Follistatin-controlled activin-HNF4 alpha-coagulation factor axis in liver progenitor cells determines outcome of acute liver failure

Lin, T.; Wang, S.S.; Munker, S.; Jung, K.; Macias-Rodriguez, R.U.; Ruiz-Margain, A.; ... ; Weng, H.L.

Received: 25 April 2021 | Revised: 1 July 2021 | Accepted: 15 August 2021

DOI: 10.1002/hep.32119

ORIGINAL ARTICLE

Follistatin-controlled activin-HNF4α-coagulation factor axis in liver progenitor cells determines outcome of acute liver failure

Tao Lin[1] | Shanshan Wang[1,2] | Stefan Munker[3] | Kyounghwa Jung[4] | Ricardo U. Macías-Rodríguez[5] | Astrid Ruiz-Margáin[5] | Robert Schierwagen[6] | Hui Liu[7] | Chen Shao[7] | Chunlei Fan[8] | Rilu Feng[1] | Xiaodong Yuan[1] | Sai Wang[1] | Franziska Wandrer[9] | Christoph Meyer[1] | Ralf Wimmer[3] | Roman Liebe[10,11] | Jens Kroll[12] | Long Zhang[13] | Tobias Schiergens[14] | Peter ten Dijke[15] | Andreas Teufel[16,17] | Alexander Marx[18] | Peter R. Mertens[19] | Hua Wang[20,21] | Matthias P.A. Ebert[22,23,24] | Heike Bantel[9] | Enrico N. De Toni[3] | Jonel Trebicka[6,25] | Steven Dooley[1] | Donghun Shin[4] | Huiguo Ding[8] | Hong-Lei Weng[1]

[1]Department of Medicine II, Section Molecular Hepatology, University Medical Center Mannheim, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

[2]Beijing Institute of Hepatology, Beijing You'an Hospital, Capital Medical University, Beijing, China

[3]Department of Medicine II, University Hospital, Campus Großhadern, LMU Munich, Munich, Germany

[4]Department of Developmental Biology, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[5]Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico city, Mexico

[6]Translational Hepatology, Medical Department I, Frankfurt University Hospital, Frankfurt, Germany

[7]Department of Pathology, Beijing You'an Hospital, Affiliated with Capital Medical University, Beijing, China

[8]Department of Gastroenterology and Hepatology, Beijing You'an Hospital, Affiliated with Capital Medical University, Beijing, China

[9]Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

[10]Clinic of Gastroenterology, Hepatology and Infectious Diseases, Heinrich Heine University, Düsseldorf, Germany

[11]Department of Medicine II, Saarland University Medical Center, Saarland University, Homburg, Germany

[12]Vascular Biology and Tumor Angiogenesis, European Center for Angioscience, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

[13]Life Sciences Institute and Innovation Center for Cell Signaling Network, Hangzhou, China

[14]Department of General, Visceral, Transplantation, Vascular and Thoracic Surgery, University Hospital, LMU Munich, Munich, Germany

[15]Oncode Institute and Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, The Netherlands

[16]Division of Hepatology, Division of Clinical Bioinformatics, Department of Medicine II, University Medical Center Mannheim, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

[17]Clinical Cooperation Unit Healthy Metabolism, Center for Preventive Medicine and Digital Health Baden-Württemberg, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

[18]Institute of Pathology, University Medical Center Mannheim, Heidelberg University, Mannheim, Germany

[19]Clinic of Nephrology and Hypertension, Diabetes and Endocrinology, Otto-von-Guericke-University, Magdeburg, Germany

Abbreviations: 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); BMOL, bipotential murine oval liver; Bhmt, betaine-homocysteine S-methyltransferase; CFP, cyan fluorescent protein; ChIP, chromatin immunoprecipitation; CON, control; F2, coagulation factor II; F5, coagulation factor V; FOXH1, forkhead box protein H1; FST, follistatin; HNF4-α, hepatocyte nuclear factor 4α; IHC, immunohistochemistry; INR, international normalized ratio; LPC, liver progenitor cell; LT, liver transplantation; MELD, Model for End-Stage Liver Disease; MHL-LF, massive hepatocyte loss–induced liver failure; Mtz, metronidazole; NTR, nitroreductase; p-SMAD2, phosphorylated Sma and Mad homolog 2; RNAi, RNA interference; SMAD, Sma and Mad homolog.

Tao Lin, Shanshan Wang, Stefan Munker, Kyounghwa Jung, Ricardo U. Macías-Rodríguez contributed equally to this work.

[20]Department of Oncology, the First Affiliated Hospital of Anhui Medical University, Hefei, China
[21]Inflammation and Immune Mediated Disease Laboratory of Anhui Province, Hefei, China
[22]Mannheim Institute for Innate Immunoscience, Mannheim, Germany
[23]Clinical Cooperation Unit Healthy Metabolism, Center of Preventive Medicine and Digital Health, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany
[24]Department of Medicine II, University Medical Center Mannheim, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany
[25]European Foundation for Study of Chronic Liver Failure, Barcelona, Spain

Correspondence
Huiguo Ding, Department of Gastroenterology and Hepatology, Beijing You'an Hospital, Affiliated with Capital Medical University, No. 8, Youanmen Wai St., 100069, Beijing, China.
Email: dinghuiguo@ccmu.edu.cn

Hong-Lei Weng, Department of Medicine II, Section Molecular Hepatology, University Medical Center Mannheim, Medical Faculty Mannheim, Heidelberg University, Theodor-Kutzer Ufer 1-3, 68167 Mannheim, Germany.
Email: honglei.weng@medma.uni-heidelberg.de

Funding information
Deutsche Forschungsgemeinschaft (WE 5009/9-1 to H.L.W.); Beijing Municipal Education Commission of Beijing Municipal Government (KZ201810025037 to H.D); Sino-German Science Center (GZ 1517 to H.L.W. and H.D. and GZ1263 to S.D.); National Natural Science Foundation of China, Ministry of Science and Technology of the People's Republic of China (81970525 to H.D. and 81870424 to S.S.W.); LiSyM Grant PTJ-FKZ (031L0043 to S.D.); Cancer Genomics Center Netherlands (to P.t.D.); National Institutes of Health (R01 DK101426 to D.S.); Deutsche Forschungsgemeinschaft (SFB TRR57 to P18, CRC 1382 to A09, to J.T.); European Union's Horizon 2020 Research and Innovation Programme (Galaxy No. 668031, MICROB-PREDICT No. 825694, and DECISION No. 847949); Eurostars (DeFiber, E!12350); IK and Societal Challenges—Health, Demographic Change and Wellbeing (731875); Cellex Foundation (PREDICT); Chinese Scholarship Council (201708080020, 201708080021, and 201706230256 to T.L., S.W., and R.F.)

Abstract

Background and Aims: In patients with acute liver failure (ALF) who suffer from massive hepatocyte loss, liver progenitor cells (LPCs) take over key hepatocyte functions, which ultimately determines survival. This study investigated how the expression of hepatocyte nuclear factor 4α (HNF4α), its regulators, and targets in LPCs determines clinical outcome of patients with ALF.

Approach and Results: Clinicopathological associations were scrutinized in 19 patients with ALF (9 recovered and 10 receiving liver transplantation). Regulatory mechanisms between follistatin, activin, HNF4α, and coagulation factor expression in LPC were investigated in vitro and in metronidazole-treated zebrafish. A prospective clinical study followed up 186 patients with cirrhosis for 80 months to observe the relevance of follistatin levels in prevalence and mortality of acute-on-chronic liver failure. Recovered patients with ALF robustly express HNF4α in either LPCs or remaining hepatocytes. As in hepatocytes, HNF4α controls the expression of coagulation factors by binding to their promoters in LPC. HNF4α expression in LPCs requires the forkhead box protein H1–Sma and Mad homolog 2/3/4 transcription factor complex, which is promoted by the TGF-β superfamily member activin. Activin signaling in LPCs is negatively regulated by follistatin, a hepatocyte-derived hormone controlled by insulin and glucagon. In contrast to patients requiring liver transplantation, recovered patients demonstrate a normal activin/follistatin ratio, robust abundance of the activin effectors phosphorylated Sma and Mad homolog 2 and HNF4α in LPCs, leading to significantly improved coagulation function. A follow-up study indicated that serum follistatin levels could predict the incidence and mortality of acute-on-chronic liver failure.

Conclusions: These results highlight a crucial role of the follistatin-controlled activin-HNF4α-coagulation axis in determining the clinical outcome of massive hepatocyte loss-induced ALF. The effects of insulin and glucagon on follistatin suggest a key role of the systemic metabolic state in ALF.

INTRODUCTION

Following liver injury, hepatocytes proliferate rapidly in order to restore liver mass and essential functions.[1] However, following massive hepatocyte loss by either massive hepatic necrosis (MHN) or submassive hepatic necrosis, the remaining hepatocytes frequently lack the proliferative capacity required to restore essential liver functions.[2] Under these life-threatening conditions, liver progenitor cells (LPCs) proliferate rapidly and differentiate into hepatocytes to restore the parenchymal compartment.[1–3] This makes LPC-mediated liver regeneration a key event that largely determines the clinical outcome of patients suffering from massive hepatocyte loss.[2]

Massive hepatocyte loss not only occurs in acute liver failure (ALF), a life-threatening condition that develops typically within 8 weeks or less from the onset of symptoms in individuals without prior liver disease,[3,4] but also in chronic liver disease with acute decompensation

(AD), which is called acute-on-chronic liver failure (ACLF).[5,6] Following massive hepatocyte loss, activated LPCs, which are morphologically presented as reactive ducts, either differentiate into hepatocytes or retain LPC identity while acquiring essential hepatocyte functions. About 75 years ago, Lucke and Mallory investigated detailed pathological features of MHN.[7,8] They found that LPC-mediated ductular reaction (DR) occurred extremely early and persisted for an extended time. A large portion of 296 autopsied patients survived for more than 1 month. Over time, DR extended from zone 1 to zones 2 and 3. However, no mature hepatocytes were detected at any sampling point, indicating that LPC-to-hepatocyte differentiation requires time. These observations suggest that in the absence of hepatocytes, LPC might temporarily perform key hepatocyte functions.

