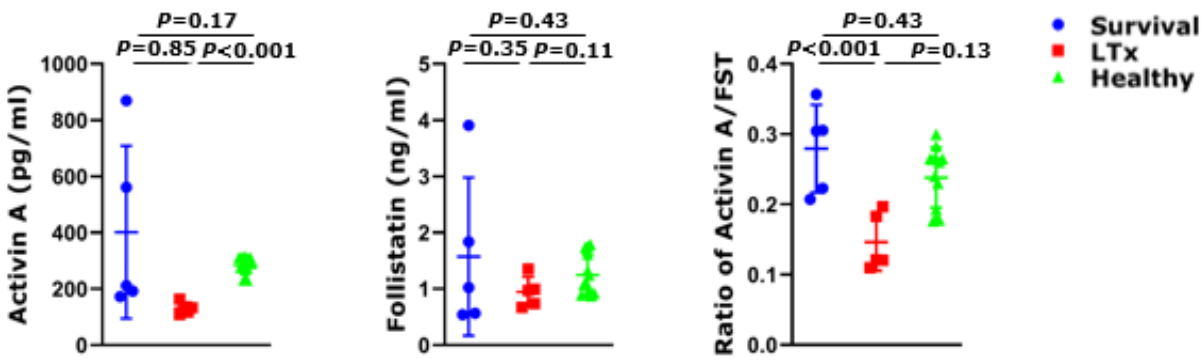
Figure 20. Activin administration or disruption of components of the Activin-SMAD-HNF4α axis did not alter the epigenetic phenotype of LPC. (**A**) Western blot analysis was performed to measure H3K4me3, H3K27me3 and H3K27ac in Activin-treated HepaRG and BMOL cells. (**B**) H3K4me3, H3K27me3 and H3K27ac were measured by Western blot in Activin-treated BMOL cells with or without RNAi for ALK4, SMAD2/3, SMAD4 and HNF4α, respectively.

3.6 Follistatin determines the Activin-HNF4α-coagulation factor axis in LPC

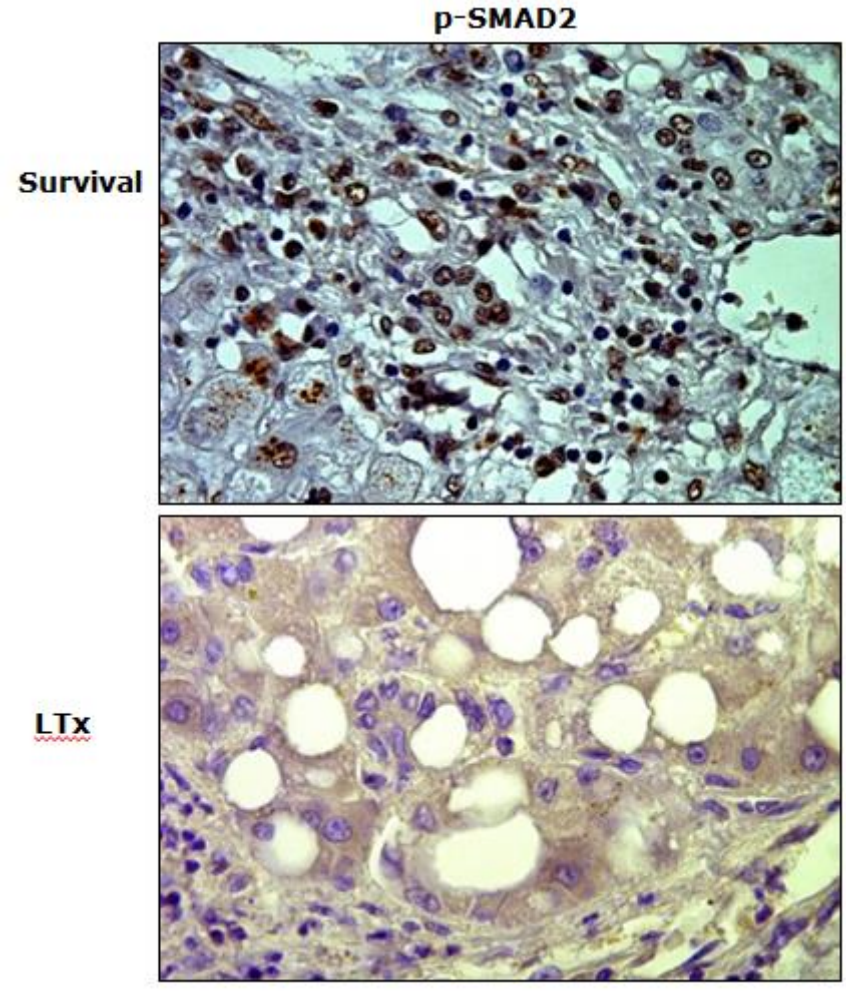
Given the key role of Activin signaling in controlling the HNF4α-coagulation factor axis in LPC, we asked whether the irreversible ACLF patients lack Activin? To this end, we measured serum Activin concentrations in the 10 ACLF patients and 13 healthy volunteers. The average serum Activin concentrations in the 5 recovered ACLF patients were similar to those in healthy controls, whereas serum Activin levels in the 5 irreversible ACLF patients were significantly reduced (**Figure 21A**). However, there was no significant difference of serum Activin levels between recovered and irreversible ACLF patients (**Figure 21A**). Notably, IHC staining showed that immune positivity of p-SMAD2 and HNF4α was robust in the recovered patients, but weak or even negative in the irreversible patients (**Figure 21B-C** and **Figure 11A**). Besides the aforementioned 10 ACLF patients enrolled in Beijing, China, we also examined p-SMAD2, FOXH1 and HNF4α expression by IHC in additional 9 ACLF patients, including 4 recovered patients, from Hannover, Germany. In line with the Beijing patients, immune positivity of p-SMAD2, FOXH1 and HNF4α was robust in the recovered patients, but weak or even negative in irreversible patients (**Figure 21D-E**). These results suggest that lack of Activin signaling, but not insufficient Activin, led to compromised Activin-p-SMAD2-HNF4α axis in the irreversible patients.

