

Liver-Enriched Transcription Factor Expression Relates to Chronic Hepatic Failure in Humans

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The mechanisms by which the liver fails in end-stage liver disease remain elusive. Disruption of the transcription factor network in hepatocytes has been suggested to mediate terminal liver failure in animals. However, this hypothesis remains unexplored in human subjects. To study the relevance of transcription factor expression in terminal stages of chronic liver failure in humans, we analyzed the expression of liver-enriched transcription factors (LETFs) hepatocyte nuclear factor (HNF)4 α , HNF1 α , forkhead box protein A2 (FOXA2), CCAAT/enhancer-binding protein (CEBP) α , and CEBP β . We then selected downstream genes responsible for some hepatic functions (ornithine transcarbamylase [OTC], cytochrome P450 3A4 [CYP3A4], coagulation factor VII [F7], cadherin 1 [CDH1], phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) [p-ERM], phospho-myosin light chain [p-MLC], low-density lipoprotein receptor-related protein 1 [LRP1]) in liver tissue from patients at different stages of decompensated liver function based upon Child-Pugh classification, Model for End-Stage Liver Disease score, and degree of inflammatory activity/fibrosis. We first examined differential expression of LETF and determined whether a relationship exists between transcript and protein expression, and liver function. We found HNF4 α expression was down-regulated and correlated well with the extent of liver dysfunction ($P = 0.001$), stage of fibrosis ($P = 0.0005$), and serum levels of total bilirubin ($P = 0.009$; $r = 0.35$), albumin ($P < 0.001$; $r = 0.52$), and prothrombin time activity ($P = 0.002$; $r = 0.41$). HNF4 α expression also correlated with CYP3A4, OTC, and F7 as well as CDH1 RNA levels. The Rho/Rho-associated protein kinase pathways, which have been implicated in the regulation of HNF4 α , were also differentially expressed, in concert with LRP1, a reported upstream regulator of RhoA function. **Conclusion:** HNF4 α and other members of the LETFs appear to be important regulators of hepatocyte function in patients with chronic hepatic failure. (*Hepatology Communications* 2018;2:582-594)

Cirrhosis of the liver is characterized by diffuse fibrosis, disruption of the normal lobular architecture of the liver with formation of regenerative nodules, and severe disruption of the vascular organization of the liver that can also result in portal hypertension.⁽¹⁾ These profound structural and vascular changes can be accompanied by hepatocellular failure and the inability of hepatocytes to perform their normal synthetic and metabolic functions.⁽²⁻⁵⁾ The causes of cirrhosis include hepatitis B virus infection, hepatitis C virus infection, alcohol-mediated Laennec's cirrhosis,

and nonalcoholic steatohepatitis (NASH)/metabolic syndrome, among others. When advanced liver cirrhosis progresses to terminal liver failure, the only definitive therapy is orthotopic liver transplantation.⁽⁶⁾ The worldwide prevalence of liver cirrhosis is roughly 25 to 400 per 100,000 subjects.⁽¹⁾ In 2015, liver disease represented the eleventh leading cause of death, with an estimated 15.8 deaths per 100,000 globally. From 2000 to 2015, mortality increased nearly 30%,⁽⁷⁾ with the majority of cases occurring during the most productive years of life, between 25 and 64 years of age.^(7,8)

Abbreviations: CEBP, CCAAT/enhancer-binding protein; Ct, comparative threshold; CYP3A4, cytochrome P450 3A4; F7, coagulation factor VII; FOXA2, forkhead box protein A2; HNF, hepatocyte nuclear factor; LETF, liver-enriched transcription factor; LRP1, low-density lipoprotein receptor-related protein 1; MELD, Model for End-Stage Liver Disease; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; OTC, ornithine transcarbamylase; p-ERM, phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr558); ROCK, Rho/Rho-associated protein kinase.

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Although the etiologic agents and events that lead to cirrhosis may be known, the mechanisms responsible for deterioration of hepatocyte function and ultimately hepatic failure are largely unknown. Chronic injury to the liver is characterized by a decrease in hepatocyte mass, ongoing oxidative stress,^(9,10) impaired mitochondrial function,^(11,12) and limited regenerative capacity.^(13,14) We have previously investigated the abnormal microenvironment associated with cirrhosis and the hepatocytes that reside within the cirrhotic liver in an experimental animal model of irreversible cirrhosis and fatal chronic liver failure that greatly resembles human disease.⁽¹³⁻¹⁶⁾ We found that hepatocytes early in the development of cirrhosis suffer an adaptive metabolic and energy shift that involves a conversion from using oxidative phosphorylation to glycolysis and that dysfunctional hepatocytes during the terminal stages of chronic liver disease are unable to sustain the needed high levels of energy produced from glycolysis.⁽¹⁵⁾ Interestingly, we found that liver-enriched transcription factors (LETFs) are stably

down-regulated in hepatocytes from animals with end-stage cirrhosis and terminal hepatic failure. We showed that forced re-expression of hepatocyte nuclear factor 4 alpha (HNF4 α) can reprogram dysfunctional hepatocytes from terminally cirrhotic livers to function again, both in culture and *in vivo*, through expansion of new hepatocytes or stem cells and without the need for regeneration.⁽¹⁶⁾

While available animal models have been extremely useful for elucidating many aspects of hepatocyte dysfunction within the cirrhotic microenvironment, the relative expression of LETF pathways and downstream genes have yet to be examined in human degenerative liver disease and correlated with clinical hepatic function. Here, we examine expression of these LETF genes from explanted human liver specimens and correlate the results with clinical parameters of liver function and stage of liver decompensation. We demonstrate that the transcription factor HNF4 α is significantly down-regulated in association with the extent of hepatic dysfunction, in concert with select

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Potential conflict of interest: Dr. Soto-Gutierrez, Dr. Bell, and Dr. Fox are inventors on a pending patent application that describes the use of transcription factors to treat chronic liver failure (US20140249209). Dr. Soto-Gutierrez, Dr. Guzman-Lepe, Dr. Takeishi, Dr. Collin de l'Hortet, Dr. Wang, and Dr. Fox are cofounders and have a financial interest in Von Baer Wolff, Inc., a company focused on biofabrication of autologous human hepatocytes from stem cell technology and programming liver failure; their interests are managed by the Conflict of Interest Office at the University of Pittsburgh in accordance with their policies. Dr. Fox, Dr. Soto-Gutierrez, Dr. Takeishi, Dr. Guzman-Lepe, Dr. Collin de l'Hortet, and Dr. Wang own stock in Von Baer Wolff. The other authors have nothing to report.