To date, liver transplantation (LT) is the only approach to rescue patients with ALF. The decision on whether patients are enrolled on the waiting list for LT is determined by the Model for End-Stage Liver Disease (MELD) score.[9] Among the three MELD parameters, the international normalized ratio (INR), which reflects coagulation function, accounts for the largest weight. With the exception of the von Willebrand factor, all coagulation proteins are synthesized in hepatocytes.[10] Hepatocyte nuclear factor 4α (HNF4α), a constitutive hepatocyte-specific lineage transcription factor, controls the expression of multiple coagulation factors through direct binding to their respective gene promoters in hepatocytes.[11,12] To date, it is unknown whether and how LPCs rapidly take over coagulation function in ALF.

In this study, we investigated potential survival mechanisms in patients with chronic liver disease suffering from massive hepatocyte loss-induced ALF. Here we defined the disease as massive hepatocyte loss–induced liver failure (MHL-LF) to distinguish it from the European Association for the Study of the Liver–Chronic Liver Failure Consortium–defined ACLF[13] and classic ALF, which usually occurs in individuals without prior liver disease.[3]

MATERIALS AND METHODS

Patients

A total of 19 patients with MHL-LF 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 an additional 9 from the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School. Liver-tissue specimens were obtained during LT or by transjugular liver biopsy (for recovered patients with MHL-LF). Given the very limited liver tissues obtained from transjugular liver biopsy, 10 liver tissues obtained from Beijing (5 recovered and 5 receiving LT) were stained by immunohistochemistry IHC staining for HNF4α and phosphorylated Sma and Mad homolog 2 (p-SMAD2). Nine liver tissues collected from Hannover (4 recovered and 5 receiving LT) were used for measuring p-SMAD2, forkhead box protein H1 (FOXH1), and HNF4α. Cytokeratin 7 was measured in all samples to identify LPCs.

Massive hepatocyte loss was diagnosed when a liver tissue presented with extensive and diffuse necrosis spanning multiple adjacent regenerative nodules.[6] AD was defined by the development of one or more major complications of liver diseases: (i) development of grade 2 to 3 ascites within < 2 weeks; (ii) HE; (iii) gastrointestinal hemorrhage; or (iv) bacterial infections (e.g., spontaneous bacterial peritonitis, spontaneous bacteremia, urinary tract infection, pneumonia, cellulitis).[14]

Detailed methods of LPC culture, human LPC isolation, cholangiocyte-to-hepatocyte differentiation in Zebrafish, Chromatin immunoprecipitation, protein complex immunoprecipitation, Western blotting, immunohistochemistry and quantitative real-time PCR and reagent information are presented in the Supporting Information.

RESULTS

Recovered patients with MHL-LF robustly express HNF4α in either LPCs or hepatocytes

To clarify why a proportion of patients survive MHL-LF, we first compared clinical parameters and liver histological alterations between 5 recovered and 5 irreversible patients with MHL-LF who received LT. All patients had known etiology and clinical duration (i.e., the interval between the first symptoms of AD and the time of tissue sampling). Both cohorts were of similar age and clinical duration ($p > 0.05$; Figure S1A). Among the 10 patients, 5 were HBV-induced MHL-LF, 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 S1A). Meanwhile, key biochemical and clinical parameters were examined 24 h before liver histological examination (transjugular liver biopsy in the recovered patients vs. liver explant in the irreversible patients). There were no statistical differences in serum alanine aminotransferase, aspartate aminotransferase, and albumin levels between the two cohorts ($p > 0.05$; Figure S1A). However, three parameters were strikingly different: total bilirubin (μmol/L, 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 1A). These results demonstrate that bilirubin metabolism and coagulation function were significantly impaired in the irreversible cohort.

Given the crucial role of HNF4α in the regulation of coagulation,[11] we subsequently performed IHC

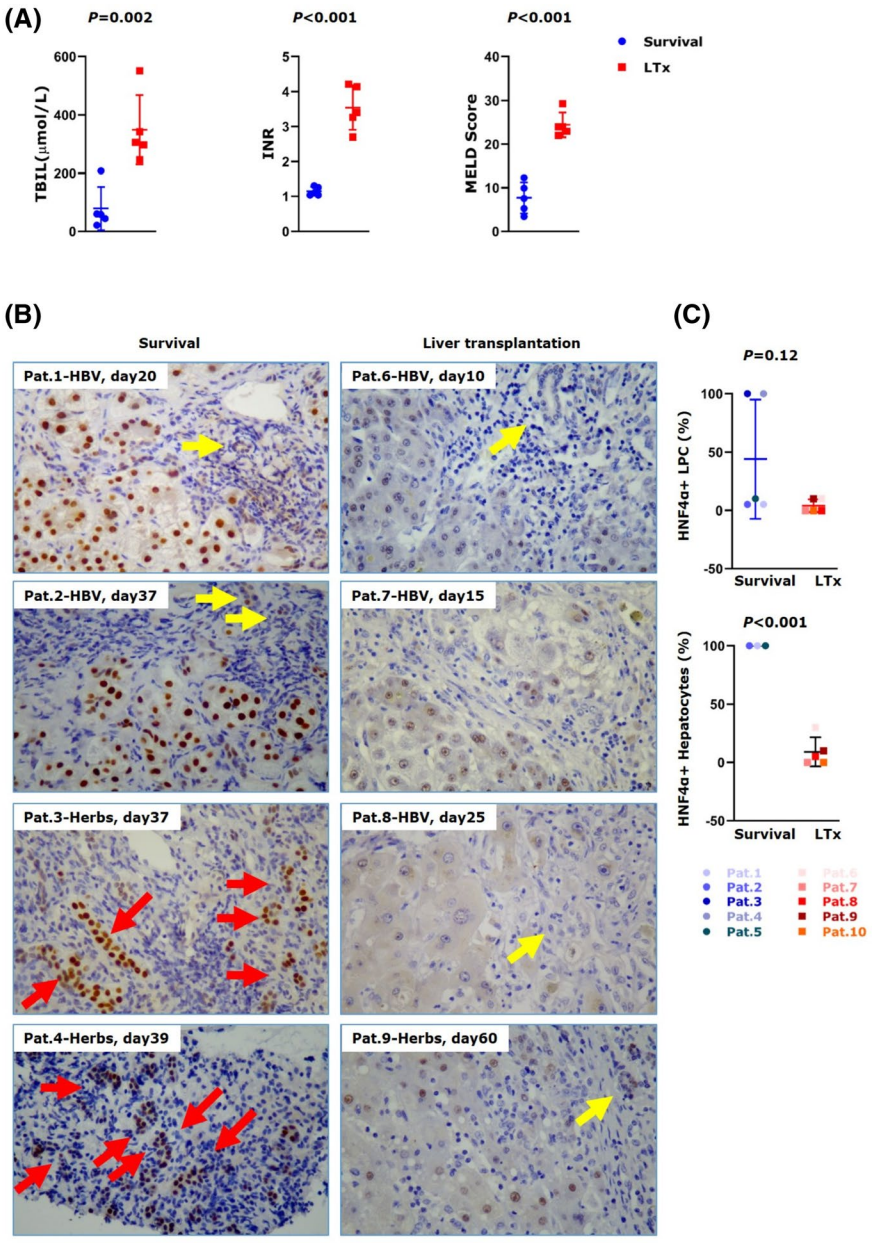

FIGURE 1 Recovered patients with massive hepatocyte loss–induced liver failure (MHL-LF) robustly express hepatocyte nuclear factor 4α (HNF4α) in either liver progenitor cells (LPCs) or hepatocytes. (A) Serum total bilirubin (TBIL), international normalized ratio (INR), and the Model for End-stage Liver Disease (MELD) scores are compared between 5 survivors and 5 patients with irreversible MHL-LF receiving liver transplantation (LTx). (B) Immunohistochemical staining for HNF4α was performed in these patients. Yellow and red arrows depict negative or positive HNF4α expression in LPCs. (C) Positive HNF4α hepatocytes and LPCs were counted in the collected patients, as described in Materials and Methods

for HNF4α in the collected liver tissues. As shown in Figure 1B, the recovered patients displayed intensive HNF4α immune positivity in the nuclei of either hepatocytes or LPCs. In areas with leftover hepatocytes, immune reactivity of HNF4α in LPCs was very weak or negative (yellow arrows in Patients 1 and 2; Figure 1B), whereas in those areas without hepatocytes, strong HNF4α immune reactivity was only observed in the nuclei of LPCs (red arrows in Patients 3 and 4; Figure 1B). In contrast to the recovered patients, the irreversible patients demonstrated rather weak immune reactivity for HNF4α in both hepatocytes and LPCs (yellow arrows depicting LPCs; Figure 1B). We quantified positive HNF4α immune reactions in LPCs and hepatocytes. In biopsied specimens collected from 5 recovered patients, 3 demonstrated remaining hepatocytes and proliferative LPCs, whereas 2 only displayed active LPCs, but no hepatocytes. In the former, all remaining hepatocytes, but not LPCs, showed strong HNF4α immune reactivity, whereas in the latter, LPCs displayed robust HNF4α immune positivity (Figure 1C). In contrast to the recovered patients, HNF4α positivity was present in a

portion of hepatocytes in the 5 irreversible patients, and only a few LPCs demonstrated a detectable HNF4α immune reaction (Figure 1C). Noteworthy, IHC showed that LPCs in patients with MHL-LF are able to express coagulation factor V (*F5*) (Figure S1B).

These results imply a potential link between HNF4α induction and the expression of coagulation factors in LPCs.

HNF4α activates expression of coagulation factors F2 and F5 by binding to their promoters in LPC

Next, we examined the effects of HNF4α on expression of coagulation factors in LPC lines: human HepaRG cells[15] and bipotential murine oval liver (BMOL) cells.[16] Chromatin immunoprecipitation (ChIP) assays revealed that HNF4α bound to the promoters of the coagulation factor *F2* and *F5* genes in both human and mouse LPCs (Figure 2A). Knockdown of HNF4α in both cell lines (>80% efficacy) significantly reduced mRNA and protein expression of coagulation factor genes *F2* and *F5* (Figure 2B).

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

SMADs-FOXH1 complex controls HNF4α expression in LPC

Next, we investigated potential transcription factors that control HNF4α expression in LPCs. In silico analysis for putative transcription factors indicated that SMAD and FOXH1 binding sites were present 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 2C). 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 2C). Immunoprecipitation further showed that FOXH1 combined with SMAD2/3-SMAD4 in HepaRG cells (Figure 2D).