ACLF is a sepsis-like clinical syndrome (Jalan et al., 2012). Sepsis patients with poor prognosis displayed high levels of follistatin, a natural antagonist of Activing (Michel et al., 1998a; Shi and Massague, 2003). Michel et al. reported that the highest follistatin increase reached approximately 40 fold of normal levels in sepsis patients. Therefore, we speculated that high levels of follistatin might lead to compromised Activin signaling in irreversible ACLF patients (Michel et al., 1998a). We examined serum follistatin concentration and calculated the ratio of Activin and follistatin in the ACLF patients and healthy volunteers. Although there was no difference of serum follistatin concentration between healthy volunteers, recovered and irreversible ACLF patients, the ratio of Activin/follistatin in the recovered ACLF patients was significantly higher than in the irreversible patients (**Figure 21A**). There was no difference of the ratio between recovered ACLF patients and health controls (**Figure 21A**).

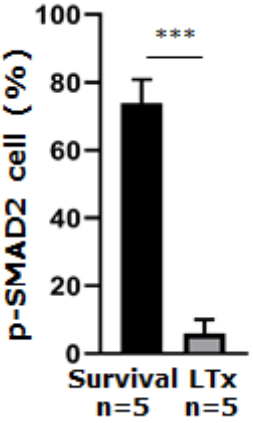
A


P=0.17
P=0.85
P<0.001
Activin A (pg/ml)
1000
800
600
400
200
0
P=0.43
P=0.35
P=0.11
Follistatin (ng/ml)
5
4
3
2
1
0
P=0.43
P<0.001
P=0.13
Ratio of Activin A/FST
0.4
0.3
0.2
0.1
0.0
Survival
LTx
Healthy