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related genes. We thus corroborate previous findings from an animal model of irreversible cirrhosis with terminal liver failure.

Materials and Methods

HUMAN TISSUE SAMPLES

Between January 2009 and March 2013, 85 liver samples were obtained from patients who underwent partial hepatectomy or liver transplantation in the Department of Surgery and Science, Kyushu University Hospital. Diseases included liver metastasis, primary hepatocellular carcinoma, and decompensated cirrhosis. The patients with liver metastasis that did not undergo preoperative chemotherapy were classified as the normal liver group control. Specimens were analyzed histologically and for gene expression. Patient demographics and clinical status are shown in Table 1. The study was conducted with the approval of the institutional ethics review boards of Kyushu University (27-245) and the University of Pittsburgh (PRO13010075). The work undertaken conforms to the provisions of the Declaration of Helsinki.

HISTOLOGIC EXAMINATION

All specimens were cut into serial slices of 5-10 mm thickness and fixed in 10% formalin. After macroscopic examination, noncancerous tissue was trimmed, embedded in a paraffin block, and cut into 4- μ m microscopic sections. Sections were stained with hematoxylin and eosin, and pathologic findings were assessed according to the Liver Cancer Study Group of Japan.^(7,8) Necroinflammatory activity and the

diagnosis of liver cirrhosis were performed according to the New Inuyama Classification⁽¹⁷⁾ and the Ishak scoring system for histologic fibrosis staging.^(18,19)

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was extracted from noncancerous liver tissue using RNeasy Mini kits (QIAGEN, Hilden, Germany) and reverse transcribed using SuperScriptIII (Invitrogen, Carlsbad, CA) following the manufacturers' instructions. We performed quantitative polymerase chain reaction with a StepOnePlus system (Applied Biosystems, Foster City, CA) using TaqMan Fast Advanced Master Mix (Life Technologies, Waltham, MA). The probes used (all from Applied Biosystems) were HNF4 α (Hs00604431_m1), forkhead box protein A2 (FOXA2) (Hs00232764_m1), CCAAT/enhancer binding protein alpha (CEBP α) (Hs00269972_s1), CEBP β (Hs00270923_s1), HNF1 α (Hs00167041_m1), ornithine transcarbamylase (OTC) (Hs00166892_m1), cytochrome P450 3A4 (CYP3A4) (Hs00604506_m1), coagulation factor VII (F7) (Hs01551992;_m1), and β -actin (Hs01060665_g1). Each sample was examined in duplicate. Gene expression levels were analyzed according to the comparative threshold cycle (Ct) method, where the amount of target was normalized to β -actin and relative to a control sample. As a relative control expression reference, we used normal human liver tissue from a liver resection performed on a patient (18 years old) without liver dysfunction, who underwent hepatectomy for a benign liver tumor. The sample reference control value was given by 2- $\Delta\Delta$ Ct. Ct indicates the polymerase chain reaction cycle number at which the amount of amplified

TABLE 1. CLINICAL PARAMETERS OF THE PATIENTS IN THIS STUDY

| Factors | All patients (n = 84) | Normal Liver (n = 20) | Child-Pugh A (n = 25) | Child-Pugh B (n = 9) | Child-Pugh C (n = 30) |
|---------------------------------------|--------------------------|--------------------------|--------------------------|-------------------------|--------------------------|
| Age, years | 61 \pm 11 | 59 \pm 2 | 67 \pm 12 | 63 \pm 4 | 57 \pm 7 |
| Sex, male, N (%) | 47 (56) | 13 (65) | 17 (68) | 4 (44) | 13 (43) |
| Etiology, N (%) | | | | | |
| Hepatitis B virus | 11 (13) | 0 (0.0) | 4 (16) | 3 (33) | 4 (13) |
| Hepatitis C virus | 36 (43) | 0 (0.0) | 15 (60) | 6 (67) | 15 (50) |
| Alcohol | 7 (8.3) | 0 (0.0) | 3 (12) | 0 (0.0) | 4 (13) |
| NASH | 7 (8.3) | 0 (0.0) | 2 (8.0) | 0 (0.0) | 5 (17) |
| Cryptogenic | 3 (3.6) | 0 (0.0) | 1 (4.0) | 0 (0.0) | 2 (6.7) |
| Metastasis | 20 (24) | 20 (100) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hepatocellular carcinoma positive (%) | 40 (48) | 0 (0.0) | 24 (96) | 7 (78) | 9 (30) |
| Histologic cirrhosis, N (%) | 39 (46) | 0 (0.0) | 0 (0.0) | 9 (100) | 30 (100) |
| Total bilirubin (mg/dL) | 2.1 \pm 2.6 | 0.74 \pm 0.46 | 0.68 \pm 0.26 | 2.4 \pm 1.2 | 4.1 \pm 3.3 |
| Albumin (g/dL) | 3.5 \pm 0.8 | 4.3 \pm 0.1 | 3.9 \pm 0.5 | 3.1 \pm 0.4 | 2.7 \pm 0.4 |
| Prothrombin time activity (%) | 75 \pm 25 | 102 \pm 2.7 | 88 \pm 14 | 68 \pm 12 | 49 \pm 9 |
| Ascites, N (%) | 33 (39) | 0 (0.0) | 0 (0.0) | 6 (67) | 27 (90) |
| MELD score | 11 \pm 6 | 6.0 \pm 0.5 | 6.2 \pm 0.4 | 12 \pm 2 | 17 \pm 4 |