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

Activin controls HNF4α and coagulation factors in LPCs

As both TGF-β and activin can activate SMAD2 and SMAD3,[17,18] 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. Quantitative PCR and western blotting analyses showed that incubation of TGF-β for 24 h significantly inhibited mRNA and protein expression of E-cadherin, HNF4α, albumin, F2, and F5 in LPCs (Figure 3A). In contrast to TGF-β, activin induced mRNA and protein expression of HNF4α, albumin, F2 and F5, but did not affect E-cadherin expression (Figure 3A). The 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 3A). Immunofluorescence showed that activin induced HNF4α expression and SB431542 decreased it in HepaRG and BMOL cells (Figure S2).

To confirm the role of activin in HNF4α expression, we knocked down the activin receptor like kinase 4 (ALK4) by small interfering RNA in HepaRG and BMOL cells (Figure 3B). Concomitant with reduced ALK4 expression, p-SMAD2 expression upon stimulation with activin was decreased in both cell lines (Figure 3B). Furthermore, ALK4 RNA interference (RNAi) significantly reduced activin-dependent mRNA and protein expression of HNF4α, F2, and F5 (Figure 3B), indicating activin-specific effects toward HNF4α, F2, and F5. Following HNF4α knockdown, activin-induced mRNA and protein expression of F2 and F5 were significantly inhibited in both HepaRG and BMOL cells (Figure 3C).

Moreover, ChIP assays showed that activin stimulation increased binding of HNF4α protein in the gene promoters of coagulation factors *F2* and *F5* (Figure 3D). Administration of SB421542 inhibited the binding (Figure 3D).

Furthermore, we examined the effect of activin in primary human LPCs isolated from healthy tissue surrounding a HCC (Figure S3). As in LPC lines, activin induced mRNA expression of HNF4α, F2, and F5 in human primary *LPCs* in vitro (Figure 3E). Upon knockdown of HNF4α in human LPCs, F2 and F5 mRNA expression was reduced (Figure 3E).

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

Based on these observations, we speculated that the activin-FOXH1-SMADs complex-HNF4α axis controls the expression of coagulation factors in LPCs. Thus, we examined the effects of SMAD2/3 and FOXH1 on expression of coagulation factors F2 and F5 in LPCs.

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 4A). ChIP assays further showed that activin administration increased, whereas SB431542 inhibited, the binding activity of SMAD2/3 and SMAD4 in the *HNF4A* gene promoter (Figure 4B).

In addition to SMADs, we investigated the role of FOXH1 in the expression of HNF4α and coagulation

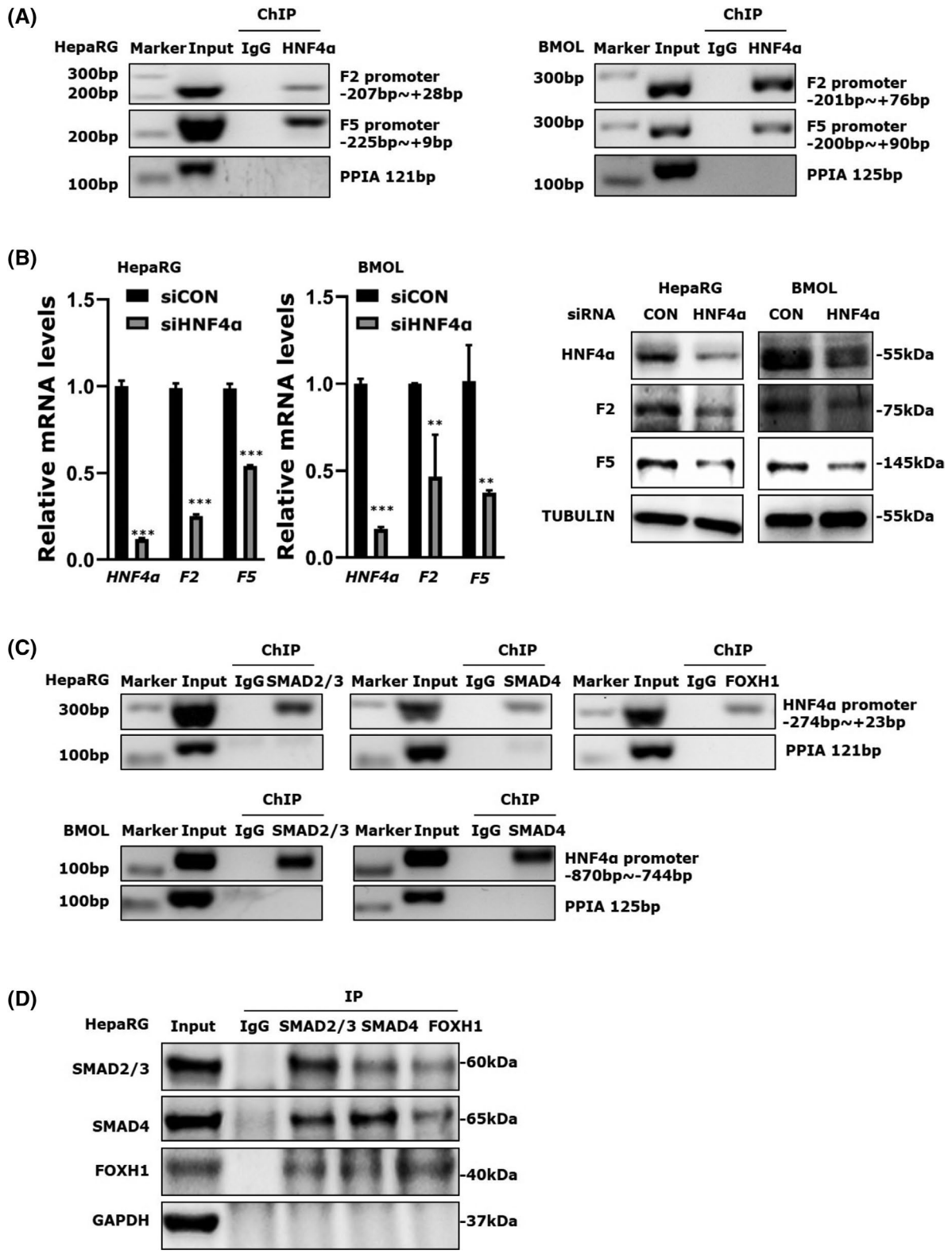

FIGURE 2 Sma and Mad homologs (SMADs)–forkhead box protein H1 (FOXH1) complex–controlled HNF4α regulates expression of coagulation factors in LPCs. (A) Chromatin immunoprecipitation (ChIP) assay was performed to measure HNF4α binding to the promoter of coagulation factor 2 (*F2*) and 5 (*F5*) genes in HepaRG and bipotential murine oval liver (BMOL) cells. (B) Quantitative PCR and western blotting were used to measure mRNA expression of coagulation factors in HepaRG and BMOL cells with or without HNF4α RNA interference (RNAi). (C) ChIP assay was performed to measure SMAD2/3, SMAD4, and FOXH1 binding to the promoter of *hnf4α* genes in HepaRG and BMOL cells. (D) Co-immunoprecipitation (Co-IP) 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. Tubulin was used as loading control in western blotting. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