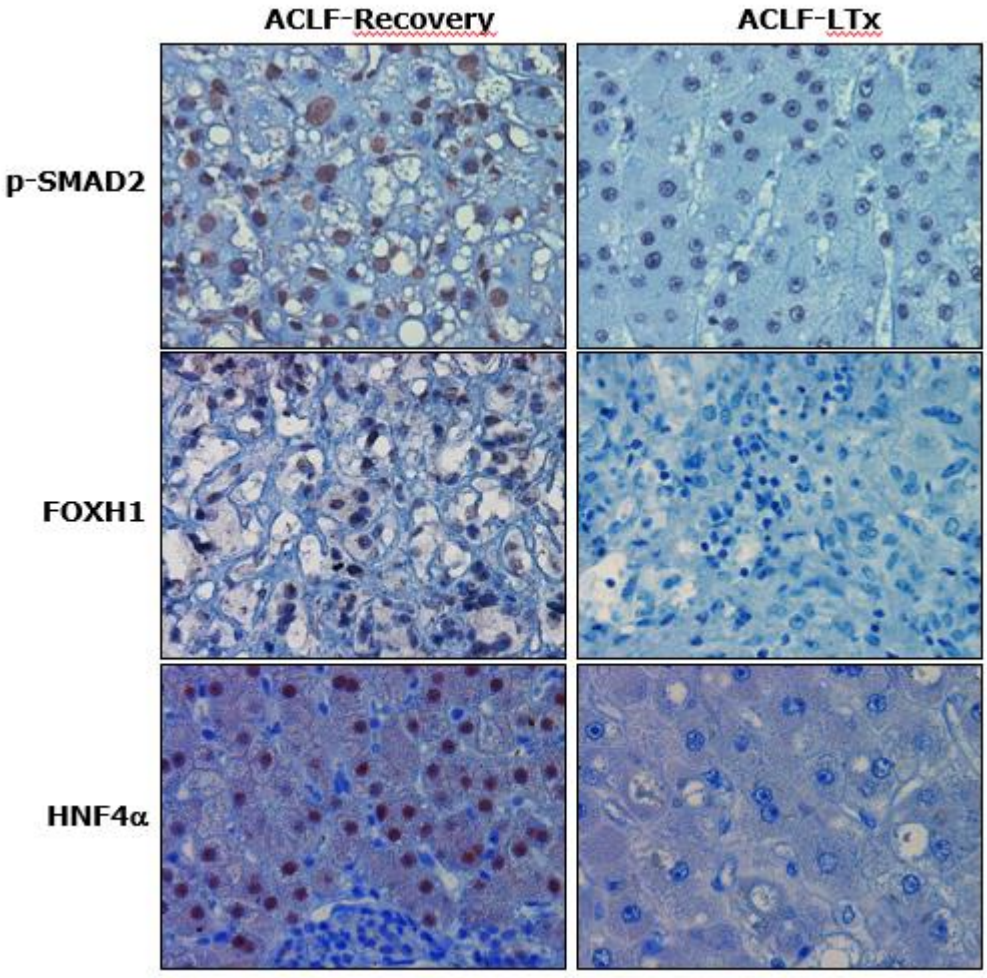
B


p-SMAD2
Survival
LTx

C


100
80
60
40
20
0
p-SMAD2 cell (%)
***
Survival
n=5
LTx
n=5

D


ACLF-Recovery
ACLF-LTx
p-SMAD2
FOXH1
HNF4α

E

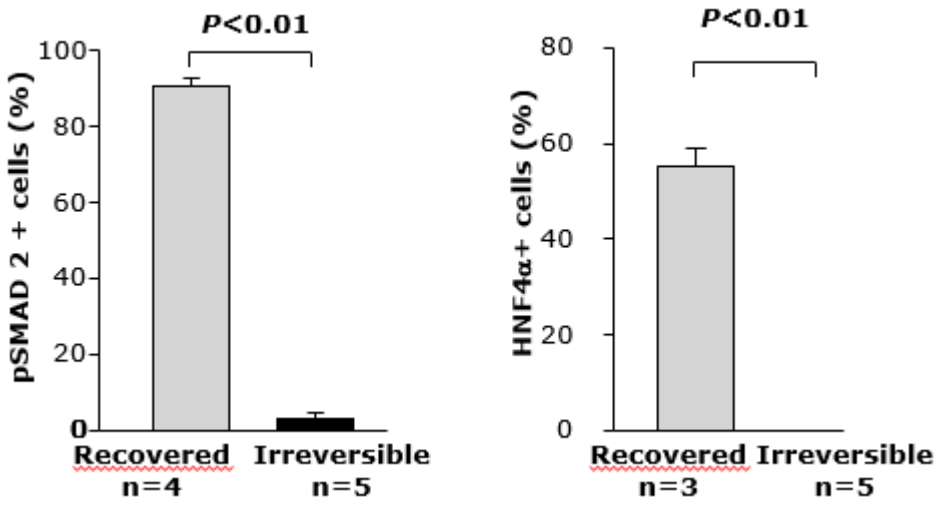


Figure 21. Insulin and glucagon regulated follistatin determines the Activin-HNF4α-coagulation factor axis in LPC. (**A**) Concentrations of Activin and follistatin were measured by ELISA in 10 ACLF patients and 13 healthy volunteers. The ratio of Activin and follistatin was calculated. (**B-C**) Immunohistochemistry for p-SMAD2 is shown in two representative ACLF patients. P-SMAD2 positive cells in 10 ACLF patients were counted. (**D-E**) Recovered ACLF patients robustly expressed p-SMAD2, FOXH1 and HNF4α. Representative images show immunohistochemical staining for p-SMAD2, FOXH1 and HNF4α, which was performed in recovered and irreversible ACLF patients enrolled in the Hannover cohort. P-SMAD2 and HNF4α positive liver cells were counted in the patients as described in the Materials and Methods section. Given that only 1 surviving and 2 irreversible ACLF patients were stained for FOXH1, semi-quantification analysis was not performed. *P < 0.05, **P < 0.01, and ****P < 0.001.

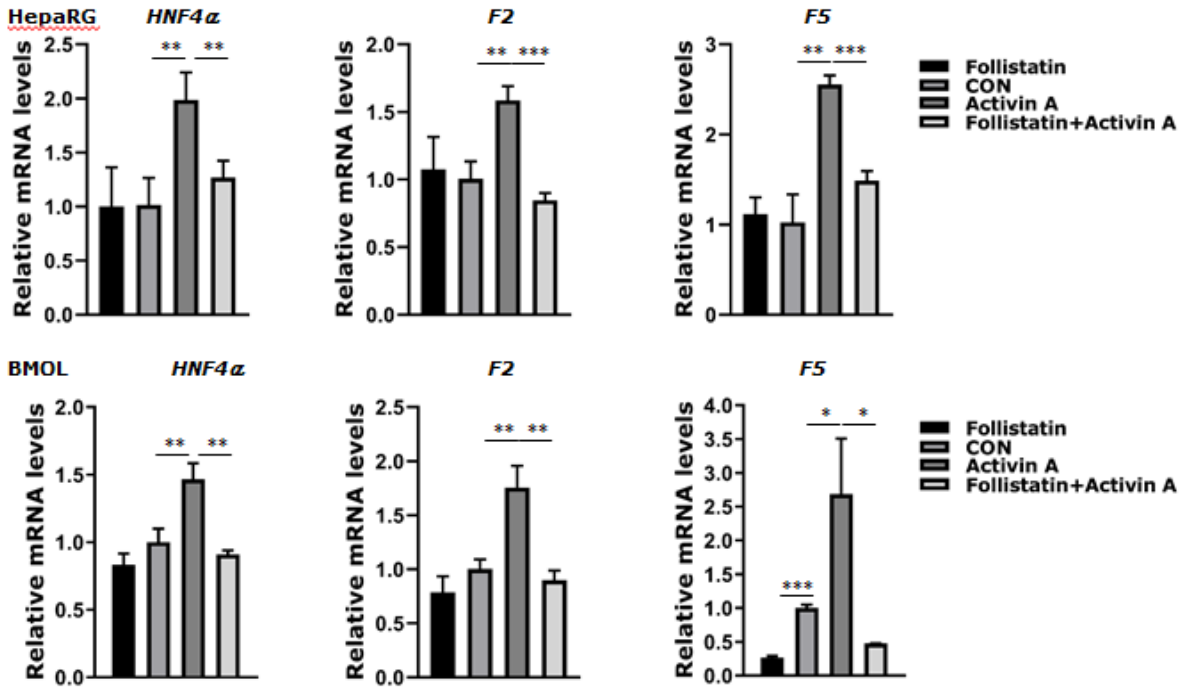
Next, we examined the effects of follistatin on Activin signaling in LPC. As shown in **Figure 22A-B**, follistatin remarkably inhibited Activin-induced p-SMAD2 expression as well as mRNA and protein expression of HNF4α, F2 and F5 in LPC. Given that follistatin is mainly produced by hepatocytes and is regulated by the ratio of glucagon and insulin (Hansen et al., 2016), we subsequently examined the effects of glucagon and insulin on follistatin in primary mouse and human hepatocytes. qPCR and Western blot analyses showed that glucagon induced mRNA and protein expression of follistatin in human and mouse primary hepatocytes (**Figure 22C-D**). The glucagon-induced follistatin expression was inhibited by insulin administration (**Figure 22C-D**).

We also measured serum levels of insulin, glucagon, and their ratio in the healthy volunteers and ACLF patients. In these cohorts of patients, we did not observe a

statistically difference of the insulin/glucagon ratio between recovered and irreversible ACLF patients, although the value of insulin/glucagon in the recovered patients was lower than in the irreversible patients (**Figure 22E**).

These results suggest that the balance between Activin and follistatin determines whether Activin signaling is capable of initiating the Activin-HNF4α-coagulation factor axis in ACLF patients.