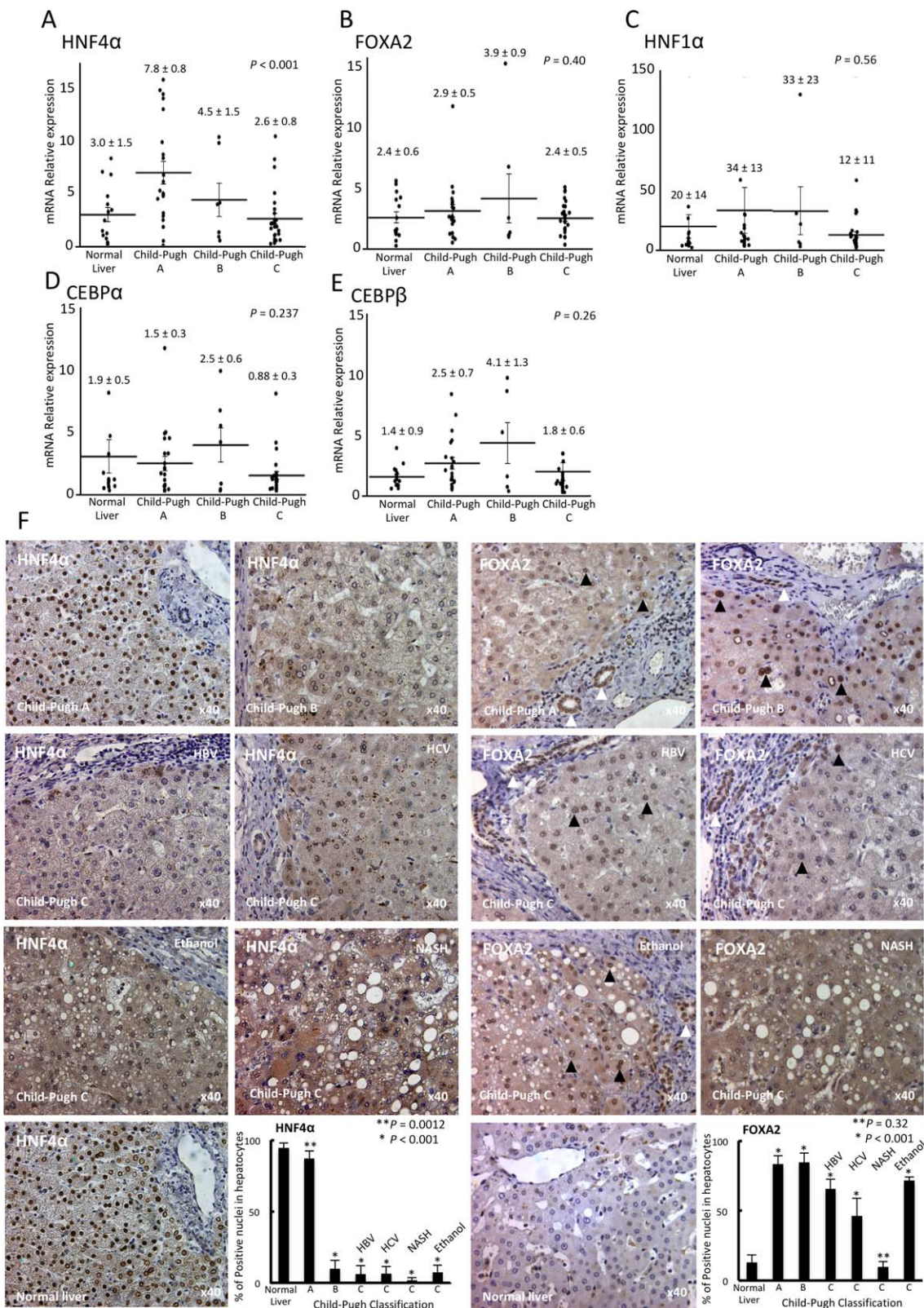


FIG. 1

target reaches a fixed threshold. The ΔCt value is determined by subtracting the average reference Ct value from the average target Ct value. The $\Delta\Delta\text{Ct}$ value involves subtraction by the ΔCt experimental control value.

IMMUNOHISTOCHEMISTRY

Paraffin-embedded liver tissue was deparaffinized with xylenes and dehydrated with ethanol. Antigen unmasking was performed by boiling in citrate buffer, pH 6.0. The slides were then incubated in 3% hydrogen peroxide, blocked with normal animal serum, and subsequently left incubating overnight at 4°C with primary antibodies; these included mouse anti-HNF4 α (41898; Abcam, Cambridge, MA), rabbit anti-FOXA2 (108422; Abcam), rabbit anti-low-density lipoprotein receptor-related protein 1 (anti-LRP1) (92544; Abcam), rabbit anti-phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) (p-ERM) (3726; Cell Signaling, Beverly, MA), and rabbit anti-myosin light chain (phosphor S20) (2480; Abcam). Tissue sections were then incubated with the secondary biotinylated antibody corresponding to the animal species of the primary antibody (BA-1000; Vector Laboratories, Burlingame, CA) and exposed to 3,3'-diaminobenzidine (SK-4105; Vector Laboratories) to visualize the peroxidase activity. Counterstaining was performed with Richard-Allan Scientific Signature Series Hematoxylin (Thermo Scientific, Waltham, MA). Control tissues were used for validation of antibodies used in this study (Supporting Fig. S1A,B). For quantification, immunoreactivities of HNF4 α and FOXA2 were independently graded by two liver pathologists, with 1,000 hepatocytes in three high-power fields being counted per sample. Normal livers ($n = 5$), Child-Pugh A ($n = 5$), Child-Pugh B ($n = 5$), and Child-Pugh C ($n = 13$) were included for these analyses.

STATISTICAL ANALYSIS

The statistical software JMP 10J (SAS Institute, Cary, NC) was used for all analyses. Data are expressed as mean \pm SEM. Continuous variables were compared using the Wilcoxon test and one-way analysis of

variance between two groups and among four groups, respectively. Additionally, the messenger RNA (mRNA) expression values in Fig. 1 were compared individually against control values using the Student t test. Regression analyses were performed to detect correlations between gene expression and clinical parameters and between relative expression of HNF4 α and the other genes expressed. Values of $P < 0.05$ were considered statistically significant.