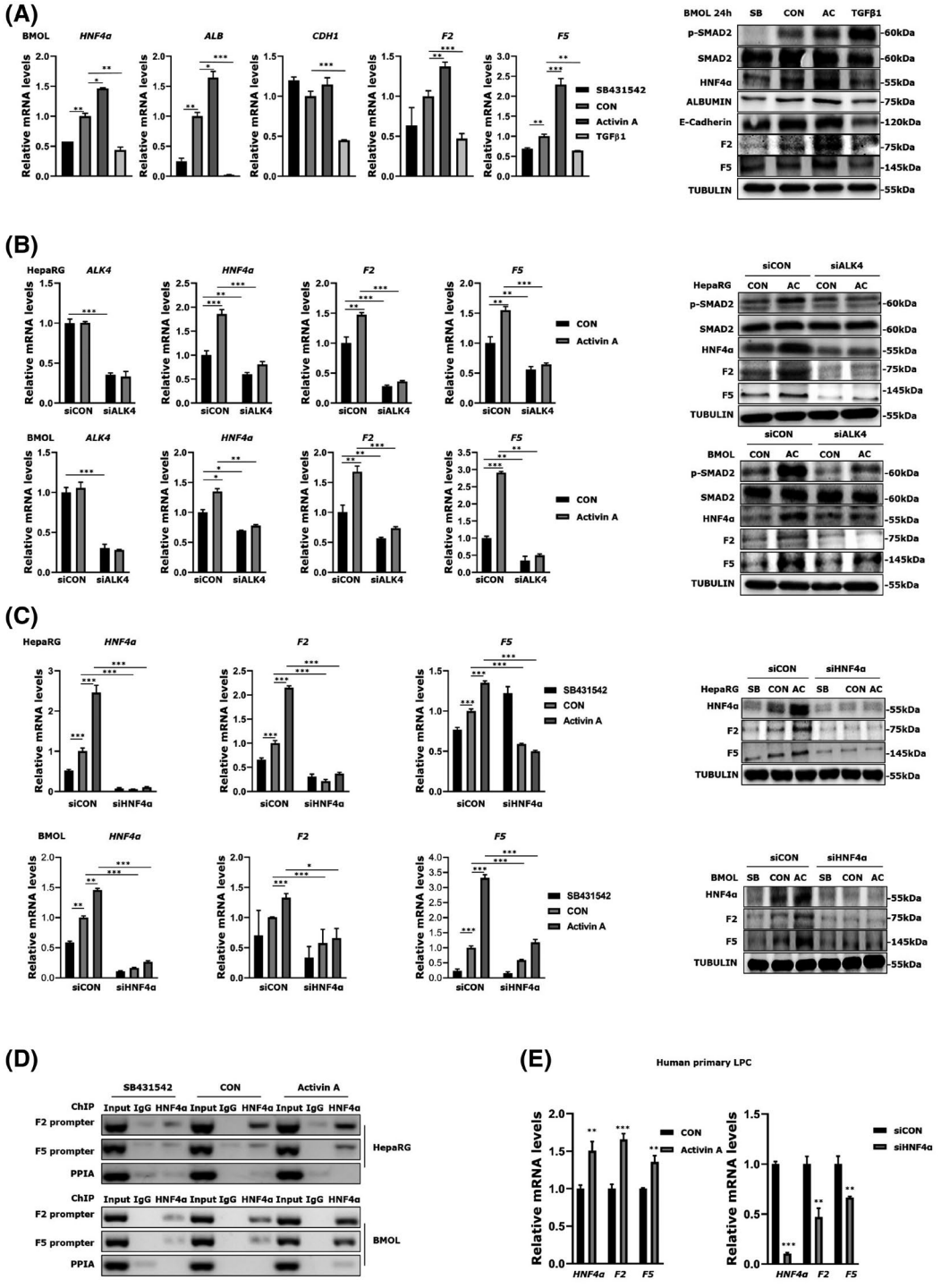

FIGURE 3 Activin, but not TGF-β, controls coagulation factors through HNF4α in LPCs. (A) Quantitative PCR and western blotting were used to measure the effects of activin (AC), TGF-β, and SB431542 (SB) administration for 24 h on mRNA and protein expression of cadherin-1 (*cdh1*)/E-cadherin, HNF4α, albumin (ALB), and F5 in BMOL cells. (B) Quantitative PCR and western blotting were performed to measure the impact of activin receptor like kinase 4 (ALK4) knockdown on mRNA and protein expression of *cdh1*/E-cadherin, HNF4α, albumin, and F5 in activin-treated HepaRG and BMOL cells. (C) Quantitative PCR 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. (D) ChIP assay was performed to examine the effect of activin A (activin) and SB431542 on the binding of HNF4α to *F2* and *F5* gene promoters in HepaRG and BMOL cells. (E) Quantitative PCR measures the effects of activin on mRNA expression of HNF4α, F2, and F5 in primary human LPCs, and mRNA expression of F2 and F5 in primary human LPCs with or without RNAi-mediated depletion of HNF4α. Expression of PPIA was used as loading control in ChIP assay. Tubulin was used as a loading control in western blotting. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. AC, activin A; CON, control; SB, SB431542

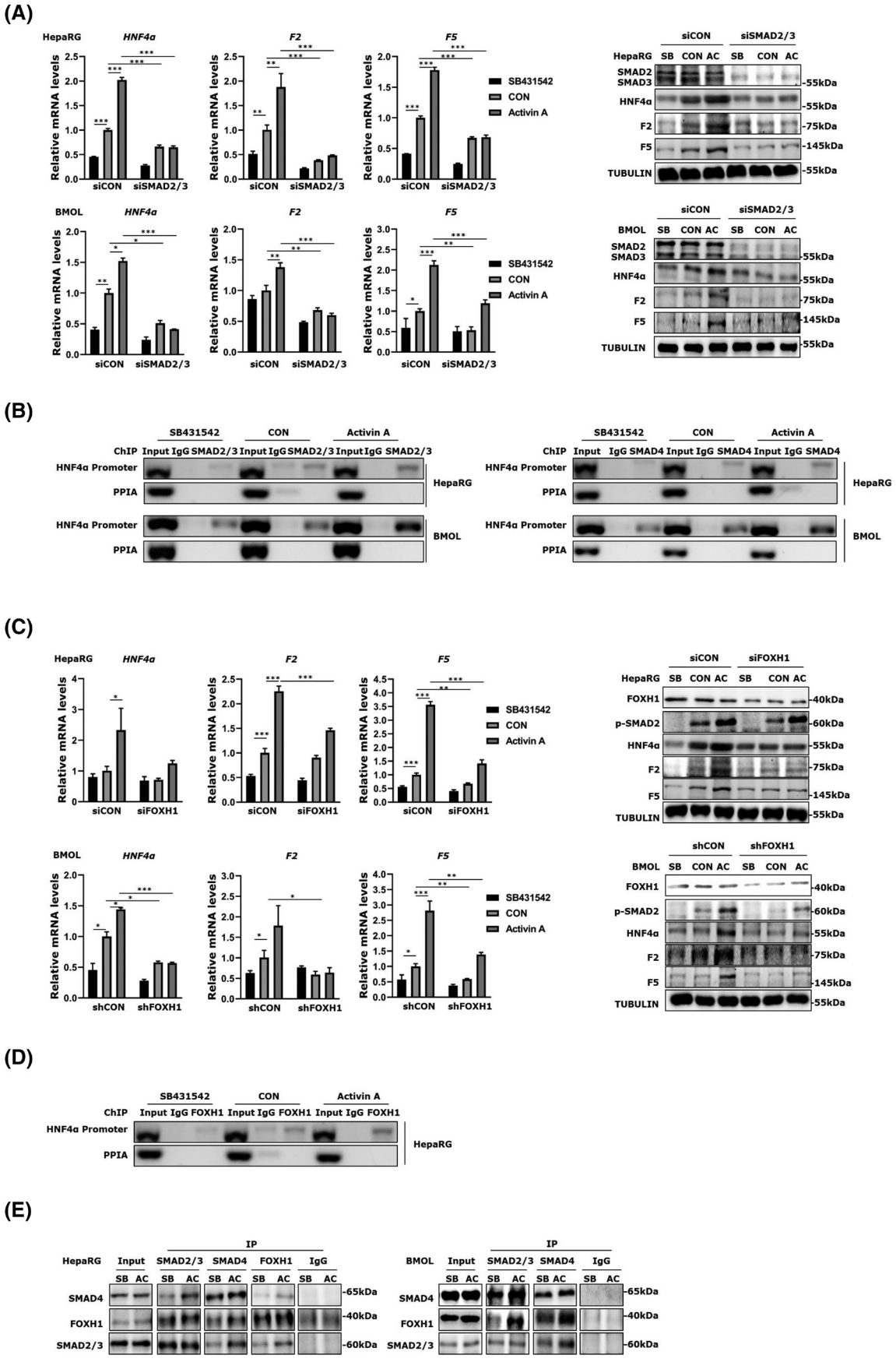
(A)
HepaRG
HNF4α
F2
F5
Relative mRNA levels
siCON
siSMAD2/3
SB431542
CON
Activin A
siCON
siSMAD2/3
HepaRG SB CON AC SB CON AC
SMAD2
SMAD3
HNF4α
F2
F5
TUBULIN
55kDa
55kDa
75kDa
145kDa
55kDa
BMOL
HNF4α
F2
F5
siCON
siSMAD2/3
BMOL SB CON AC SB CON AC
SMAD2
SMAD3
HNF4α
F2
F5
TUBULIN
55kDa
55kDa
75kDa
145kDa
55kDa
(B)
SB431542
CON
Activin A
ChIP Input IgG SMAD2/3 Input IgG SMAD2/3 Input IgG SMAD2/3
HNF4α Promoter
PPIA
HepaRG
HNF4α Promoter
PPIA
BMOL
SB431542
CON
Activin A
ChIP Input IgG SMAD4 Input IgG SMAD4 Input IgG SMAD4
HNF4α Promoter
PPIA
HepaRG
HNF4α Promoter
PPIA
BMOL
(C)
HepaRG
HNF4α
F2
F5
Relative mRNA levels
siCON
siFOXH1
SB431542
CON
Activin A
siCON
siFOXH1
HepaRG SB CON AC SB CON AC
FOXH1
p-SMAD2
HNF4α
F2
F5
TUBULIN
40kDa
60kDa
55kDa
75kDa
145kDa
55kDa
BMOL
HNF4α
F2
F5
shCON
shFOXH1
shCON
shFOXH1
BMOL SB CON AC SB CON AC
FOXH1
p-SMAD2
HNF4α
F2
F5
TUBULIN
40kDa
60kDa
55kDa
75kDa
145kDa
55kDa
(D)
SB431542
CON
Activin A
ChIP Input IgG FOXH1 Input IgG FOXH1 Input IgG FOXH1
HNF4α Promoter
PPIA
HepaRG
(E)
IP
HepaRG Input SMAD2/3 SMAD4 FOXH1 IgG
SB AC SB AC SB AC SB AC SB AC
SMAD4
FOXH1
SMAD2/3
65kDa
40kDa
60kDa
IP
BMOL Input SMAD2/3 SMAD4 IgG
SB AC SB AC SB AC SB AC
SMAD4
FOXH1
SMAD2/3
65kDa
40kDa
60kDa

FIGURE 4 Activin controls coagulation factor expression in LPCs through the SMADs-FOXH1 complex-controlled HNF4α. (A) Quantitative PCR 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. (B) ChIP assay was performed to examine the role of activin and SB431642 on the binding of SMAD2/3 and SMAD4 to the *HNF4A* gene promoters in indicated LPCs. (C) Quantitative PCR 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. (D) ChIP assay examined the role of activin and SB431642 in the binding of FOXH1 to the *HNF4A* gene promoters in HepaRG cells. (E) Co-IP was performed to measure SMADs-FOXH1 complex formation in HepaRG and BMOL cells. The expression of PPIA was used as loading control in ChIP assay. Tubulin was used as loading control in western blotting. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

factors in LPCs. Knockdown of FOXH1 significantly inhibited activin-induced mRNA and protein expression of HNF4α, F2, and F5 in both HepaRG and BMOL cells (Figure 4C). ChIP assays revealed that activin administration increased the FOXH1 binding to the *HNF4A* gene promoter in HepaRG cells, which was significantly reduced by SB431542 (Figure 4D). Co-immunoprecipitation analyses showed that activin increased FOXH1 complex forming with SMAD2/3-SMAD4 in HepaRG and BMOL cells, which was inhibited by SB431542 (Figure 4E).

These results demonstrate an activin-SMADs-FOXH1 complex-HNF4α-coagulation factor axis in LPCs.