A



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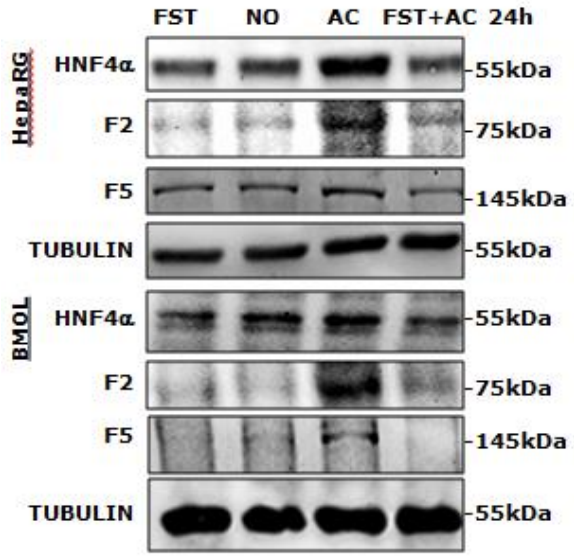
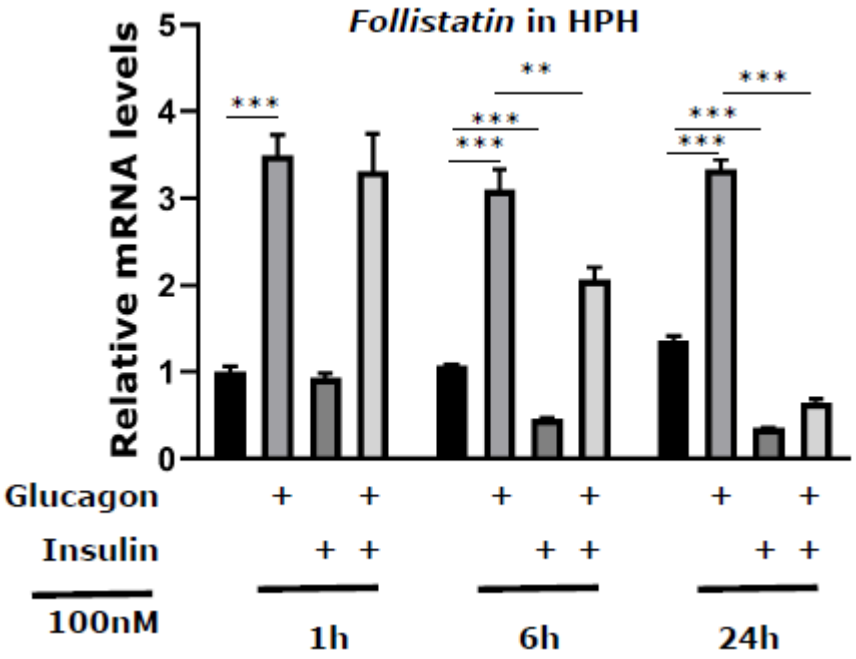
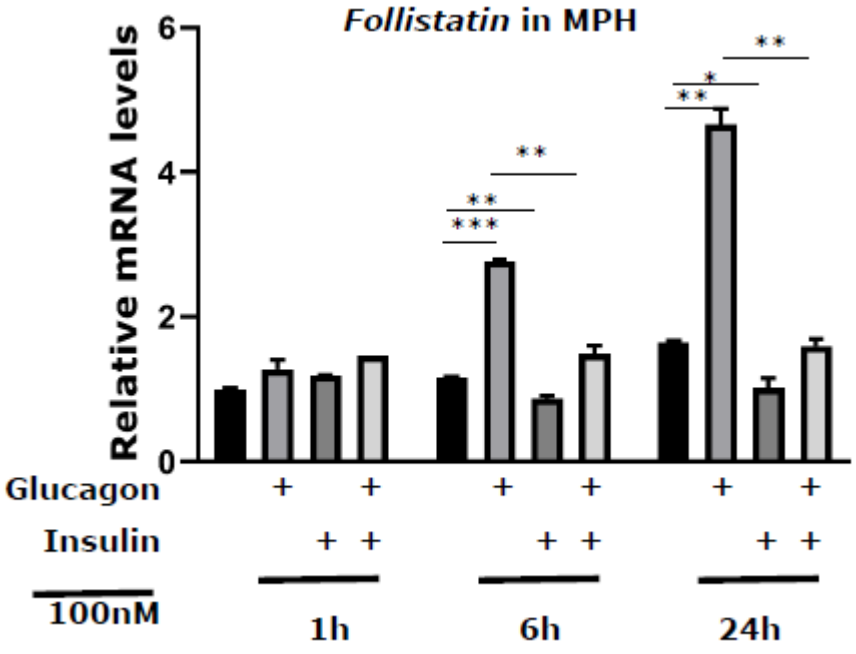
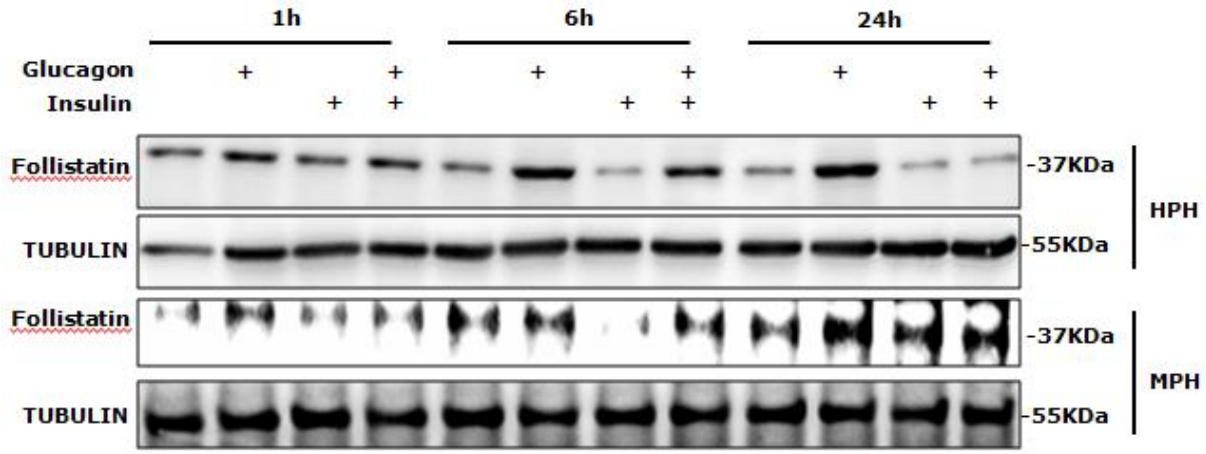