Results

CLINICAL LIVER FUNCTION AND LETF EXPRESSION

Previous observations in animals with terminal chronic hepatic failure indicated that disruption of the transcription factor network and cellular dedifferentiation likely mediate hepatocyte decompensation. This observation led us to assess the relationship between LETF expression (HNF4 α , FOXA2, HNF1 α , CEBP α , and CEBP β) and clinical evidence of liver dysfunction in human cirrhotic livers where gene expression was correlated with the Child-Pugh score. There was a significant difference in expression of HNF4 α among specimens from patients with normal liver and those with Child-Pugh A, Child-Pugh B, and Child-Pugh C liver disease (3.0 ± 1.5 versus 7.8 ± 0.8 versus 4.5 ± 1.5 versus 2.6 ± 0.8 ; $P < 0.001$) using one-way analysis of variance to determine differences among groups (Fig. 1A). Additionally, we performed individual comparisons of each group versus the normal liver control group. This analysis revealed that only mRNA expression of HNF4 α in the Child-Pugh A group was significantly higher than that in the normal liver group (7.8 ± 0.8 versus 3.0 ± 1.5 ; $P = 0.008$). However, the mRNA expression of HNF4 α in the Child-Pugh B or Child-Pugh C group did not differ from that in normal liver (4.5 ± 1.5 or 2.6 ± 0.8 versus 3.0 ± 0.9 ; $P = 0.32$ or 0.65 , respectively) (Fig. 1A). Moreover, HNF4 α expression correlated significantly with serum total bilirubin level ($r = -0.35$,

FIG. 1. Expression levels of liver-enriched transcription factors, grouped by the Child-Pugh classification. Gene expression levels were analyzed according to the Ct method with the amount of target normalized to β -actin and relative to a control sample. The relative expression of (A) HNF4 α , (B) FOXA2, (C) HNF1 α , (D) CEBP α , and (E) CEBP β were analyzed using real-time reverse-transcription polymerase chain reaction. The expression values were compared using one-way analysis of variance among four groups. To compare the mRNA expression values from each experimental group against controls, the Student t test was used. (F) HNF4 α and FOXA2 expression in liver samples (normal livers [$n = 5$], Child-Pugh A [$n = 5$], Child-Pugh B [$n = 5$], and Child-Pugh C [$n = 13$]) were analyzed by immunohistochemistry, and immunoreactivity was quantified in hepatocyte nuclei (magnification $\times 40$). Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus. Data are expressed as mean \pm SEM.

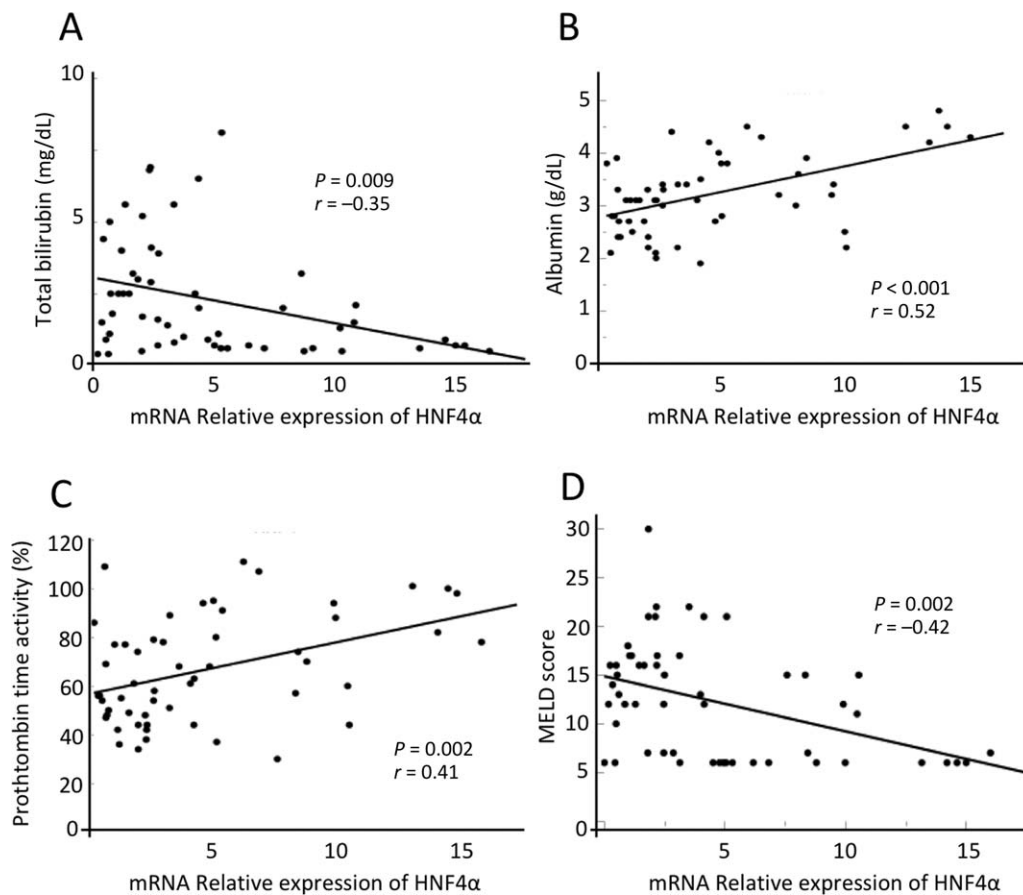


FIG. 2. Regression analyses between HNF4 α expression and clinical parameters. Gene expression levels in Child-Pugh A, B, and C patients' samples were analyzed according to the Ct method with the amount of target normalized to β -actin and relative to a control sample. Scatterplots are shown depicting the correlation between the expression of (A) HNF4 α and total bilirubin level, (B) serum albumin, (C) prothrombin time activity, and (D) MELD score.

$P = 0.009$), serum albumin levels ($r = 0.52$, $P < 0.001$), and prothrombin time activity ($r = 0.41$, $P = 0.002$) among the patients analyzed (Fig. 2). In fact, HNF4 α mRNA values correlated inversely and significantly with the Model for End-Stage Liver Disease (MELD) score of disease severity ($r = 0.42$, $P = 0.002$). However, gene expression levels of FOXA2, HNF1 α , CEBP α , and CEBP β showed no significant difference in the livers among patients with Child-Pugh A, B, and C scores (FOXA2, 2.4 ± 0.6 versus 2.9 ± 0.5 versus 3.9 ± 0.9 versus 2.4 ± 0.5 , $P = 0.40$; HNF1 α , 20 ± 14 versus 34 ± 13 versus 33 ± 23 versus 12 ± 11 , $P = 0.56$; CEBP α , 1.9 ± 0.5 versus 1.5 ± 0.3 versus 2.5 ± 0.6 versus 0.88 ± 0.3 , $P = 0.237$; CEBP β , 1.4 ± 0.9 versus 2.5 ± 0.7 versus 4.1 ± 1.3 versus 1.8 ± 0.6 , $P = 0.26$) (Fig. 3B-E). In addition, FOXA2, HNF1 α , CEBP α , and CEBP β had no

significant correlation with serum total bilirubin level, serum albumin levels, prothrombin time activity, or MELD score (Supporting Figs. S2-S3). These facts indicate that only loss of HNF4 α expression is associated with the progression to terminal chronic hepatic failure in humans.