Zebrafish Hnf4a mutant exhibits reduced expression of coagulation factor *f2* but normal cholangiocyte-to-hepatocyte conversion

To investigate the role of HNF4α in the regulation of coagulation factor genes in LPCs *in vivo*, we used a zebrafish model for LPC-mediated liver regeneration, *Tg(fabp10a:CFP-NTR)*^{s931}, in which cholangiocytes rapidly and robustly contribute to hepatocytes through LPCs following severe hepatocyte ablation.[19] The *Tg(fabp10a:CFP-NTR)* line expresses bacterial nitroreductase (NTR) fused with cyan fluorescent protein (CFP) under the hepatocyte-specific *fabp10a* promoter. NTR converts metronidazole (Mtz) into a cytotoxic drug; thus, Mtz treatment ablates cells that express NTR[20,21] (e.g., hepatocytes in *Tg[fabp10a:CFP-NTR]* larvae). As previously described, treating *Tg(fabp10a:CFP-NTR)* larvae with Mtz from 3.5 to 5 days post-fertilization for 36 h ablates nearly all hepatocytes.[19] During this ablation, cholangiocytes dedifferentiate into LPCs; following Mtz washout, LPCs rapidly differentiate into hepatocytes or cholangiocytes.[19] We examined the expression of two coagulation factor genes, *f2* and *f5*, and *gc* (albumin counterpart in zebrafish) in regenerating (R) larvae at 6 and 24 h following Mtz washout (R6h and R24h, respectively) by in situ hybridization. At R24h, all LPCs were differentiated into either hepatocytes or cholangiocytes, whereas at R6h, this differentiation was minimal.[19] There was no difference in *gc* and *f5* expression in regenerating livers among *hnf4a*$^{+/+}$, *hnf4a*$^{+/-}$, and *hnf4a*$^{-/-}$ larvae; however, *f2* expression was undetectable in *hnf4a*$^{-/-}$ larvae at both R6h and R24h (Figure 5A,B). These results suggest that *hnf4a* regulates *f2* expression in both LPC and regenerating hepatocytes.

Using whole-mount immunostaining, we examined the expression of hepatocyte marker betaine-homocysteine S-methyltransferase (Bhmt) at R24h. The Notch reporter *Tg(Tp1:H2B-mCherry)*^{s939} line, which expresses a nuclear red fluorescent protein under the control of an element containing 12 recombination signal binding protein for immunoglobulin kappa J region binding sites,[22] was used to reveal cholangiocytes (H2B-mCherry^{strong}) and cholangiocyte-derived hepatocytes (H2B-mCherry^{weak}) in the regenerating liver. Bhmt and *Tp1:H2B-mCherry* expression appeared normal in *hnf4a*$^{-/-}$ larvae (Figure 5C), suggesting that lack of *hnf4a* does not affect LPC-to-hepatocyte differentiation in zebrafish.

Epigenetic phenotype of LPCs is not altered by activin signal transduction and HNF4α expression

We also examined the role of the activin-HNF4α axis in the expression of epigenetic hallmarks, such as H3K4me3, H3K27me3 and H3K27ac, in HepaRG and BMOL cells. As shown in Figure S4A, methylation patterns were not affected following 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 in BMOL cells (Figure S4B).

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

Activin signaling is negatively controlled by follistatin in MHL-LF

Given the key role of activin signaling in controlling the HNF4α-coagulation factor axis in LPCs, we asked whether patients with irreversible MHL-LF lack activin. To this end, we measured serum activin concentrations in the 10 patients with MHL-LF described in Figure 1 and in 13 healthy volunteers. The average serum activin concentrations in the 5 recovered patients with MHL-LF were similar to those in healthy controls,

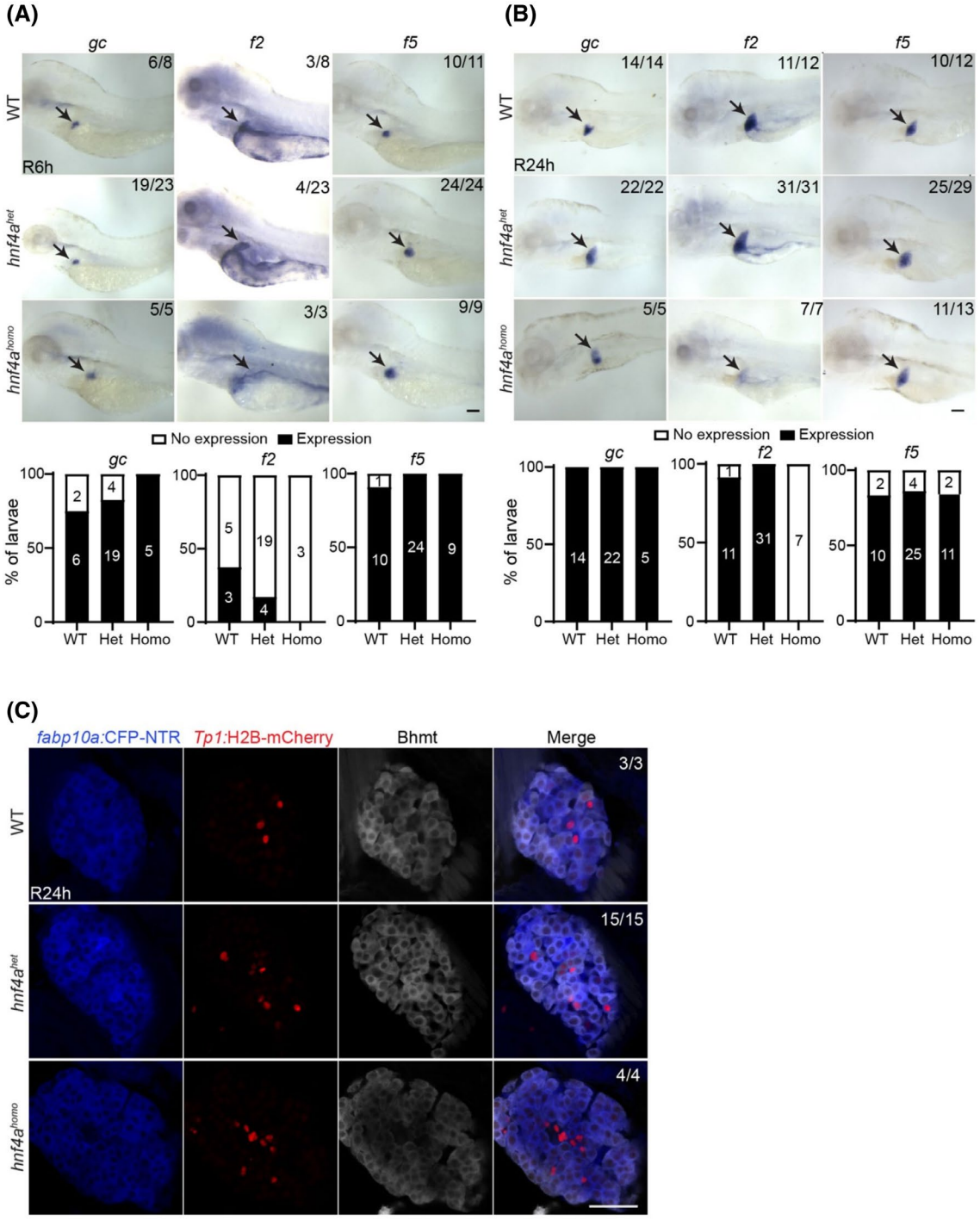

FIGURE 5 Zebrafish hnf4a mutants exhibit the reduced expression of coagulation factor 2, but normal cholangiocyte-to-hepatocyte conversion. (A,B) Whole-mount *in situ* hybridization images show the expression of *gc*, *f2*, and *f5* in regenerating livers (arrows) at R6h (A) and R24h (B). Graphs show the percentage of larvae that express gc (albumin counterpart in zebrafish), f2, or f5 in the regenerating liver. (C) Single-optical section images show the expression of fabp10a:CFP-NTR (blue), Tp1:H2B-mCherry (red), and betaine-homocysteine S-methyltransferase (Bhmt; gray) in regenerating livers at R24h. Numbers in the upper-right corner indicate the proportion of larvae exhibiting the expression/phenotype pattern shown. Scale bars: 100 μm (A,B) and 50 (C) μm. CFP, cyan fluorescent protein; NTR, nitroreductase; R, regenerating; WT, wild type

whereas serum activin levels in the 5 patients with irreversible MHL-LF were significantly reduced (Figure 6A). However, there was no significant statistic difference of serum activin levels between recovered patients and patients with irreversible MHL-LF (Figure 6A). In 3 of 5 recovered patients, serum activin concentrations were at the same level as in the 5 irreversible patients (Figure 6A). Notably, IHC staining showed strong immune positivity for p-SMAD2 and HNF4α in all recovered patients, but weak or even negative staining in the