C


Follistatin in HPH
Relative mRNA levels
0
1
2
3
4
5
***
***
***
**
***
***
***
Glucagon
Insulin
100nM
+ + + + + + + + + + + +
1h
6h
24h


Follistatin in MPH
Relative mRNA levels
0
2
4
6
***
**
**
**
**
*
Glucagon
Insulin
100nM
+ + + + + + + + + + + +
1h
6h
24h

D



E

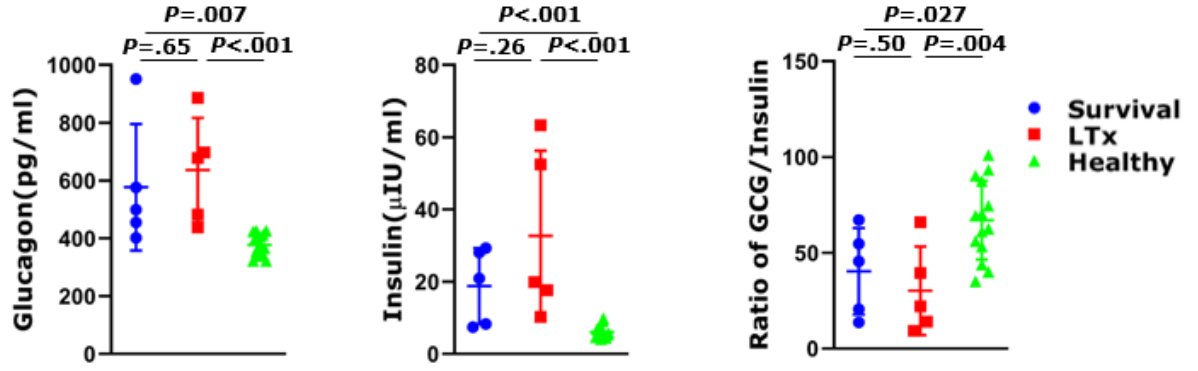


Figure 22. (**A-B**) qPCR and Western blot were used to measure mRNA and protein expression of HNF4α and F5 in HepaRG and BMOL cells with Activin and/or follistatin treatment for 24h. (**C-D**) qPCR and Western blot were used to measure mRNA and protein expression of follistatin in human primary hepatocytes (HPH) and mouse primary hepatocytes (MPH) with insulin and/or glucagon treatment. (**E**) Serum insulin and glucagon concentrations were measured in 10 ACLF patients and 13 healthy volunteers by ELISA. The ratio of insulin and glucagon was calculated.

4 DISCUSSION

4.1 Running hepatocyte function by LPC is critical for the survival of ACLF patients

In an ACLF patient with massive loss of hepatocytes, the maintenance of essential hepatocyte function for survival is dependent on the number and function of remaining hepatocytes and activated LPC (Weng et al., 2015). In contrast to ALF, a similar disease occurring in healthy people before the liver insult, ACLF occurs in patients with chronic liver diseases, in particular liver cirrhosis (Arroyo et al., 2020; Weng et al., 2015). In cirrhotic patients, a large portion of hepatocytes show decompensation before acute deterioration (Alastair D. Burt, 2012b). It has been reported that around 70% of hepatocyte buds are derived from LPC in cirrhotic patients (Stueck and Wanless, 2015). Therefore, the takeover of hepatocyte functions by LPC might precede ACLF under circumstances of liver cirrhosis. Following massive hepatocyte loss, expansion of activated LPC morphologically presents as rapid ductular reaction (Li et al., 2015; Lucke, 1944). Over time, a large amount of LPC diffuses from zone 1 to zone 3 (Li et al., 2015; Lucke, 1944). These cells will differentiate into mature hepatocytes over time (Weng et al., 2015). However, finalizing the process of LPC-to-hepatocyte differentiation needs at least one month, which depends on the microenvironment of the disease (Weng et al., 2015). Therefore, before LPC are capable of differentiation into sufficient numbers of hepatocytes, the performance of key hepatocyte functions by LPC is crucial for the survival of ACLF patients. The present study indeed found that LPC express hepatocyte-specific proteins, e.g. coagulation factor F5 (**Figure 11**) and albumin (data not shown) in ACLF patients. More impressively, hepatocyte-specific lineage transcription factor HNF4α is expressed in LPC, particularly in the recovered ACLF patients. These results imply that LPC might perform key hepatocyte functions by expressing HNF4α in ACLF.

4.2 The Activin-HNF4α-coagulation factor axis in LPC

For an ACLF patient, maintaining essential coagulation function is indispensable for survival. A recent study shows that the serum plasminogen levels is a promising biomarker predicting the progression of ACLF patients (Wu et al., 2020), indicating the relevance of coagulation factors in determining the clinical outcome of ACLF. In a

health person, most coagulation factors are synthesized only by hepatocytes (Alastair D. Burt, 2012a). Coagulation factor genes are controlled by several master hepatic transcription factors, including HNF4α (Chiang, 2009; Odom et al., 2004). In hepatocytes, HNF4α regulates the transcription of coagulation factors through binding to their gene promoters (Safdar et al., 2012). The expression of both HNF4α and F5 in LPC suggested that HNF4α might be one of the key transcription factors controlling expression of coagulation factors in LPC. We confirmed this hypothesis by ChIP assays: HNF4α initiates the transcription of multiple coagulation factors, such as F2 and F5, through binding to their gene promoters in LPC. These results explain as to why ACLF patients with high levels of HNF4α in LPC have a normal INR ratio.