HNF4 α and FOXA2 expressions were then examined by immunohistochemistry. HNF4 α immunoreactivity was observed in almost all nuclei from normal ($n = 5$) and Child-Pugh A liver samples ($n = 5$), as expected. However, HNF4 α was not observed in hepatocytes from Child-Pugh B ($n = 5$) and C liver samples ($n = 13$) (Fig. 1F) independent of etiology (hepatitis B virus, hepatitis C virus, alcohol-mediated Laennec's cirrhosis, and NASH). FOXA2, a LETF normally related to biliary epithelial cell specification in human adult normal livers but observed in hepatocytes

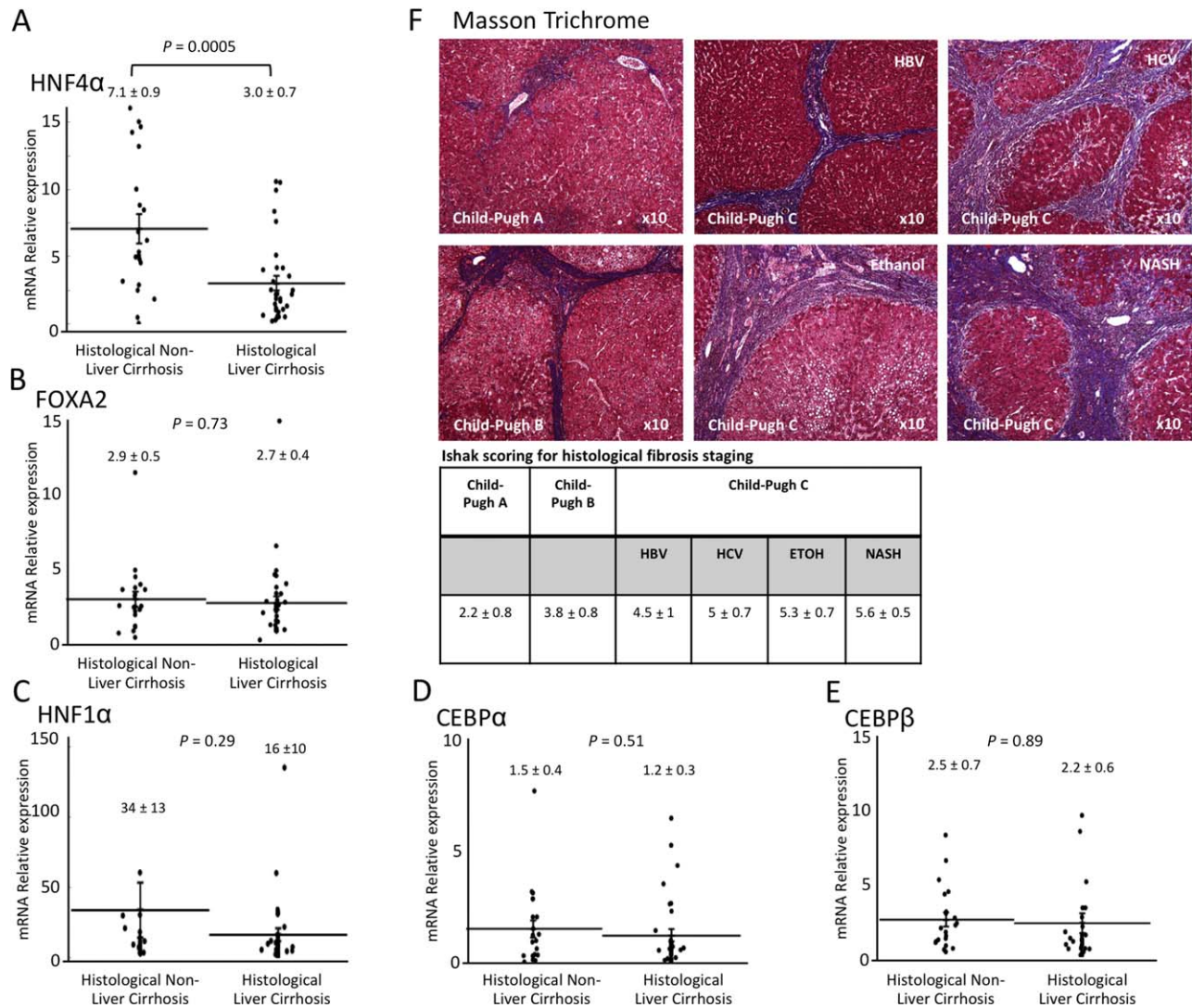


FIG. 3. Comparison of liver-enriched transcription factors and histologic cirrhosis. Livers were stained with Masson Trichrome and evaluated for fibrosis using the New Inuyama and Ishak classification. Data were analyzed according to the Ct method with the amount of target normalized to β -actin and relative to a control sample. The relative expression of (A) HNF4 α , (B) FOXA2, (C) HNF1 α , (D) CEBP α , and (E) CEBP β was compared between histologic liver cirrhosis and non-liver cirrhosis. (F) Liver fibrosis in each Child-Pugh classification (A, B, and C) and etiology was evaluated using Masson Trichrome staining and scored by Ishak scoring for histologic fibrosis (magnification $\times 10$). Abbreviations: ETOH, ethanol; HBV, hepatitis B virus; HCV, hepatitis C virus.

of diseased human livers,⁽²⁰⁾ was strongly expressed in duct structures from normal liver samples as expected, and Child-Pugh A and B liver samples showed FOXA2 was confined mainly to duct-like structures. However, weak expression was also observed in the majority of hepatocytes, and there was strong expression in a minority of hepatocytes. In Child-Pugh C liver samples, FOXA2 was more variable and weak in

hepatocytes; notably, NASH–Child-Pugh C liver samples showed weak expression in a minority of hepatocytes (Fig. 1F). The findings related to specific transcription factor expression relate to nuclear localization (the nucleus is the site in which transcription factors exert their effects on gene expression); however, weak cytoplasmic staining was often observed, especially in patient samples with Child-Pugh B and C.