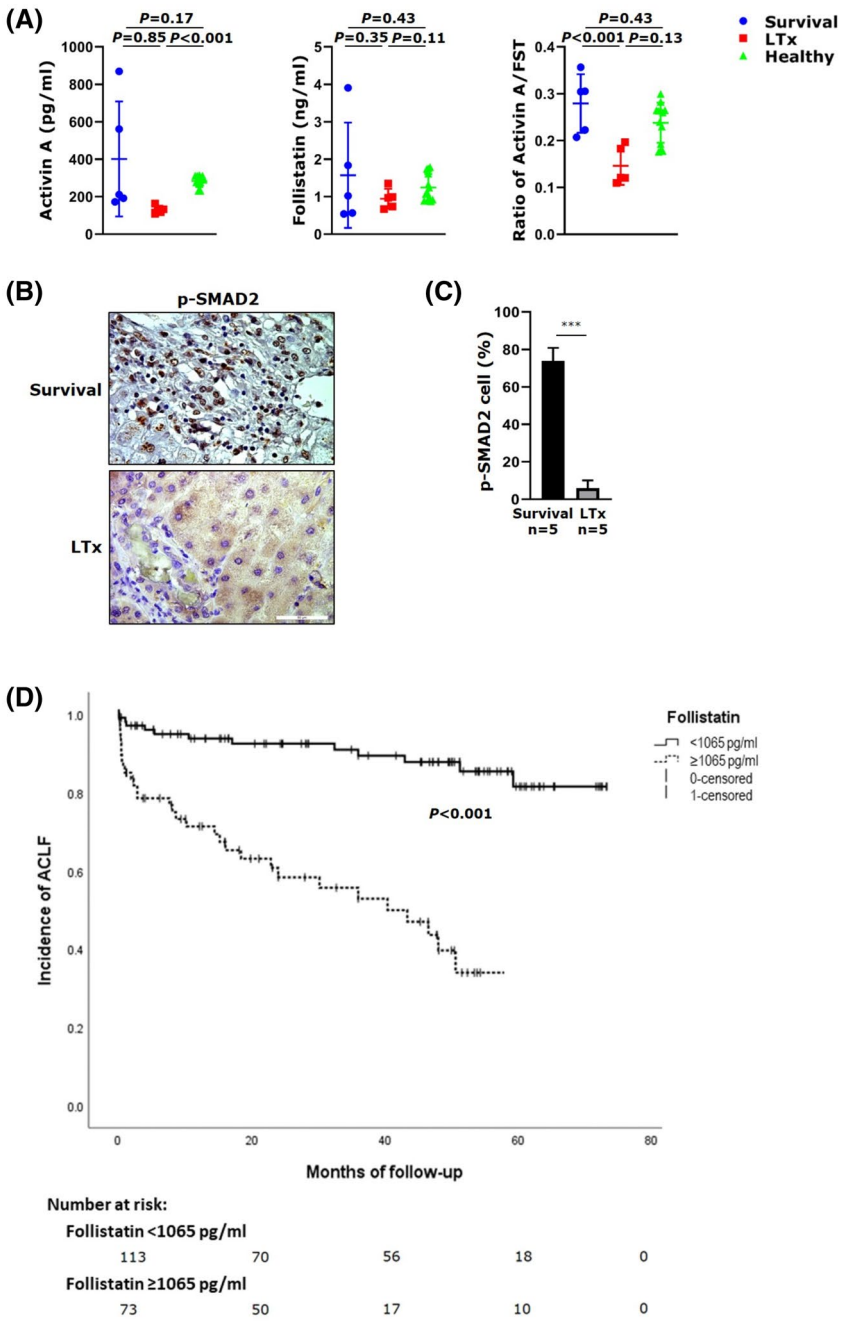

FIGURE 6 Follistatin (FST) negatively controls activin signaling and predicts the incidence of acute-on-chronic liver failure (ACLF). (A) Concentrations of activin and FST were measured by ELISA in 10 patients with MHL-LF and 13 healthy volunteers. The ratio of activin and FST was calculated. (B) Immunohistochemistry for p-SMAD2 is shown in 2 representative patients with MHL-LF. (C) Positive number of phosphorylated SMAD2 (p-SMAD2) in 10 patients with MHL-LF was counted. (D) Kaplan-Meier curves analysis was performed to calculate the incidence of ACLF in 186 patients with cirrhosis, as described in the Materials and Methods

irreversible patients (Figures 1B and 6B,C). In addition to the aforementioned 10 patients with MHL-LF enrolled in Beijing, we also examined p-SMAD2, FOXH1, and HNF4α expression by IHC in an additional 9 patients with MHL-LF, including 4 recovered patients from Hannover. In line with the Beijing patients, immune positivity of p-SMAD2, FOXH1, and HNF4α was robust in the recovered patients, but weak or undetectable in the patients receiving LT (Figure S5).

MHL-LF induces severe local inflammation and sepsis-like systemic inflammatory response.[3,6] Patients with sepsis with poor prognosis display high levels of follistatin (FST), a natural antagonist of activin.[17,23] In patients with sepsis, FST increases up to 40-fold of normal levels.[23] Therefore, we speculated that high levels of FST might antagonize activin signaling in patients with irreversible MHL-LF. We measured serum FST concentrations and calculated the ratio

of activin to FST in the 10 patients with MHL-LF and 13 healthy volunteers. Although there were no significant differences of serum FST concentrations between groups, the ratio of activin to FST in the recovered patients with MHL-LF was significantly higher than in the irreversible patients (Figure 6A). There was no difference in the ratio between recovered patients with MHL-LF and healthy controls (Figure 6A).

Given that most patients with MHL-LF enrolled in this study had cirrhosis, we further determined the role of FST in cirrhosis by performing a prospective study in Mexico. We examined serum concentrations of FST in 186 patients with cirrhosis (86 with compensated cirrhosis, 52 with AD, and 48 with ACLF; Table S1). Table S2 lists the characteristics of the 48 patients with ACLF. The three patient cohorts had similar age and etiologies (Table S1). There was a significant difference in gender among the three cohorts. Compared to the patients with compensated cirrhosis and AD, the prevalence of male patients was significantly higher in ACLF (Table S1). FST levels were lowest in patients who remained compensated during follow-up, significantly higher in those who developed AD and ACLF, with the highest values being observed in the latter group. Clinical severity scores were higher in AD and ACLF (Table S1). Death rates were significantly higher (i.e., about 4-fold) in ACLF than in any other group.

FST levels positively correlated with severity scores, Child-Pugh (R = 0.395; p < 0.0001), and MELD score (R = 0.410; p < 0.0001) (Table S1). To obtain a cutoff value for FST, a receiver operating characteristic (ROC) curve analysis was performed, in which the best cutoff value was ≥ 1065 pg/mL with an area under the ROC of 0.711, sensitivity of 75%, and specificity of 65.2%. In the Kaplan-Meier curve analysis, FST was significantly associated with the development of ACLF. Patients with FST < 1065 pg/mL had a mean incidence of ACLF at 64.0 ± 2.5 months compared to those with FST ≥ 1065 pg/mL, where the mean incidence occurred at 31.7 ± 3.3 months (p < 0.0001; Figure 6D). In a further Cox regression analysis, FST remained independently associated with the incidence of both ACLF and survival (Table S3).

These results suggest that FST deserves further investigation as a potential predictive biomarker for the incidence and mortality of ACLF.

Glucagon and insulin control hepatocellular FST, which is critical for an activated activin-HNF4α-coagulation axis in LPCs

FST is primarily produced by hepatocytes, and its expression is regulated by the ratio of glucagon and insulin.[24] Therefore, we examined the effects of glucagon and insulin on FST expression in primary human and mouse hepatocytes. Quantitative PCR and western blotting analyses showed that glucagon induced mRNA and protein expression of FST in hepatocytes (Figure 7A,B). The glucagon-induced FST expression was inhibited by insulin administration (Figure 7A,B). Next, we examined the effects of FST on activin-treated HepaRG and BMOL cells. As shown in Figure 7C,D, FST remarkably inhibited activin-induced mRNA and protein expression of HNF4α, F2, and F5 in LPCs.

We also measured serum levels of insulin, glucagon, and their ratio in healthy volunteers and 10 patients with MHL-LF. Among patients, we did not observe a statistically significant difference of the insulin/glucagon ratio between recovered and irreversible patients with MHL-LF, although the value of insulin/glucagon in the recovered patients was lower than in the irreversible patients (Figure S6).

These results suggest that the balance between activin and FST determines whether activin signaling is capable of initiating the activin-HNF4α-coagulation factor axis in patients with MHL-LF. We highlight the regulatory model in a scheme (Figure 8).

DISCUSSION

Why and how some patients can survive massive hepatocyte loss, while others cannot, is a key clinical question. We provide the following findings to answer this question[1]: (i) In patients with MHL-LF, LPCs take over and carry out key hepatocyte functions, such as coagulation[2]; (ii) expression of coagulation factors in LPCs depends on HNF4α[3]; (iii) HNF4α expression in LPCs is driven by activin signaling[4]; (iv) activity of an intact activin-HNF4α-coagulation factor regulatory axis in LPCs largely determines the clinical outcome of patients with MHL-LF[5]; and (v) activin signaling is negatively regulated by FST, a hepatocyte-derived inhibitor controlled by the insulin-to-glucagon ratio.

In patients with massive hepatocyte loss, the maintenance of vital hepatocyte functions depends on sufficient abundance and function of remaining hepatocytes and activated LPCs.[2] In contrast to classic ALF, MHL-LF occurs in patients with chronic liver diseases, in particular liver cirrhosis.[2] Patients with cirrhosis have a unique liver condition characterized by a substantial portion of hepatocytes damaged and incapacitated before acute deterioration.[10] It addition, approximately 70% of hepatocyte buds are derived from LPCs in patients with cirrhosis.[25] Therefore, performance of hepatocyte functions by LPCs might precede MHL-LF in patients with liver cirrhosis. Following MHL and rapid ductular reaction,[6,7] differentiation into mature hepatocytes takes approximately 1 month.[2] During this time, performance of vital liver functions by LPCs is crucial for patient survival. We found that LPCs in patients with MHL-LF indeed express hepatocyte-specific proteins, such as coagulation factor F5 (Figure S1B). Impressively, the

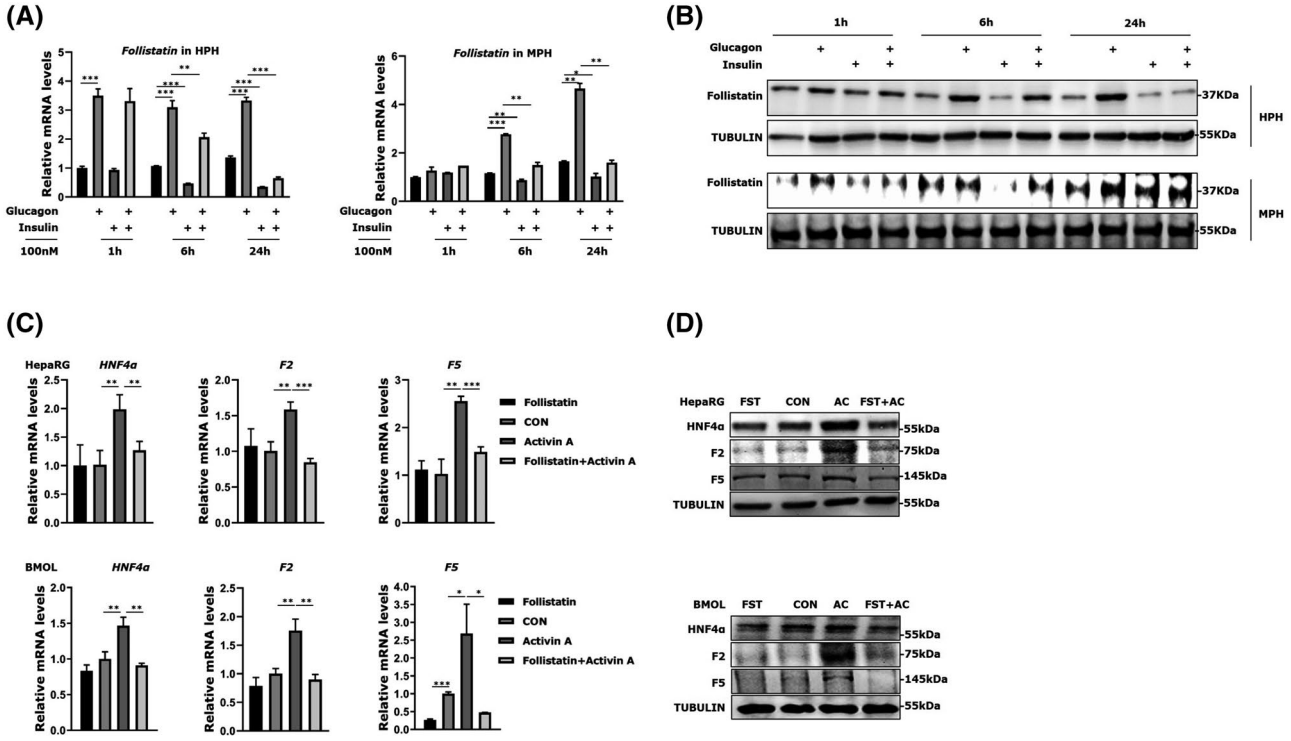

FIGURE 7 Insulin-regulated and glucagon-regulated FST determines the activin-HNF4α-coagulation factor axis in LPCs. (A,B) Quantitative PCR and western blot were used to measure mRNA and protein expression of FST in human primary hepatocytes (HPHs) and mouse primary hepatocytes (MPHs) with insulin and/or glucagon treatment. (C,D) Quantitative PCR and western blot were used to measure mRNA and protein expression of HNF4α and F5 in HepaRG and BMOL cells with activin and/or FSTs treatment for 24 h. Tubulin was used as loading control in western blotting. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