How do LPC initiate HNF4α expression? We found that the formation of transcription factor FOXH1-SMADs complex is essential to initiate HNF4α transcription in LPC. Although upstream signal driving FOXH1 in LPC is unknown to date, the formation of SMAD complex suggest the requirement of either TGF-β or Activin signal in initiating HNF4α transcription. A recently study shows that in mouse embryonic stem cells, signal-independent binding of SMAD3 and FOXH1 prime differentiation gene promoters for activation while Nodal signal-driven SMAD2:SMAD4 binds to the promoters preloaded with SMAD3:FOXH1 to activate transcription (Aragon et al., 2019). In LPC, whether the initiation of HNF4α transcription also requires SMAD3 and FOXH1 prime needs further investigation. Interestingly, Activin and TGF-β play an opposite role in regulating HNF4α in LPC: Activin upregulates and TGF-β inhibits HNF4α expression. Further experiments based on ALK4 knockdown confirmed the key role of Activin in HNF4α expression in LPC. Why Activin and TGF-β lead to opposite effects on HNF4α expression is worthy of future investigation.

4.3 Taking over hepatocyte function or initiating differentiation?

The Activin-driven FOXH1-SMAD2/3/4 complex plays a crucial role in mediating embryonic stem cell-to-mesoderm differentiation through upregulating master differentiation genes, e.g., Gsc and Mixl (Xi et al., 2011). *In vitro*, Activin initiates embryonic stem cell-to-mesoderm differentiation through SMAD2-mediated H3K27me3 reduction (Wang et al., 2017). This raises an interesting question: Does

Activin directly initiate LPC-to-hepatocyte differentiation in ACLF rather than merely inducing hepatocyte functions? Hepatocyte-ablated zebrafish provide a model to clarify this issue. Administration of Mtz completely destroys the hepatocytes of zebrafish livers (Choi et al., 2014). Until hepatocytes generate from LPCs through LPC-to-hepatocyte differentiation, which occurs between R6h and R24h (Choi et al., 2014), LPCs might run hepatocyte functions. We found that lack of *hnf*4α did not affect the LPC-to-hepatocyte differentiation, but remarkably reduced the expression of the coagulation factor gene *f2* at both R6h and R24h (data not shown). Consistent with these zebrafish data, Activin stimulation or disruption of any components of the FOXH1-SMAD2/3/4 complex-HNF4α axis did not alter epigenetic phenotypes of LPC. These results suggest that HNF4α is a key transcription factor required for the expression of coagulation factor gene in both LPC and hepatocytes. However, the Activin- HNF4α axis is dispensable for the LPC-to-hepatocyte differentiation.

In ACLF patients, lack of hepatocytes does not denote poor prognosis. As shown in **Figure 11**, two recovered patients do not display any hepatocytes in the examined tissues. However, the proliferative LPC demonstrate robust HNF4α expression, indicating that these cells are actively running hepatocyte functions. In contrast to the recovered patients, all examined irreversible patients possess sufficient hepatocytes and active LPC. However, both cell types lack HNF4α expression while the patients show high INR and cholestasis. These findings suggest that performance of sufficient essential functions by either the remaining hepatocytes or LPC is more important for the survival of ACLF patients than restoring parenchymal cell numbers.

In clinical practice, whether morphologically successful liver regeneration denotes a good clinical outcome of ACLF patients is a controversial issue. Very frequently, histological examination in explanted livers reveals a successful LPC-mediated liver regeneration, featured as robust hepatocyte-like cells (Weng et al., 2015). It is debated whether these patients should receive LTx. Whether these patients with “successful hepatic regeneration” will recover spontaneously over time? In contrast to the “excellent” histological features, clinical manifestation and parameters, including MELD scores, demonstrate severe disease, which indicates to perform LTx in these patients. Based on the current study, receiving LTx is a correct decision for these patients because these “successfully” regenerated hepatocytes do not perform essential functions due to a lack of key regulatory networks, e.g., the Activin-HNF-4α

axis.

4.4 The Activin and follistatin ratio determines a successful Activin-HNF4α-coagulation factor axis in LPC

Given the key role of Activin signaling in the regulation of coagulation factors in LPC, we doubted whether the absence of the Activin-HNF4α-coagulation factor axis in irreversible ACLF patients is due to lack of Activin. We found that serum Activin concentrations in most recovered patients were similar to those in the irreversible patients. However, p-SMAD2 levels in hepatocytes and LPC of the recovered patients were remarkably higher than those in the irreversible patients. This observation indicated that the Activin signaling in irreversible patients might be inhibited. Based on three reasons, we hypothesized that follistatin might be the factor that inhibits the Activin signaling in irreversible ACLF patients: Firstly, follistatin is a natural inhibitor of Activin (Hansen and Plomgaard, 2016). Secondly, follistatin is synthesized mainly in hepatocytes (Hansen and Plomgaard, 2016). Massive hepatocyte death might release huge amount of follistatin. Thirdly, follistatin is a key reproduction hormone that suppresses the follicle stimulating hormone (FSH) (Hansen and Plomgaard, 2016). According to the life history theory, growth, reproduction, and maintenance are three fundamental biological programs in humans (Wang et al., 2019). In favorable environments, the synthesis and release of follistatin in hepatocytes is strictly regulated by the glucagon-to-insulin ratio to promote investment in growth and reproduction (Hansen and Plomgaard, 2016). In harsh environments, such as severe infection and immune defense, the function of follistatin is required to transfer from reproduction and divert to the defense (Wang et al., 2019). Circulating follistatin concentration in sepsis patients even increased to 40 fold of the normal level (Michel et al., 1998b). Therefore, high levels of follistatin suggest a trade-off inasmuch as the host temporarily sacrifices growth and reproduction function in order to spend more energy on supporting the immune defense.