HISTOLOGIC MEASURES OF LIVER CIRRHOSIS AND LETF EXPRESSION

We next evaluated whether the histologic findings and extent of fibrosis correlated with LETF expression. To accomplish this, we first evaluated the extent of fibrosis using Masson trichrome staining (Fig. 3F) and the New Inuyama classification.⁽¹⁷⁾ Cases were divided into two groups: those with histologic liver cirrhosis ($n = 39$) and those without liver cirrhosis ($n = 25$). We found that the level of HNF4 α gene expression directly correlated with liver cirrhosis ($P = 0.0005$) (Fig. 3A). However, there was no significant difference in HNF1 α , CEBP α , CEBP β , or FOXA2 expression in cirrhotic and noncirrhotic liver specimens (FOXA2, 2.7 ± 0.4 versus 2.9 ± 0.5 , $P = 0.73$; HNF1 α , 16 ± 10 versus 34 ± 13 , $P = 0.29$; CEBP α , 1.2 ± 0.3 versus 1.5 ± 0.4 , $P = 0.051$; CEBP β , 2.2 ± 0.6 versus 2.5 ± 0.7 , $P = 0.89$) (Fig. 3A-E).

Additionally, patient liver specimens were also quantitatively scored using the Ishak scoring system for fibrosis and correlated with the Child-Pugh score. An inverse relationship between the amount of fibrosis and hepatic function (Fig. 3F) was observed, indicating that the amount of liver fibrosis directly correlated with hepatocyte function and HNF4 α gene expression.

REGULATION OF HNF4 α AND DOWNSTREAM EFFECTORS OF LIVER FUNCTION

To determine the extent to which HNF4 α serves as a master regulator of liver function, HNF4 α expression was correlated with the expression of three genes that are important indicators of hepatic function and known to be regulated by HNF4 α : *OTC*, *CYP3A4*, and *F7* (Fig. 4A-D). The expression of HNF4 α correlated with the expression of *OTC* ($r = 0.37$, $P = 0.0064$),

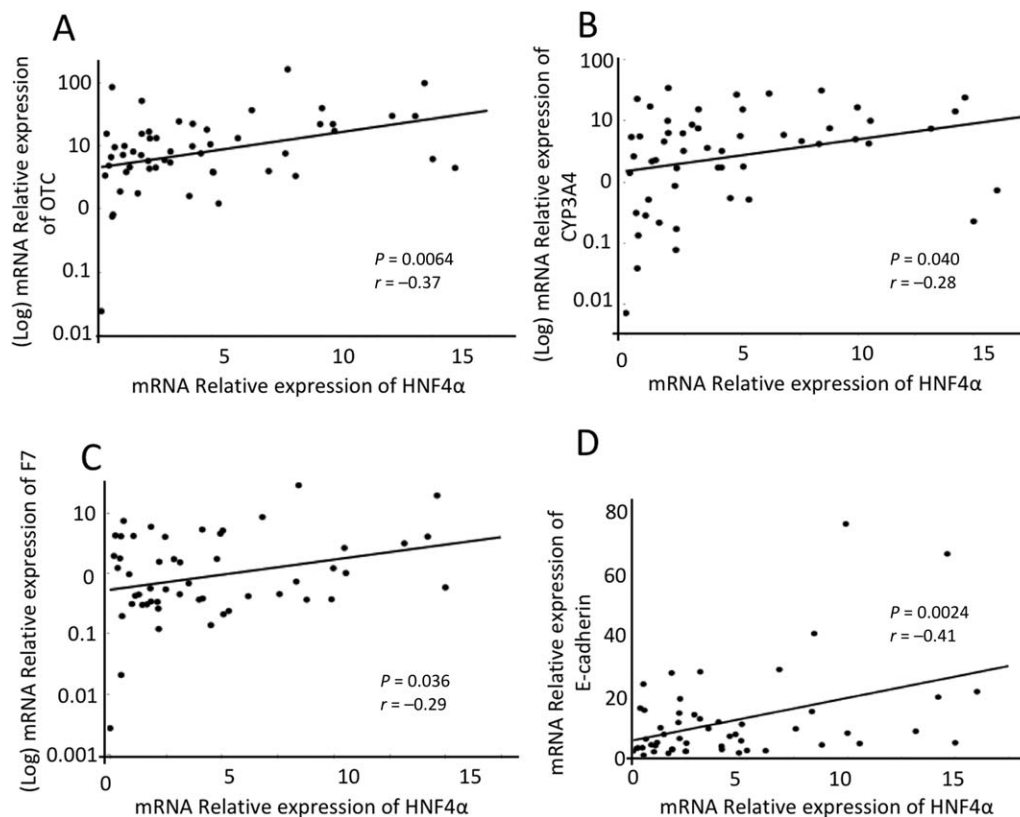


FIG. 4. HNF4 α expression and liver function genes. Gene expression levels in Child-Pugh A, B, and C patients' samples were analyzed according to the Ct method with the amount of target normalized to β -actin and relative to a control sample. Scatterplots are shown depicting the correlation between the expression of (A) HNF4 α and *OTC*, (B) *CYP3A4*, (C) *F7*, and (D) *E-cadherin*.