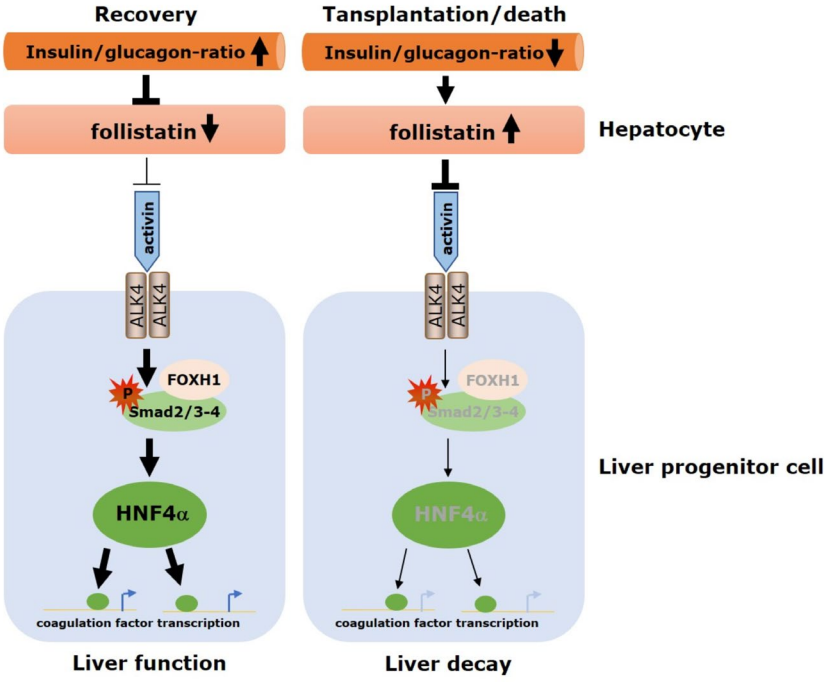

FIGURE 8 Hormone-controlled activin-HNF4α-coagulation factor axis in LPCs

recovered patients with MHL-LF express HNF4α in either hepatocytes or LPCs depending on the abundance of the respective cell type. These results imply that in MHL-LF, LPCs might rescue key hepatocyte functions by forced expression of HNF4α before differentiation into hepatocytes.

For patients with MHL-LF, coagulation function is indispensable for survival. A recent study showed that the serum plasminogen level is a promising prognostic biomarker in patients with ACLF.[26] In normal physiological circumstances, most coagulation factors are synthesized by hepatocytes under the control of master hepatic transcription factors, including HNF4α.[10,11,27] Co-expression of HNF4α and F5 in LPCs suggests that HNF4α might be one of the key transcription factors controlling coagulation factors expression in these cells. We confirmed this hypothesis by ChIP assays: HNF4α initiates the transcription of F2 and F5 through binding to their promoters in LPCs. These results explain why patients with MHL-LF with high levels of HNF4α in LPCs have a normal INR.

How do LPCs initiate HNF4α expression? We found that the formation of transcription factor FOXH1-SMADs complex is essential to initiate HNF4α transcription in LPCs. Although the upstream signal controlling FOXH1 expression in LPCs is not known yet, the formation of SMAD complex suggests the requirement of either TGF-β or activin signal in initiating HNF4α transcription. Interestingly, activin and TGF-β play an opposing role in regulating HNF4α in LPCs: Activin up-regulates, whereas TGF-β inhibits, HNF4α expression. Further experiments based on ALK4 knockdown confirmed the key role of activin in HNF4α expression in LPCs.

The activin-driven FOXH1-SMAD2/3/4 complex plays a crucial role in mediating embryonic stem cell–to-mesoderm differentiation through up-regulating master differentiation genes (e.g., goosecoid, mix paired-like homeobox).[28] In vitro, activin initiates embryonic stem cell–to-mesoderm differentiation through SMAD2-mediated H3K27me3 reduction.[29] This raises an interesting question: Does activin directly initiate LPC-to-hepatocyte differentiation rather than merely inducing hepatocyte functions in patients with MHL-LF? Rodents cannot survive massive hepatic necrosis, as they do not receive intensive life support. Therefore, no rodent animal model is available for mimicking the pathophysiology of MHL-LF. Fortunately, hepatocyte-ablated zebrafish provide an elegant model to investigate MHL-LF.[19] We observed that lack of the *hnf4a* gene did not affect the LPC-to-hepatocyte differentiation, but remarkably reduced the expression of the coagulation factor gene *f2* at both R6h and R24h. Consistent with these zebrafish data, activin stimulation or disruption of any component of the FOXH1-SMAD2/3/4 complex-HNF4α axis did not alter epigenetic phenotypes of LPCs. These results suggest that HNF4α is a key transcription factor required for the expression of coagulation factor genes in both LPCs and hepatocytes, but is dispensable for LPC-to-hepatocyte differentiation.

In patients with MHL-LF, a lack of hepatocytes does not denote a poor prognosis. As shown in Figure 1, 2 recovered patients do not display any hepatocytes in the examined tissues. However, the proliferating LPCs demonstrate robust HNF4α expression, indicating that these cells are performing hepatocyte functions. In contrast to the recovered patients, all irreversible patients examined possess sufficient hepatocytes and active LPCs. 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 LPCs is more important than restoring parenchymal cell numbers for survival. Consistent with our findings, several previous studies observed that the extent of necrosis does not directly affect the final clinical outcome of patients with MHL-LF.[30,31]

In clinical practice, it is a controversial issue whether morphologically successful liver regeneration denotes a good clinical outcome of patients with MHL-LF. Frequently, histological examination in explanted livers reveals a successful LPC-mediated liver regeneration.[2] The question is whether these patients should receive LT. Will patients showing “successful hepatic regeneration” recover over time? In contrast to the “excellent” histological features, clinical manifestation and functional parameters, including MELD score, may demonstrate severe disease and thus the need to perform LT. Based on the current study, performing LT is the correct decision, because the regenerated hepatocytes do not perform essential functions due to a lack of key regulatory networks, such as the activin-HNF-4α axis.

Given the key role of activin signaling in the regulation of coagulation factors in LPCs, we asked whether repression of the activin-HNF4α-coagulation factor axis in patients with irreversible MHL-LF is due to a lack of activin. However, serum activin concentrations in recovered patients were not significantly different from those in the irreversible patients. In remarkable contrast, p-SMAD2 levels in hepatocytes and LPCs of the recovered patients were considerably higher than those in the irreversible patients. This observation indicated that the activin signal in the latter might be inhibited. We hypothesized that FST might be the responsible inhibitory factor in patients with irreversible MHL-LF, based on three reasons: First, FST is a natural inhibitor of activin.[32] Second, FST is synthesized mainly in hepatocytes[32]; hence, massive hepatocyte death might release copious amounts of FST. Third, FST is a key reproduction hormone that suppresses the follicle stimulating hormone.[32] According to the life history, theory, growth, reproduction, and maintenance are three fundamental biological programs in humans.[33] In favorable environments, the synthesis and release of FST in hepatocytes is strictly regulated by the glucagon-to-insulin ratio to promote investment in growth and reproduction.[32] In harsh environments, such as severe infection, FST is required for diversion of resources from reproduction to the defense arm.[33] Circulating FST concentration in sepsis patients increased to 40-fold of the normal level.[34] Therefore, high levels of FST 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.

Lin et al. examined the FST-to-activin ratio 16 patients with ALF and reported that the ratio in surviving patients with ALF was significantly higher than that in nonsurvivors.[35] When we measured serum FST levels in 10 patients with MHL-LF, there was no significant difference between the recovered and the irreversible patients. In contrast to Lin et al.'s observation, the activin/FST ratio in the recovered patients was remarkably higher than in the irreversible patients, suggesting that the activin-to-FST ratio is the crucial determinant of robust p-SMAD2 expression.

In addition to regulating coagulation factor expression, high levels of FST reflect an emergency condition that requires the host to re-allocate energy resources toward maintenance by inhibiting reproduction.[33] Our prospective clinical study in 186 patients with cirrhosis showed that serum FST level not only reflects the mortality in patients with MHL-LF, but also the incidence of cirrhosis and AD progressing to ACLF. These results imply that serum FST is a reliable parameter to identify emergency condition in patients.

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 FST.[24] Consistent with previous studies, our in vitro experiments confirmed that glucagon induced and insulin inhibited expression and secretion of FST in hepatocytes, and thus regulated the activin-HNF4α-coagulation factor axis in LPCs. In the circumstance of sepsis, high levels of glucagon are required to maintain high levels of blood glucose.[36] To guarantee sufficient energy supply for priority organs and cells, such as the brain and immune cells, insulin resistance occurs in major metabolic tissues such as adipose tissue, skeletal muscle, and hepatocytes.[36] Hepatic insulin resistance thus abrogates the inhibitory effect of insulin on FST, disrupting the regulation of the FST synthesis by the glucagon/insulin ratio. This might explain why we did not observe significant differences of insulin and glucagon levels between the recovered patients and patients with irreversible MHL-LF.