To date, few studies have examined follistatin levels in ACLF patients. In this study, we firstly measured serum follistatin levels in 10 ACLF patients receiving histological examination. In this cohort, there was no difference of circulation follistatin

concentration between the recovered and the irreversible patients. However, the Activin/follistatin ratio in the recovered patients was remarkably higher than the irreversible patients. These results suggest that Activin/follistatin ratio is a crucial factor leading to robust p-SMAD2 expression in the recovered patients and lack of the Activin signaling in the irreversible patients.

4.5 Insulin and glucagon influence the Activin-HNF4α-coagulation factor axis through follistatin

As two key systemic regulators, insulin and glucagon not only regulate energy allocation in different organs, but also control the synthesis of hormones such as follistatin (Hansen et al., 2016). Consistent with previous study (Hansen et al., 2016), our *in vitro* experiments confirmed that glucagon induced and insulin inhibited expression and secretion of follistatin in hepatocytes and thus regulated the Activin-HNF4α-coagulation factor axis in LPC. Under circumstances of sepsis, high levels of glucagon are required to maintain high levels of blood glucose. To guarantee sufficient energy supply for priority organs e.g., the brain and immune cells, insulin resistance occurs in major metabolic tissues such as adipose tissue, skeletal muscle and hepatocytes. Insulin resistance in hepatocytes compromises inhibitory effect of insulin on follistatin and thus disrupts the ratio of glucagon/insulin to control follistatin synthesis. This might explain why we did not observe differences of insulin and glucagon levels between the recovered and irreversible ACLF patients.

4.6 The Activin-HNF4α-coagulation factor axis and beyond

It is noteworthy that the Activin-HNF4α axis is not the only signaling network regulating coagulation factors in ACLF patients. Even in irreversible patients lacking Activin signal and HNF4α expression, coagulation factor expression in the liver is still detectable. Repression of the Activin-HNF4α axis leads to insufficient, but not totally lacking coagulation function. Additional non-Activin-HNF4α regulatory networks need to be clarified in the future.

On the other hand, the effects of the Activin-HNF4α axis are not limited to controlling

coagulation factor expression. HNF4α is a master transcription factor in the regulation of the transcriptional program in the liver, given that HNF4α binds to more than 40% of actively-transcribed genes in hepatocytes. We performed RNA sequencing in Activin- and/or HNF4α-dependent transcriptome alteration of LPC. Bioinformatics analyses show that the Activin-HNF4α axis profoundly impacts LPC metabolism of drugs, steroid hormones, chemicals, and xenobiotics as well as the expression of transmembrane transporters, which are essential for bile acid delivery (data not shown). The detailed mechanisms require further investigation.

Taken together, the current study provides evidence for a critical role of the Activin-HNF4α axis in LPC, required to take over key hepatocyte functions such as coagulation in circumstances of massive hepatocyte loss and thus is crucial for the survival of ACLF patients. This regulatory axis is inhibited by hepatocyte-derived follistatin secretion, which in turn is governed by the systemic balance between insulin and glucagon. These results further suggest that acute-on-chronic liver failure is not merely a disease with a failed liver. Repairing a failed liver also relies on the recovery of systemic homeostasis. Besides the mechanistic analyses, the current study demonstrates a promising power of serum follistatin levels to predict the incidence and mortality of ACLF. If histological examination is available, evaluation of HNF4α expression in liver tissues might also be a potential new approach to predict the clinical outcome of ACLF patients.

In addition, this study raises several unanswered questions: (1) Why do Activin and TGF-β lead to opposite effects on HNF4α? (2) What is the upstream signal driving FOXH1 expression in LPC? (3) Besides regulating coagulation factor expression, which hepatocytes functions are also controlled by the Activin-HNF4α axis in ACLF? (4) Besides the Activin-HNF4α axis, how do the non-Activin-HNF4α networks contribute to regulate coagulation function in ACLF?

5 SUMMARY

The regulatory model is depicted in a schematic diagram (**Figure 23**). Why and how a patient with massive hepatocyte loss is capable of surviving is a key clinical question. The current study provides the following findings, which partly explain this issue: (1) LPC take over and run key hepatocyte functions, e.g. coagulation, in ACLF patients suffering from massive loss of hepatocytes; (2) Expression of coagulation factors in LPC depends on hepatocyte lineage transcription factor HNF4α, which is usually expressed in hepatocytes; (3) HNF4α expression in LPC is driven by Activin signal; (4) Whether LPC possess an intact Activin-HNF4α-coagulation factor regulatory axis largely determines the clinical outcome of ACLF patients; (5) The Activin signaling is negatively regulated by follistatin, a hepatocyte-derived Activin inhibitor controlled by the insulin-to-glucagon ratio.