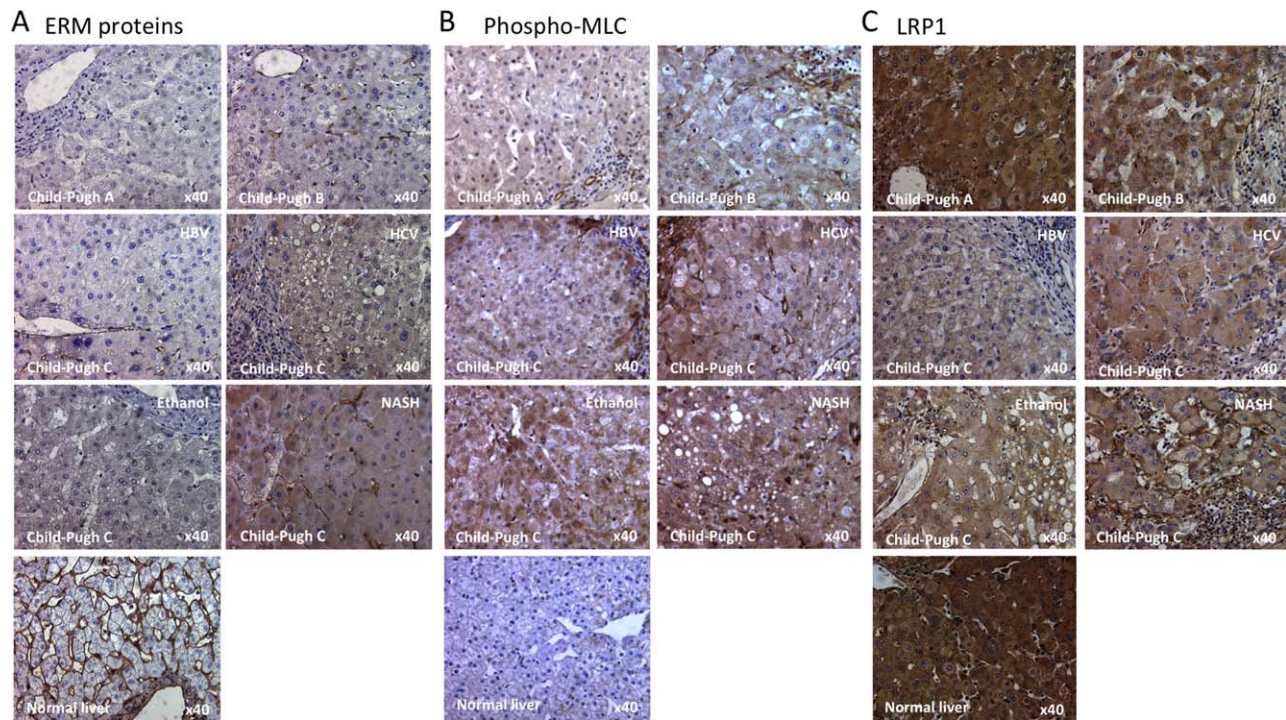


FIG. 5. Immunohistochemistry of mechanosignaling molecules in human livers grouped by the Child-Pugh classification. The expressions of (A) p-ERM, (B) phospho-MLT, and (C) LRP1 were analyzed by immunohistochemistry (magnification $\times 40$). Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus.

CYP3A4 ($r = 0.28$, $P = 0.040$), and F7 ($r = 0.29$, $P = 0.036$). However, the expression of other LETFs did not correlate significantly with these hepatocyte-specific genes (Supporting Fig. S4A-C). Indeed, these three hepatocyte-specific genes were severely down-regulated in association with low expression of HNF4 α , indicating that HNF4 α definitely affects the expression of many liver-specific target genes involved in protein secretion, amino acid production, and xenobiotic and drug metabolism. However, the molecular changes that promote the loss of HNF4 α in cirrhotic livers in chronic hepatic failure remain undefined. Thus, we evaluated the role of cell-cell and cell-matrix mechanotransduction cascade-related pathways in this process. As fibrosis is a dominant feature in the cirrhotic liver and the scarring causes cell membrane changes, we evaluated the expression of cadherin 1 (CDH1), a gene that encodes the cell-cell adhesion protein E-cadherin. Linear regression analysis showed that E-cadherin significantly correlated with HNF4 α expression ($r = 0.41$, $P = 0.0024$) (Fig. 4D). We further examined the activity of Rho/Rho-associated protein kinase (ROCK), a known mechanotransducer, and its downstream target p-ERM (Fig. 5A), using

immunohistochemistry, and found no activity in hepatocytes from the Child-Pugh A and Child-Pugh B specimens. However, p-ERM immunoreactivity was present in the cytoplasm of hepatocytes from Child-Pugh C specimens. Moreover, we confirmed ROCK activation by examining the expression of phospho-myosin light chain and found that immunoreactivity was present in the cytoplasm of hepatocytes from Child-Pugh B and Child-Pugh C specimens (Fig. 5B). Finally, as the loss of LRP1, a marker of mature hepatocytes that regulates surface urokinase receptor⁽²¹⁾ and integrins,⁽²²⁾ also reportedly leads to increased RhoA/ROCK function,⁽²³⁾ we examined the staining pattern of LRP1 in relation to Child-Pugh status. LRP1 staining inversely correlated with that of p-ERM as would be expected if LRP1 regulated RhoA/ROCK (Fig. 5F).

Discussion

Hepatic transcription factors are key regulators of normal liver function.⁽²⁴⁾ Hepatocyte-specific gene transcription is stimulated by the concerted action of

HNF 1, CEBP α , CEBP β (formerly called HNF2), FOXA (previously known as HNF3), HNF4, and HNF6.⁽²⁵⁾ Current literature indicates that HNF4 α is the master hepatic regulator and controls numerous functions that include maintenance of the hepatic epithelium and morphogenesis,^(26,27) glucose and fatty acid metabolism, synthesis of blood coagulation factors, detoxification (CYPP450 activity),^(15,28) preservation of hepatocyte polarity, and maintenance of differentiation.^(29,30) HNF4 α even controls the expression of other liver-enriched factors, such as HNF1 α ,⁽²⁴⁾ and can reestablish liver function and differentiated hepatocyte-specific characteristics when reintroduced in models of liver cirrhosis and hepatocellular carcinoma.^(15,16,24,28,31)

We now show levels of HNF4 α , HNF1 α , CEBP α , CEBP β , and FOXA2 expression in human livers with and without cirrhosis and with varying degrees of liver failure as defined by the Child-Pugh score. We previously demonstrated that levels of HNF4 α decreased in end-stage hepatocytes from animals with cirrhosis and terminal hepatic failure⁽¹⁴⁾ and showed that forced re-expression of HNF4 α can immediately convert senescent and irreversibly dysfunctional hepatocytes derived from terminal livers to reestablish normal function.⁽¹⁶⁾ In the present study, we show that patients with cirrhosis of the liver express significantly lower levels of HNF4 α in their livers than those without cirrhosis.

In addition, we showed significant mRNA down-regulation of HNF4 α in Child-Pugh C decompensated livers compared to mRNA expression in Child-Pugh A compensated livers. Importantly, HNF4 α mRNA expression in Child-Pugh C decompensated livers was not significantly different when compared to normal liver samples. However, HNF4 α localization to the nucleus was significantly reduced in Child-Pugh B and Child-Pugh C livers. These findings might indicate an essential role for HNF4 α in preserving normal liver function. Its expression is significantly up-regulated at the onset of injury (Child-Pugh A) to potentially maintain liver homeostasis; however, as liver disease progresses, HNF4 α protein expression is diminished in the nuclei of hepatocytes and/or rendered ineffective, leading to impaired regulation and hepatic function. Although several LETFs were down-regulated in severe liver disease (Child-Pugh C), only HNF4 α expression directly correlated with liver function and disease progression. For instance, protein expression of FOXA2 in biliary endothelial cells remained consistent in all liver samples examined in this study. However, in liver samples from patients

with Child-Pugh A, B, and C, a substantial number of hepatocytes became positive for FOXA2, supporting that reprogramming of biliary endothelial cells to hepatocytes or vice versa is also operative in human liver.^(20,32)

Our findings indicate that failure of the hepatic transcription system appears to be a pathologic finding in liver disease progression. These findings suggest that any correlation between chronic liver failure in cirrhosis in humans and HNF4 α -dependent hepatic functions is not regulated at the level of HNF4 α transcription but rather at the level of protein expression and/or nuclear localization.