It is noteworthy that the effects of the activin-HNF4α axis are not limited to controlling coagulation factor expression. HNF4α is a hepatic master transcription factor that binds to more than 40% of actively transcribed genes in hepatocytes.[11] RNA sequencing of activin-treated LPCs with or without HNF4α disruption revealed that the activin-HNF4α axis profoundly affects the 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). On the other hand, the activin-HNF4α axis is not the only signaling network regulating coagulation factors. Even in irreversible patients lacking activin signaling and HNF4α expression, coagulation factor expression in the liver is still detectable. Repression of the activin-HNF4α axis leads to repression, but not total abrogation, of coagulation function. Additional regulatory networks governing coagulation need to be clarified in the future.

Taken together, the current study highlights a requirement of the activin-HNF4α axis in LPCs for the assumption of vital hepatocyte functions, such as coagulation, in circumstances of massive hepatocyte loss, and therefore plays a crucial role for the survival of patients. This regulatory axis is inhibited by hepatocyte-derived FST secretion. Distinct from the physiological conditions, in which FST production is regulated by the systemic balance between insulin and glucagon, excess FST in MHL-ALF is locally released by massive hepatic necrosis. On the other hand, MHL-induced severe inflammatory response results in insulin resistance in leftover hepatocytes, which leads to glucagon producing abundant FST in these cells. In clinical practice, histological examination is very difficult to perform in MHL-LF. Our results indicate that serum FST levels might be a surrogate marker reflecting the extent of hepatocyte death and hepatic insulin resistance, which point to the danger of coagulopathy and clinical deterioration. The hypothesis requires further confirmation in the future.

ACKNOWLEDGMENT

The authors thank Dr. Peter Heinrich, Joan Massagué, Paul Romeo, and George Yeoh for kindly providing valuable comments, plasmids, and BMOL cells.

CONFLICT OF INTEREST

Jonel Trebicka has received speaking and/or consulting fees from Gore, Bayer, Alexion, MSD, Gilead, Intercept, Norgine, Grifols, Versantis, and Martin Pharmaceutical.

AUTHOR CONTRIBUTIONS

Conception, design, and hypothesis: Hong-Lei Weng. *Clinical design and organization:* Huiguo Ding and Jonel Trebicka. *Patients' sampling, serum measurement, and pathological experiments:* Shanshan Wang, Stefan Munker, Ricardo U. Macías-Rodríguez, Astrid Ruiz-Margáin, Robert Schierwagen, Hui Liu, Chen Shao, Xiaodong Yuan, Chunlei Fan, Franziska Wandrer, Enrico N. De Toni, Heike Bantel, and Huiguo Ding. In vitro *experiments:* Tao Lin, Shanshan Wang, Stefan Munker, Rilu Feng, Sai Wang, and Xiaodong Yuan. *Animal experiments:* Kyounghwa Jung and Donghun Shin. *Drafting the article:* Tao Lin and Hong-Lei Weng. *Data discussion, reviewing, and editing the article critically:* Stefan Munker, Christoph Meyer, Roman Liebe, Jens Kroll, Long Zhang, Peter ten Dijke, Andreas Teufel, Alexander Marx, Peter R. Mertens, Hua Wang, Matthias P. A. Ebert, Heike Bantel, Enrico N. De Toni, Jonel Trebicka, Steven Dooley, Donghun Shin, Huiguo Ding, and Hong-Lei Weng.

ORCID

Ricardo U. Macías-Rodríguez https://orcid.org/0000-0002-7637-4477
Jonel Trebicka https://orcid.org/0000-0002-7028-3881

REFERENCES

1. Michalopoulos GK. Principles of liver regeneration and growth homeostasis. Compr Physiol. 2013;3:485–513.
2. Weng HL, Cai X, Yuan X, Liebe R, Dooley S, Li H, et al. Two sides of one coin: massive hepatic necrosis and progenitor cell-mediated regeneration in acute liver failure. Front Physiol. 2015;6:178.
3. Lefkowitch JH. The pathology of acute liver failure. Adv Anat Pathol. 2016;23:144–58.
4. Bernal W, Wendon J. Acute liver failure. N Engl J Med. 2013;369:2525–34.
5. Stravitz RT, Lefkowitch JH, Fontana RJ, Gershwin ME, Leung PSC, Sterling RK, et al. Autoimmune acute liver failure: proposed clinical and histological criteria. Hepatology. 2011;53:517–26.
6. Li H, Xia Q, Zeng BO, Li S-T, Liu H, Li QI, et al. Submassive hepatic necrosis distinguishes HBV-associated acute on chronic liver failure from cirrhotic patients with acute decompensation. J Hepatol. 2015;63:50–9.
7. Lucke B. The pathology of fatal epidemic hepatitis. Am J Pathol. 1944;20:471–593.
8. Lucke B, Mallory T. The fulminant form of epidemic hepatitis. Am J Pathol. 1946;22:867–945.
9. European Association for the Study of the Liver. EASL clinical practice guidelines: liver transplantation. J Hepatol. 2016;64:433–85.
10. Alastair D, Burt BCP, Ferrel LD. MacSween's Pathology of the Liver, 6th edn. Eidingburg: Churchill Livingstone Elsevier; 2012.
11. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, et al. Control of pancreas and liver gene expression by HNF transcription factors. Science. 2004;303:1378–81.
12. Safdar H, Cheung KL, Vos HL, Gonzalez FJ, Reitsma PH, Inoue Y, et al. Modulation of mouse coagulation gene transcription following acute in vivo delivery of synthetic small interfering RNAs targeting HNF4alpha and C/EBPalpha. PLoS ONE. 2012;7:e38104.
13. Arroyo V, Moreau R, Jalan R. Acute-on-chronic liver failure. N Engl J Med. 2020;382:2137–45.
14. Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. Gastroenterology. 2013;144:1426–37, e1–e9.
15. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A. 2002;99:15655–60.
16. Tirnitz-Parker JE, Tonkin JN, Knight B, Olynyk JK, Yeoh GC. Isolation, culture and immortalisation of hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented diet. Int J Biochem Cell Biol. 2007;39:2226–39.
17. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 2003;113:685–700.
18. Heldin CH, Moustakas A. Signaling receptors for TGF-beta family members. Cold Spring Harb Perspect Biol. 2016;8:a022053.
19. Choi TY, Ninov N, Stainier DY, Shin D. Extensive conversion of hepatic biliary epithelial cells to hepatocytes after near total loss of hepatocytes in zebrafish. Gastroenterology. 2014;146:776–88.
20. Curado S, Anderson RM, Jungblut B, Mumm J, Schroeter E, Stainier DY. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. Dev Dyn. 2007;236:1025–35.
21. Pisharath H, Rhee JM, Swanson MA, Leach SD, Parsons MJ. Targeted ablation of beta cells in the embryonic zebrafish pancreas using *E. coli* nitroreductase. Mech Dev. 2007;124:218–29.
22. Ninov N, Borius M, Stainier DY. Different levels of Notch signaling regulate quiescence, renewal and differentiation in pancreatic endocrine progenitors. Development. 2012;139:1557–67.
23. Michel K, Roth S, Trautwein C, Gong W, Flemming P, Gressner AM. Analysis of the expression pattern of the latent transforming growth factor beta binding protein isoforms in normal and diseased human liver reveals a new splice variant missing the proteinase-sensitive hinge region. Hepatology. 1998;27:1592–9.
24. Hansen JS, Rutti S, Arous C, Clemmesen JO, Secher NH, Drescher A, et al. Circulating follistatin is liver-derived and regulated by the glucagon-to-insulin ratio. J Clin Endocrinol Metab. 2016;101:550–60.
25. Stueck AE, Wanless IR. Hepatocyte buds derived from progenitor cells repopulate regions of parenchymal extinction in human cirrhosis. Hepatology. 2015;61:1696–707.
26. Wu D, Zhang S, Xie Z, Chen E, Rao Q, Liu X, et al. Plasminogen as a prognostic biomarker for HBV-related acute-on-chronic liver failure. J Clin Invest. 2020;130:2069–80.
27. Chiang JY. Hepatocyte nuclear factor 4alpha regulation of bile acid and drug metabolism. Expert Opin Drug Metab Toxicol. 2009;5:137–47.
28. Xi Q, Wang Z, Zaromytidou AI, Zhang XH, Chow-Tsang LF, Liu JX, et al. A poised chromatin platform for TGF-beta access to master regulators. Cell. 2011;147:1511–24.
29. Wang LU, Xu X, Cao Y, Li Z, Cheng H, Zhu G, et al. Activin/Smad2-induced histone H3 Lys-27 trimethylation (H3K27me3) reduction is crucial to initiate mesendoderm differentiation of human embryonic stem cells. J Biol Chem. 2017;292:1339–50.
30. Hanau C, Munoz SJ, Rubin R. Histopathological heterogeneity in fulminant hepatic failure. Hepatology. 1995;21:345–51.
31. Chenard-Neu MP, Boudjema K, Bernuau J, Degott C, Belghiti J, Cherqui D, et al. Auxiliary liver transplantation: regeneration of the native liver and outcome in 30 patients with fulminant hepatic failure–a multicenter European study. Hepatology. 1996;23:1119–27.
32. Hansen JS, Plomgaard P. Circulating follistatin in relation to energy metabolism. Mol Cell Endocrinol. 2016;433:87–93.
33. Wang A, Luan HH, Medzhitov R. An evolutionary perspective on immunometabolism. Science. 2019;363:eaar3932.
34. Michel U, Shintani Y, Nau R. Serum follistatin concentrations are increased in patients with septicaemia. Clin Endocrinol. 1998;48:413–7.
35. Lin SD, Kawakami T, Ushio A, Sato A, Sato S-I, Iwai M, et al. Ratio of circulating follistatin and activin A reflects the severity of acute liver injury and prognosis in patients with acute liver failure. J Gastroenterol Hepatol. 2006;21:374–80.
36. Van Wyngene L, Vandewalle J, Libert C. Reprogramming of basic metabolic pathways in microbial sepsis: therapeutic targets at last? EMBO Mol Med. 2018;10.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Lin T, Wang S, Munker S, Jung K, Macías-Rodríguez RU, Ruiz-Margáin A, et al. Follistatin-controlled activin-HNF4α-coagulation factor axis in liver progenitor cells determines outcome of acute liver failure. Hepatology. 2022;75:322–337. doi:10.1002/hep.32119