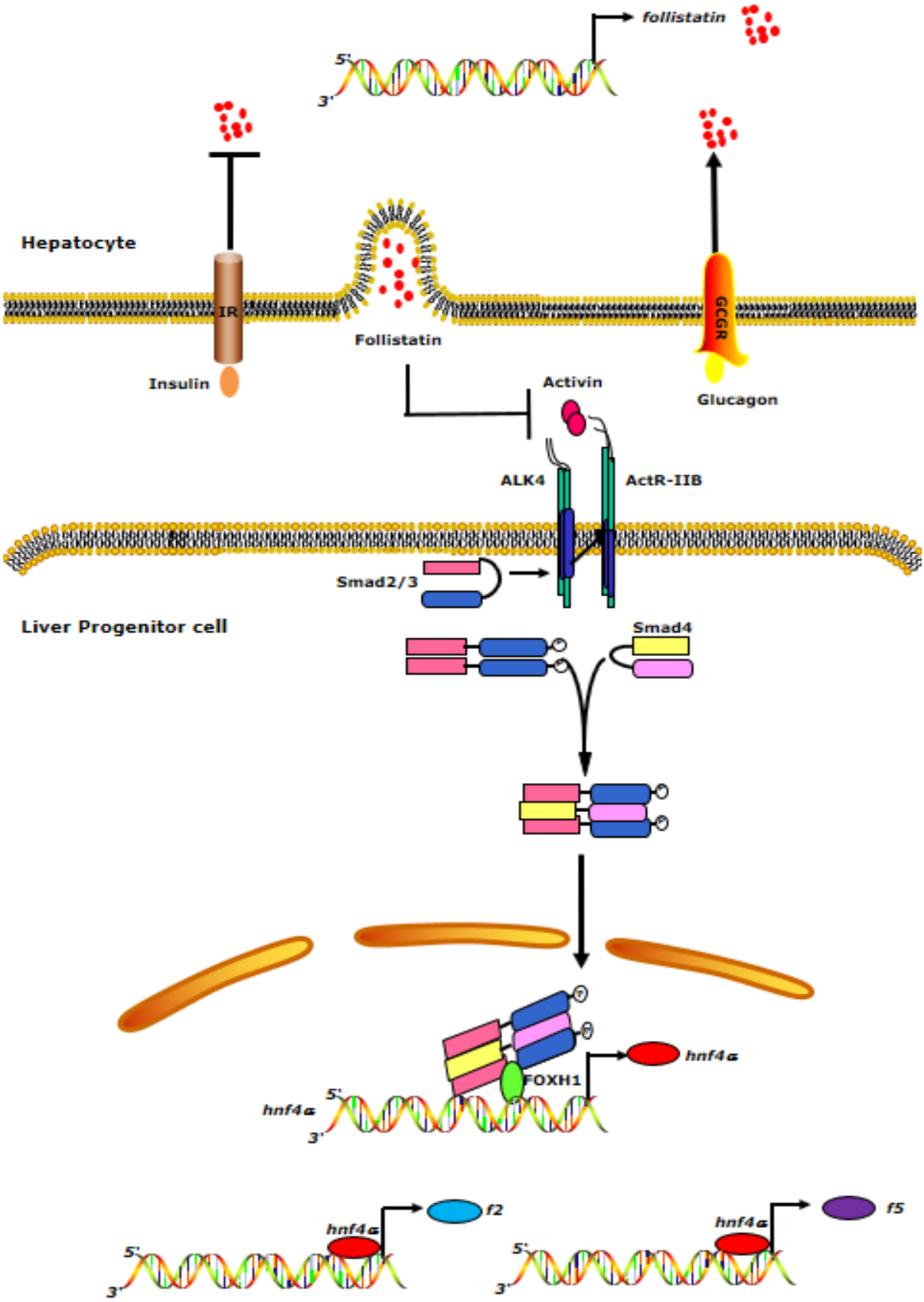


Figure 23. A scheme depicts hormone-controlled the Activin-HNF4α-coagulation factor axis in LPC.

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8 CURRICULUM VITAE

8.1 Personal information

Name:	Tao Lin
Date of birth:	June 2, 1988
Nationality:	Chinese
Marital state:	Unmarried
Email:	Tao.Lin@medma.uni-heidelberg.de
Address:	PETTENKOFERSTRASSE 19, 68169, Mannheim

8.2 Education

Aug.2017 – present	Ph.D. student (Dr.sc.hum.) Medizinischen Fakultät Mannheim Ruprecht-Karls-University Heidelberg
Ph.D. project:	Activin drives liver progenitor cells to take over coagulation function through upregulating HNF4α in acute-on-chronic liver failure
Sep. 2012 – Jul. 2015	Master of Medical Microbiology College of Life Sciences, Wuhan University
Master project:	Global quantitative proteomics analysis of human glioma cells profiled host protein expression in response to EV71 infection & mAbs against EV71 were produced by VLPs
Sep. 2008 – Jul. 2012	Bachelor of Biotechnology College of Bioengineering, Hubei University of Technology

8.3 Scholarship

Ph.D. scholarship from China Scholarship Council

8.4 Publications

- “Global quantitative proteomics analysis of human glioma cells profiled host protein expression in response to Enterovirus type 71 infection”. Zhang LK, **Lin T** et al. Proteomics 2015 Nov;15(22):3784-96
- “Monoclonal neutralizing antibodies against EV71 screened from mice immunized with yeast-produced virus-like particles”. **Lin T** et al. Virol Sin. 2015, 30 (3): 1-6.
- "SOX9 expression decreases survival of patients with intrahepatic cholangiocarcinoma by conferring chemoresistance”. Xiaodong Yuan, Jun Li, Cédric Coulouarn, **Tao Lin** et al. Br J Cancer. 2018 Nov; Epub 2018 Nov 13.
- "Human skin-derived ABCB5+ stem cell injection improves liver disease parameters in Mdr2KO mice”. Hartwig V, Dewidar B, **Lin T** et al. Arch Toxicol. 2019 Sep; Epub 2019 Aug 21.
- "Follistatin controlled Activin-HNF4α-coagulation factor axis in liver progenitor cells determines clinical outcome of acute liver failure". **Lin T** et al., Journal Hepatology. 2021 (under review)

8.5 Conference presentations and posters

- 2019 GASL Poster presentation:
  Transcription factor TRIM33 controls liver progenitor cell towards hepatocyte differentiation through synergizing with phosphorylated Smad2/3 in liver cirrhosis
- 2019 EASL Poster presentation:
  Transcription factor TRIM33 controls liver progenitor cell towards hepatocyte differentiation through synergizing with Smad2/3 following massive parenchymal loss
- 2019 DGVS Oral presentation:
  Loss of functional Activin-HNF4α-coagulation axis in patients with high MELD score determines impossible recovery from acute-on-chronic liver failure
- 2019 Signal Transduction – Receptors, Mediators and Genes, Oral presentation and Poster:

Activin signal-driven lineage determination transcription factor HNF4α is pivotal in liver progenitor cell-mediated liver regeneration

- 2020 GASL Oral and Poster presentation:
  Activin-driven fate determination is pivotal for liver progenitor cells to take over hepatocytic function in acute-on-chronic liver failure
- 2020 EASL Poster presentation:
  Functional Activin-HNF4α-coagulation axis in liver progenitor cells determines MELD score and outcome of acute-on-chronic liver failure

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