To confirm the role of LETFs in liver disease progression, we examined the possible correlation of HNF4 α and other LETFs with clinical measures of hepatic function. We chose serum albumin, total bilirubin, and prothrombin time activity as indicators of overall hepatic function, although this only partially covers the many functions of the liver. These clinical markers, however, affect bile metabolism and indicate the capacity of the hepatocyte to synthesize proteins.⁽³³⁾ Significant correlations were found between the levels of serum albumin and total bilirubin with expression of HNF4 α .

Regulation of serum albumin has previously been noted to be associated with HNF4 α as well as with the expression of HNF1 α and the FOXA family of transcription factors.⁽²⁵⁾ Biliary metabolism has also been linked to HNF4 α expression as some enzymes involved in bile acid synthesis, such as 7 α -hydroxylase and 12 α -hydroxylase, have promoters that are bound directly by HNF4 α ^(34,35) and the expression of bile acid transporter proteins, such as organic anion transporting polypeptide-C and Na⁺-taurocholate cotransporting polypeptide, is indirectly regulated by HNF4 α -mediated transactivation of HNF1 α .⁽³⁶⁾ Unfortunately, regulation of the LETFs is complex and has not been completely delineated in humans, although LETF regulatory networks that control the expression of liver function genes have been published based on microarray data.⁽¹⁴⁾

In our study, we evaluated hepatic function directly through expression of *OTC*, *CYP3A4*, and *F7*, which are three genes that loosely reflect metabolic, detoxification, and synthetic capacities of the liver. Interestingly, all three are downstream targets of HNF4 α and their expression correlated strongly with HNF4 α expression, although statistical significance was obtained only with *OTC* and *CYP3A4*. These findings emphasize the role of HNF4 α in maintaining essential

liver functions and, as described previously regarding its down-regulation in decompensated liver disease, may explain the mechanism responsible for progression of cirrhosis to organ failure.

While the mechanism by which HNF4 α and other LETFs are down-regulated in cirrhosis has not yet been delineated, there are several possible candidate factors and pathways. For instance, there is evidence that nuclear localization of HNF4 α can be regulated at the posttranslational level by phosphorylation^(37,38) and acetylation,⁽³⁹⁾ while SUMOylation can regulate HNF4 α protein degradation and stability.⁽⁴⁰⁾ At the transcriptional level, Desai et al.⁽³⁰⁾ recently showed that hepatic HNF4 α expression and its target genes are regulated by extracellular matrix rigidity; they found a threshold level of stiffness beyond which expression of HNF4 α and liver function genes are lost. The extracellular matrix signals through a mechanotransduction cascade in which cells sense the stiffness of their environment through integrin clustering, activation of focal adhesion kinase, and further activation of intracellular signaling pathways involving the ROCK pathway.⁽⁴¹⁻⁴³⁾ Activation of the ROCK pathway by mechanical cues leads to phosphorylation and activation of effector proteins, such as the ERM proteins, which induces actin stabilization, smooth muscle contraction, and transcriptional regulation.⁽⁴¹⁾ Our study shows a significant increase in the phosphorylated ERM proteins, indicative of ROCK activation, coincident with loss of E-cadherin, HNF4 α , and other LETFs in more severe liver failure. Although it is not clear how Rho/ROCK signaling affects gene expression, it has been found to interact with YAP/TAZ transcriptional regulators.⁽⁴⁴⁾ Importantly, it was previously shown that loss of LRP1, a protein essential for normal hepatic development,⁽⁴⁵⁾ a regulator of lipid metabolism in mature livers,⁽⁴⁶⁾ and a known regulator of other mechanotransducers,^(21,22) also activates the Rho/ROCK pathway in Schwann Cells.⁽²³⁾ In this paper, we similarly show an inverse correlation between LRP1 levels and Rho/ROCK activation in hepatocytes. Interestingly, while little is known regarding the transcriptional regulation of LRP1, the [harmonizome ENCODE transcription factor database](#) indicates that LRP1 is a potential downstream transcriptional target of HNF4 α . This suggests there may be a biological loop involving HNF4 α , LRP1 expression, and suppression of Rho/ROCK that is disrupted in high-grade liver failure.

E-cadherin is a cell-cell junction protein that has many signaling partners in common with the integrin-

focal adhesion kinase signaling pathway; it is involved in mechanical transduction and is greatly affected by fibrosis. Molecules involved in the genesis of liver fibrosis, such as transforming growth factor beta, are also implicated in the loss of E-cadherin, and knockout of *CDH1*, the gene encoding E-cadherin, has been shown to induce an inflammatory response and periductal fibrosis.^(47,48) Furthermore, fibrocystin-deficiency diseases, such as congenital hepatic fibrosis, down-regulate E-cadherin expression.⁽⁴⁹⁾ Interestingly, E-cadherin is a direct transcriptional target of HNF4 α and loss of HNF4 α results in loss of E-cadherin expression.^(26,27,50) While there is no known link between mechanical transduction signaling and regulation of LETFs in fibrosis, two intracellular kinases involved in integrin- and E-cadherin-mediated signaling, e.g., proto-oncogene tyrosine-protein kinase Src (c-Src) and protein kinase C, can phosphorylate HNF4 α and other nuclear receptors, and their activation can lead to a decrease in stability and a loss of nuclear localization and transactivation functions.^(37,51)

In summary, gene and protein expression analysis of liver samples from patients with cirrhosis and worsening liver function demonstrate that liver disease disrupts the normal expression and activity of LETFs, especially HNF4 α . Clinical data and MELD score correlations point to the loss of hepatic transcriptome expression with liver decompensation and suggest a possible mechanism responsible for disease progression in cirrhosis and terminal liver failure. Further studies are needed to more fully characterize how each LETF might affect liver function and to delineate molecules that directly regulate their expression levels.

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